Part I:
B Cell Development and Function in Mice with a Targeted λ1 Light Chain Gene Insertion

Part II:
Novel Approaches for Cre-mediated Inducible Gene Alteration in Mice

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Meiner Mutter

C Part II) Novel approaches for Cre-mediated inducible gene alteration in mice

A Opening remark

This thesis was performed in a laboratory that focuses on B cell development and function *in vivo* and has developed conditional gene targeting as a tool to analyze gene function in the murine immune system. I completed two independent projects that reflect both the biological and technical scope of the lab. In the first project I generated a mouse strain that carries an immunoglobulin (Ig) λ1 light chain gene insertion. I analyzed the impact of a pre-rearranged λ1 light chain on Igλ rearrangement and Igλ versus Igκ isotype exclusion as well as the fate of B cells that escape isotype exclusion and consequently express two distinct B cell receptor specificities. The second project describes two novel approaches in conditional gene targeting: one allows unidirectional genetic inversion in mice and the other involves siRNA-mediated inducible gene inactivation in ES cells. Both methods are useful additions to the toolbox of mouse genetics and will hopefully find a broad range of biological applications.

B Part I) B cell development and function in mice with a targeted λ1 light chain gene insertion

B 1 INTRODUCTION

B lymphocyte development is a highly regulated process that leads to the generation of a diverse repertoire of B cell receptor (BCR) antigen specificities. Multiple alleles can potentially contribute to the formation of the BCR. However, a tightly controlled sequence of chromatin remodeling and somatic recombination events ensures that any given B cell expresses a single BCR specificity, which can then be recruited into an antigen-specific immune response.

B 1.1 The B cell antigen receptor

The BCR consists of two identical immunoglobulin heavy (IgH) chains and two identical Ig light (IgL) chains, which are covalently linked by disulfide bonds, and are brought to the cell surface only in combination with the signal transducing Igα/Igβ heterodimer. Each Ig molecule has a constant (C) and a variable (V) region. The variable regions of IgH and IgL chains contain hypervariable regions, which form the antigen binding sites of the BCR and are therefore termed complementarity determining regions (CDRs). The constant regions define the isotype of the Ig molecule and are subdivided into Cκ and Cλ in the case of IgL and Cμ, Cδ, Cγ1, Cγ2a, Cγ2b, Cγ3, Cε and Cα in the case of IgH. While no functional differences have been reported for Cκ and Cλ, different IgH constant regions have different signaling properties when membrane-bound and mediate distinct effector functions when secreted. Switch from IgM to IgG, IgA or IgE is an irreversible somatic recombination event termed Ig class switch recombination. Expression of IgD, on the other hand, is the consequence of alternative splicing and can therefore occur together with IgM expression. Alternative splicing is further responsible for the generation of the secreted form of Ig molecules (for reviews see (Manis et al., 2002; Rajewsky, 1996)).

B 1.2 B cell development

In the mouse, B cells develop in the fetal liver during embryogenesis and in the bone marrow after birth. IgH and IgL chains are assembled from various gene segments by tightly regulated somatic recombination events (see chapter 1.3) at consecutive developmental stages during B cell development. These stages can be distinguished by differential expression of surface antigens. IgH rearrangements occur in pro B cells ($B220^{low}$, $CD43^{+}$, $CD25^{-}$, Ig^{-}) and, if productive, promote a phase of proliferative expansion and subsequent IgL rearrangement in pre B cells ($B220^{low}$, $CD43^{-}$, $CD25^{+}$, Ig^{-}). Upon productive IgL rearrangement, B cells express IgM on the cell surface and are considered immature B cells ($B220^{low}$, $CD43^{-}$, $CD25^{-}$, IgM^{+}) until they complete maturation with co-expression of IgD (reviewed in (Hardy and Hayakawa, 2001; Rolink and Melchers, 1996)). Mature B cells can be subdivided into several, functionally and phenotypically distinct subpopulations: follicular (FO) B cells or B2 cells, B1 cells, marginal zone (MZ) B cells, plasma cells and memory B cells.

FO B cells are IgM^{+}, IgD^{bright} and are typically long-lived with life spans ranging from a few weeks to several months and possibly years (Fulop et al., 1983; Forster and Rajewsky, 1990; Osmond, 1993; Hao and Rajewsky, 2001). FO B cells compose the vast majority of the peripheral B cell pool and recirculate through all lymphatic organs. Upon antigen encounter, they become activated and undergo further differentiation into plasma cells or memory B cells. While plasma cells can form in response to both T cell dependent (TD) and T cell independent (TI) antigens, the generation of memory B cells is normally dependent on T cell help and takes place in specialized morphological structures termed germinal centers (reviewed in (MacLennan, 1994)). Memory B cells have generally undergone class-switch recombination, are long-lived and their survival appears to be independent of cognate antigen (Schittek and Rajewsky, 1990; Maruyama et al., 2000). Memory B cells are further characterized by somatically mutated BCRs, which have been selected for optimal affinity to the immunizing antigen following somatic hypermutation in germinal centers. This process is termed affinity maturation and is driven by BCR-antigen interaction (reviewed in (Diaz and Casali, 2002)). A subset of memory B cells terminally differentiates into antibody-secreting plasma cells, which preferentially home to the bone marrow where they were shown to persist for several months or longer (Benner et al., 1981; Manz et al., 1997). Similar to memory B cells, long-lived

plasma cells appear to be maintained in an antigen-independent manner (Manz et al., 1998; Slifka et al., 1998). While long-lived plasma cells represent a small, specialized plasma cell subset that is thought to confer long term protection against the immunizing antigen, most plasma cells form early during an immune response in order to provide rapid protection against the invading pathogen. Such extrafollicular plasma cells are generated during both TD and TI immune responses in the medullary cords of lymph nodes and in foci in the red pulp of the spleen. The majority of these cells survive for approximately three days. Some were, however, shown to become long-lived and are thought to reside either in the splenic red pulp or in the bone marrow (Sze et al., 2000). Extrafollicular plasma cells can be class-switched, but do normally not show signs of somatic hypermutation (reviewed in (Cyster, 2003; MacLennan et al., 2003)).

Another important source of serum Ig are B1 cells, which were first identified by Hayakawa et al. (Hayakawa et al., 1983) as a B cell subset that expresses the T cell marker Ly-1/CD5 (Hayakawa et al., 1983). B1 cells are generated during early B cell development, mainly from fetal liver precursor B cells and are activated in a polyclonal, T cell independent fashion (reviewed in (Su and Tarakhovsky, 2000)). Activation can be triggered by self-antigen and activated B1 cells are therefore considered a major source of natural autoantibodies. B1 cell derived serum Ig is predominantly of the μ and □β isotypes (reviewed in (Hayakawa and Hardy, 2000)). B1 cells are generally $Mac1^{+}$, $CD43^{+}$, IgM^{bright} and IgD^{low}, have self-renewing capacity and are predominantly located in the peritoneum. It is still unclear whether these cells represent a distinct B cell lineage or whether B1 and B2 B cells are derived from the same precursor cells. The observation that recognition of auto-antigen in the bone marrow favored the generation of B1 cells indicates that ligand-BCR interaction may play a role in B1 lineage decision. ((Hayakawa et al., 1999; Hayakawa et al., 2003)). Moreover, certain antigen receptor specificities appear to preferentially direct B1 cell development (Arnold et al., 1994; Lam and Rajewsky, 1999).

MZ B cells represent a similarly specialized B cell subset and are found exclusively in the spleen within the marginal zones that surround B cell follicles. MZ B cells are defined as IgM^{high}, IgD^{low}, $CD21^{high}$ and $CD23^{low}$. In contrast to B2 cells, MZ B cells and B1 cells have the unique capacity to generate effector cells in early stages of the immune response, mostly against TI antigens, and are thus thought to be the first line of defense for antigens that are scavenged efficiently in either the peritoneum or the

marginal zone (Martin and Kearney, 2000a; Martin et al., 2001). The generation of MZ B cells was shown to depend on several BCR-associated signaling molecules and was further suggested to be influenced by the BCR specificity (Martin and Kearney, 2000b). Thus, like in B1 cell formation, antigenic selection might play a role in MZ B cell differentiation.

While the role of BCR-ligand interaction in the generation and maintenance of individual B cell subsets is still poorly understood, it is clear that surface expression of the BCR is essential for the survival of most B cell subsets (Lam et al., 1997). The requirement of BCR expression in the memory B cells subset and in plasma cells is, however, still controversial.

B 1.3 V(D)J recombination

To guarantee a diverse B cell repertoire, which is able to respond to an unlimited spectrum of foreign antigens, vertebrates have evolved a combinatorial approach to assemble immunoglobulin variable regions from a variety of distinct gene segments. In mice, these gene segments comprise several hundred variable (V), 14 diversity (D) and 4 joining (J) segments in the IgH locus, ~100 V and 4 functional J segments in the Igκ locus and 3 V and 3 functional J segments in the Igλ locus (Figure 1). V, D and J segments are combined during early lymphocyte differentiation by a common V(D)J recombinase that consists of the Recombination Activating Gene products RAG1 and RAG2 (Oettinger et al., 1990; Schatz et al., 1989) and recognizes conserved recombination signal sequences (RSS) flanking the V, D and J segments. Imprecise joining and N nucleotide addition mediated by Terminal deoxynucleoside transferase (TdT) represent an additional means of variable region diversification during V(D)J recombination. IgH rearrangement can occur on two IgH alleles, while Ig light chains can be generated from two Igκ and two Igλ alleles. Any given B cell expresses only one of the two allelic IgH loci and one of the multiple IgL loci as proteins and thus carries an Ig molecule of single specificity. This phenomenon is termed allelic or (κ versus λ) isotype exclusion (reviewed in (Bassing et al., 2002)). While allelic exclusion at the IgH loci is extremely efficient and B cells that express two distinct IgH chains are virtually absent, allelic and isotype exclusion at the IgL loci appear to be less stringent as 1-2% of mature B cells were shown to express more than one light chain in mice (Casellas et al., 2001; Gollahon et al., 1988). The reason

for this discrepancy is unknown. It may, however, be related to the fact that pre B cells can undergo several rounds of IgL rearrangement in order to alter the specificity of the BCR. This process is termed receptor editing and is discussed in further detail in chapter 1.6.

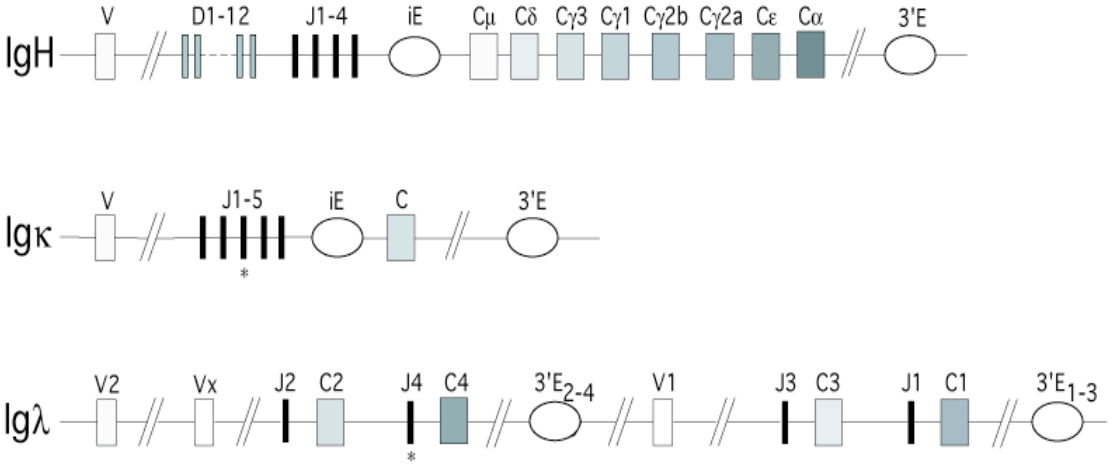


Figure 1. Genomic map of the mouse Ig loci. Exons are shown as rectangles and comprise variable (V), diversity (D), joining (J) and constant region (C) gene segments. Enhancers are depicted as ovals; iE, intronic enhancer; 3'E, e' enhancer. Map is not drawn to scale.

B 1.4 Ig locus accessibility

Generally, tissue-specific and developmentally regulated Ig rearrangement is ensured by Ig locus-specific enhancers, which render the Ig locus accessible for DNA binding proteins such as transcription factors and the RAG1/RAG2 complex. Ig loci normally harbor two types of enhancers: intronic enhancers, which are located in the intron between the last J exon and the first C exon, and 3' enhancers, which can be found 3' of the last C exon (reviewed in (Chen and Alt, 1993; Gorman and Alt, 1998)). However, no intronic enhancers have been identified in the Ig locus. Germline transcripts from unrearranged Ig loci that initiate upstream of V, D or J segments can be detected in B cell progenitors that are in the process of rearranging the respective Ig loci (Corcoran et al., 1998; Schlissel et al., 1991; Yancopoulos and Alt, 1985). Recently, Nussenzweig and colleagues showed that the level of V germline

transcription needs to exceed a certain threshold before a V segment becomes susceptible to rearrangement, thus providing evidence for a functional association of germline transcription with rearrangement (Casellas et al., 2002). Moreover, the intronic Ig enhancer (iE) and the 3' Ig enhancer (3'E) were found to be important for Ig transcription and rearrangement (Gorman et al., 1996; Takeda et al., 1993; Xu et al., 1996; Meyer and Neuberger, 1989). Mice that lack both enhancers do not undergo detectable Ig rearrangements and lack of either 3'E or iE alone causes a quantitatively comparable decrease in Ig germline transcription and Ig rearrangements (Inlay et al., 2002; Gorman et al., 1996). The introduction of a phosphoglycerol kinase (PGK)-promoter driven *neo*^{R} gene 5' of the J1 segment led to a substantial increase in both J1 germline transcription and V1 J1 rearrangement and thus demonstrates a similar linkage between the two processes in the Ig locus (Sun and Storb, 2001).

Besides cis-regulatory elements, epigenetic DNA modifications were shown to play an important role in the regulation of Ig rearrangement. These modifications are inheritable changes in chromatin structure that do not affect the genetic information itself. Epigenetic changes include histone modifications, such as acetylation and methylation, as well as DNA methylation. Methylation of CpG DNA islands is now recognized to be a main player in establishing a silent chromatin state and acts through the recruitment of proteins that modify nucleosomes, such as methyl-CpG binding proteins and histone deacetylases (reviewed in (Jaenisch and Bird, 2003)). It has been demonstrated many years ago, that DNA undermethylation correlates with Ig expression and B cell differentiation (Storb and Arp, 1983). Recently, Mostoslavsky et al. found that the initiation of Ig rearrangements coincides with demethylation in the J region and that only one of the two Ig alleles is subject to demethylation in the majority of B cells (Mostoslavsky et al., 1998). Interestingly, Ig germline transcription seems to be unaffected by DNA methylation since it was shown to occur in a biallelic fashion (Singh et al., 2003). Demethylation and germline transcription may therefore be independently required to mark an Ig allele for rearrangement and RAG1/2 accessibility. Further chromosome modifications that have been correlated with Ig rearrangement include histone hyperacetylation (Johnson et al., 2003; McMurry and Krangel, 2000) and asynchronous chromosomal replication, which is established during emryogenesis and appears to 'mark' one

chromosome for rearrangement by an as yet unknown mechanism (Mostoslavsky et al., 2001).

1.5 Regulation of IgL rearrangement

Despite extensive research on IgL locus accessibility and rearrangement, the developmental regulation of IgL rearrangement is still poorly understood. In mice, B cells that express Igλ are 15-20 times more frequent than those expressing Igκ. In humans, the frequencies of κ and λ expressing B cells are similar, yet in both mice and humans, κ^+ B cells generally carry the Igκ locus in germline configuration, while the vast majority of λ^+ B cells has inactivated its Igκ loci by either non-functional VκJκ joints or deletion of the κ constant region (Cκ) gene (Brauninger et al., 2001; Hieter et al., 1981; Takeda et al., 1993; Yamagami et al., 1999). Cκ deletion is the consequence of a recombination event that occurs between an RSS located either in the Jκ-Cκ intron or at the 3' end of a non-rearranged Vκ gene and a downstream "rearranging" sequence called RS in mice (Durdik et al., 1984) and κ-deleting element (Kde) in humans (Siminovitch et al., 1985). However, in some cells, IgL rearrangement is initiated at the Igλ locus as shown by a small fraction of λ^+ B cells that carry non-functional Igλ rearrangements (Coleclough et al., 1981; Yamagami et al., 1999; Zou et al., 1993).

An ordered and a stochastic model were put forward to explain these findings (Hieter et al., 1981; Coleclough et al., 1981). The ordered model proposes regulated opening of IgL loci with Igκ being accessible for rearrangements prior to Igλ. The stochastic model predicts that both IgL loci are accessible at the same time with the probability of rearrangements being higher for Igκ than for Igλ (reviewed in (Gorman and Alt, 1998)). The analyses of several mouse mutants with impaired Igκ rearrangement and/or expression demonstrated, however, that inactivation of the Igκ locus causes a tenfold increase in λ^+ B cells and were therefore incosistent with either of the two models. Inactivation of Igκ was achieved by replacing the intronic κ enhancer (iEκ) (Takeda et al., 1993), the Cκ gene segment (Zou et al., 1993) or Jκ and Cκ gene segments (Chen et al., 1993) with a *neo*^{R} gene. While the former manipulation causes complete silencing of Vκ→Jκ rearrangements, the two latter mutations exert only a mild effect on Igκ rearrangement but abolish expression of a functional κ light chain.

The drastic increase in λ-expressing B cells in these mice led to the proposal of negative regulatory elements in the germline Igκ locus that would actively suppress κ rearrangements and be artificially disrupted in the mutant alleles. In wild-type (WT) mice, inactivation of such elements upon Igκ rearrangement was suggested to increase the probability of Igλ rearrangements (Chen et al., 1993; Zou et al., 1993).
Taken together, current knowledge suggests that Igκ is generally rearranged prior to Igλ and that this phenomenon may be controlled by an Igκ-derived negative regulatory signal that interferes with Igλ rearrangement and/or transcription. Whether or not the unrearranged Igκ locus negatively regulates the transcriptional accessibility of Igλ is investigated in the work presented here.

B 1.6 B cell tolerance and receptor editing

The random assembly of a diverse BCR repertoire imposes a major problem on the immune system in that self-reactive receptor specificities have to be eliminated. In order to achieve 'tolerance' to self-antigens, auto-reactive B cells can be deleted, functionally silenced in a state called 'anergy' or undergo a process known as receptor editing (reviewed in (Buhl et al., 2000)). Receptor editing describes the alteration of a given BCR specificity via continuing Ig rearrangements. The fraction of immature B cells that undergo editing in wild-type mice was estimated to be between 20% to 30% (Casellas et al., 2001). In humans, 55-75% of all antibodies that are expressed in immature B cells were shown to be autoreactive, and receptor editing appears to play an important role in the removal of these cells (Wardemann et al., 2003). Thus, receptor editing represents a major mechanism of B cell tolerance. The Igκ locus allows multiple rounds of Vκ-Jκ rearrangements (see Figure 1) and is consequently the most frequently edited Ig locus. Ongoing Igκ rearrangements lead to the inactivation of previous VJκ joints and can ultimately inactivate the respective Igκ allele by RS recombination (see above). These cells can be rescued by Igλ rearrangements and the generation of λ^+ B cells has therefore been directly correlated with receptor editing (Retter and Nemazee, 1998). Whether λ^+ B cells themselves undergo receptor editing has however not yet been conclusively demonstrated. The unique composition of the Igλ locus does not allow the deletion of previously rearranged Igλ joints (see Figure 1) and editing of λ^+ B cells is expected to yield B

cells that express two distinct Ig light chains. As mentioned earlier, κ/λ double positive B cells can be generated at very low numbers and are a potential threat to the immune system, since specific immune responses may activate these cells and lead to the production of unspecific and potentially autoreactive 'bystander' Igs (Li et al., 2002). Both the extent of receptor editing in λ1$^+$ B cells and the fate of B cells, which express two distinct BCR specificities as a consequence of receptor editing, are addressed in this thesis.

B 1.7 Objectives of this study

In humans and mice, Igλ rearrangements occur more frequently than Igκ rearrangements and previous results suggested that the unrearranged Igκ locus negatively regulates Igλ transcription and/or rearrangement. The aim of my thesis was to test whether the unrearranged Igκ locus does indeed provide a negative regulatory signal that interferes with Igλ expression. I therefore generated a mouse mutant, in which a VJκ1-joint has been inserted into its physiological position in the Igκ locus, and analyzed its impact on Igλ rearrangement and isotype exclusion. I also addressed the fate of B cells that escape isotype exclusion in this mouse model via receptor editing in κ$^+$ B cells. Specifically, I analyzed how the expression of two distinct BCR specificities affects B cell development and B cell activation upon antigen encounter.

B 2 MATERIALS AND METHODS

B 2.1 Molecular Biology

B 2.1.1 Competent cells and iolation of plasmid DNA

Competent *Escherichia coli* DH5 cells were prepared as described by Inoue et al. (Inoue et al., 1990) and used in heat shock transformations of plasmid DNA. DNA ligation was performed using NEB T4 Ligase or NEB Quick Ligase according to the maufacturer's protocol. Plasmid DNA was isolated from transformed *Escherichia coli* DH5 bacteria by alkaline lysis (Birnboim, 1983) according to the protocol of (Zhou et al., 1990). Plasmid DNA of a higher purity was obtained with QIAGEN columns (QIAGEN) following the supplier's instructions.

B 2.1.2 Molecular cloning

Molecular cloning was performed according to standard protocols (Sambrook et al., 1989). Enzymes were obtained from the following companies: Roche, GIBCO-BRL, New England Biolabs (NEB), Stratagene and Takara.

B 2.1.3 Isolation of genomic DNA from ES cells and mouse organs

Cells were lysed over night at 56°C in lysis buffer (100 mM Tris-HCl, pH 8.5; 5 mM EDTA; 200 mM NaCl; 0.2% SDS; 500 mg/ml *Proteinase K*). Subsequently, DNA was precipitated by the addition of an equal volume of isopropanol. DNA was pelleted by centrifugation, washed in 70% EtOH and resuspended in double distilled (dd) H_2O. To extract DNA from ES cell clones, that were grown in 96-well tissue culture dishes, plates were treated according to the protocol of (Pasparakis and Kollias, 1995). Mouse tissue was incubated for 6 h to o/n at 56°C in lysis buffer. Undissolved debris was pelleted and the supernatant was mixed with an equal volume of isopropanol to precipitate the DNA. The DNA was washed in 70% EtOH, dried and resuspended in ddH_2O.

B 2.1.4 Agarose gel electrophoresis and DNA gel extraction

Separation of DNA fragments by size was achieved by electrophoresis in agarose gels (0.7% - 2.5%; 1 x TAE (Sambrook et al., 1989); 0.5 mg/ml ethidium bromide). DNA

fragments were isolated from agarose gel slices using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. Size markers for agarose gel electrophoresis were obtained from GIBCO-BRL (1 kb marker) and NEB (2-Log ladder).

B 2.1.5 DNA sequencing

DNA fragments were sequenced with the 'Taq DyeDeoxyTerminator Cycle Sequencing Kit' (Applied Biosystems), which is a modification of the Sanger et al. Protocol (Sanger et al., 1977). The fluorescently labelled DNA pieces were separated and analysed with ABI373A and ABI377 systems (Applied Biosystems) with the help of S. Willms.

B 2.1.6 Purification of RNA and reverse transcription

Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's protocol. All RNA work was done using DEPC-treated H_2O. cDNA was synthesized from 20,000 cells using oligo-dT primer and Thermoscript RT-PCR System (Invitrogen) according to the manufacturer's instructions. 1/10 of the cDNA template and serial dilutions thereof were subjected to PCR (see chapter 2.1.8)

B 2.1.7 Quantification of nucleic acids

The concentration of nucleic acids was determined by measuring the absorption of the sample at 260 nm and 280 nm in a spectrophotometer (Pharmacia). An OD_{260} of 1 corresponds to approximately 50 µg/ml for double stranded DNA or 40 µg/ml for RNA and single stranded DNA. Purity of nucleic acids can be estimated by the ratio OD_{260}/OD_{280}, pure preparations of DNA and RNA show a ratio of 1.8 and 2.0, respectively.

B 2.1.8 Polymerase chain reaction (PCR)

PCR (Mullis and Faloona, 1987; Saiki et al., 1985) was used to genotype mice for the presence of the targeted allele or the respective wild-type allele, and to amplify fragments for sequencing, molecular cloning or the generation of Southern blot probes. In addition, PCR served to amplify cDNA for analytic purposes. Reactions were performed either in PTC-200 machines (MJ-Research) or Trio-thermocyclers (Biometra).

PCR reactions were generally performed in a total volume of 50 µl using the following reaction mix: 25 pmol of each primer, 0.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (GIBCO-BRL or Eppendorf), 10 mM dNTPs (Pharmacia), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10-100 ng template DNA. Amplification started with denaturation for 3 min at 94°C followed by 30-35 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 30 sec. A final extension step was performed at 72°C for 10 min. Amplification of fragments that were subsequently used for sequencing or molecular cloning were performed using Expand High Fidelity PCR System (Roche) according to the manufacturer's instructions.

Name	**Sequence (5'-3')**	**Location**	**□**
5'NXF1	TGCCAGAGCGGCCGCTGCTAGTAACAATAAGAGTGG	5' of V□1	s*
3'NFX1	GTT CTA GAG TGA CAA TAG TAA CGA	5' of V□1	as*
5'XF-3	TAA AAA GAA AAA AAA CAT AGG	5' of V□1	s
3'XF-2	CCA AGA TTG GGT TAA TGT ATC	5' of V□1	as
5'XbaI/XhoI	CAG AAA TGC AAG CCC AGG AAG	3' of C□1	s
3'XbaI/XhoI	TTA CTG GGG AAC ACA CTA CAC	3' of C□1	as
VJ□1-int	TTG TGA CTC AGG AAT CTG CA	V□1 exon	s
VJ□1-ext	GGG TAT GCA ACA ATG CGC ATC TTG TC	V□1-LI*	s
LAMINFOR	GGA GCA GTC TGA AAT GAG ACA AAG CAT	J□1-C□1 intron	as
C□1	CTC GGA TCC TTC AGA GGA AGG TGG AAA CA	C□1 exon	as
5'J□1#2	ACA GCC AAA ACT CAT CCA TAC CCA	5' of J□1	s
Ms□	GAT ATT GTG ATG ACC CAG TCT	V□ exon	s
C□E	ACA CTC ATT CCT GTT GAA GCT CTT	C□ exon	as
m-□-actin-T	CCT AAG GCC AAC CGT GAA AAG	□-actin exon 2	s
m-□-actin-B	TCT TCA TGG TGC TAG GAG CCA	□-actin exon 4	as

Table I. Complete list of PCR primers. * abbreviations: LI, leader intron; s, sense strand; as, antisense strand.

***Allele* or cDNA**	**Primer pair (s/as)**	**Product [bp]**
VJ□1i	VJ□1-int/LAMINFOR	543
Ig□GL	5'J□1#2/LAMINFOR	1068
m-□-actin	m-□-actin-T/m-□-actin-B	646
□1 light chain	VJ□1-int/C□1	378
□ light chain	Ms□/C□E	~600*

Table II. Genotyping and RT-PCR products. * Ms□ is a promiscuous primer and amplifies most VJ□-joints.

B 2.1.9 Southern blot analysis

5-15 □g DNA were digested o/n with 50 to 100 U of the appropriate restriction enzyme. Subsequently, the DNA fragments were resolved by agarose gel electrophoresis and transferred onto Hybond^{TM}-N^{+} (Amersham) or GeneScreenPlus (Dupont) nylon membranes by an alkaline capillary transfer as described (Chomczynski and Qasba, 1984). Membranes were baked at 80°C for 1 hour to covalently link the DNA with the membrane. Membranes were then equilibrated in 2x SSC (Sambrook et al., 1989) and prehybridized o/n in hybridization solution (1M NaCl, 1% SDS, 10% dextran sulfate, 50 mM Tris-HCl pH 7.5, 250 □g/ml sonicated salmonsperm DNA) at 65°C.

To generate Southern probes, 25 to 60 ng of probe DNA were radioactively labeled with 2.5 mC □^{32}P-dATP (Amersham) using the Ladderman^{TM} Labeling Kit (Takara), which is based on the principle of random primed oligolabeling [Feinberg, 1984 #215}. Unincorporated radiolabeled nucleotides were removed with MicroSpinTM S-200HR columns (Pharmacia) to reduce background during hybridization. The probe was denatured for 5 min at 95°C before it was added to the hybridization solution. Hybridization was performed for at least 8 h at 65°C in a rotating cylinder (Hybaid). After hybridization stringency washes were performed in the following order: 2x SSC/0.1 % SDS, 1x SSC/0.1 % SDS and 0.2 x SSC/0.1 % SDS, if necessary. All washes were done at 65°C under gentle agitation for 15 min to 1 hour. After each wash the filter was monitored with a Geiger-counter. The washes were stopped when specific signals of 20 to 100 cps were detectable. Then, the filter was sealed in a plastic bag and exposed to X-ray film (Kodak XAR-5 or BioMAX MR; Eastman

Kodak) at –70°C. Films were developed in an automatic developer (Agfa). Alternatively, the filter was exposed at RT to a phosphoimager screen (Fuji) that was analyzed on a Bio-Imaging Analyser (Fuji Bas 1000; Fuji).

The following probes were used for Southern blotting:

5'V1: This probe is located 5' of the Vλ1 gene outside the homology region and was used as external probe to identify homologous recombination in ES cells. 5'V1 was generated by PCR using primers 5'XF-3 and 3'XF-2 (see Table I), phage clone KX39 (covering 15 kb upstream of Vλ1; gift from Ursula Storb) served as template.

3'C1: This probe is located downstream of the Cλ1 exon in the long arm of homology and was used to distinguish homologous recombination from random integration in targeted ES cells. 3'C1 was generated by PCR using the primers 5'XbaI/XhoI and 3'XbaI/XhoI (see Table I), cosmid cos2 (gift from Ursula Storb (Miller et al., 1988)) was used as template.

RS-probe: This probe is located 5' of the Igκ RS element and was used to analyze RS recombination. RS-probe was excised from plasmid pBM-RS (Durdik et al., 1984) using BamHI and EcoRI.

5'Jλ-probe: This probe is located 5' of Jλ1 and was used to analyze Igλ rearrangement. 5'Jλ1 was excised from plasmid pSP-Ig8 (Pelanda et al., 1996) using EcoRI.

IL-4 probe: This probe hybridizes to the IL-4 gene and was used to normalize for DNA content. IL-4 probe was excised from pIL-4T (Kuhn et al., 1991) using SmaI.

B 2.2 Cell Biology

B 2.2.1 Embryonic stem cell culture

The VJ□1i allele was generated through homologous recombination in Bruce-4 ES cells (Kontgen et al., 1993). Culturing and transfection of ES cells was performed according to published protocols (Pasparakis and Kollias, 1995; Torres and Kuhn, 1997). In brief, ES cells were cultured in ES cell medium (DMEM, 15 % FCS, 2 mM sodium pyruvate, 2 mM L-glutamine, 1x non essential amino acids, 1:1000 LIF, 0.1 mM 2-□-mercaptoethanol) on a layer of mitotically inactive embryonic feeder (EF) cells. LIF (Leukemia inhibitory factor, generated in our laboratory) was added to ensure the maintenance of ES cell omnipotency in culture. Only FCS batches that have been tested to promote ES cell growth and prevent premature *in vitro* differentiation were used for ES cell culture. To obtain mitotically inactive EF cells, fibroblasts from day 13 mouse embryos (prepared by Angela Egert) were passaged up to three times in EF cell medium (DMEM, 10% FCS, 2 mM sodium pyruvate, 2 mM L-glutamine) and subsequently treated with mitomycin-C (10 □g/ml for 2 h). ES and EF cells were grown in tissue culture dishes (Falcon, Greiner) and kept at 37°C in a humid atmosphere with 7.5% CO_2. ES cells were passaged before colonies became confluent. Colonies were washed once with PBS and then treated with trypsin (0.05 % trypsin, 0.02 % EDTA in PBS; GIBCO-BRL) at 37°C until the cells detached from the dish. Optimal conditions for trypsinization of Bruce-4 cells were achieved by supplementing the solution with 1% chicken serum. This single cell suspension was then used for passaging, transfecting or freezing. ES cells were frozen in FCS containing 10 % DMSO at –80°C. Long time storage occurred in liquid nitrogen. For transfection, 0.6 - 1 x 10^7 ES cells were mixed with 30 to 40 □g of DNA in 800 µl PBS (GIBCO-BRL) and electroporated at RT (500 mF, 230V). Transfection time constants ranged from 7 to 10 s. After transfection, ES cells were transferred onto an embryonic feeder layer. From day 2, cells were kept in G418-containing ES cell medium (150-200 □g/ml) to select for integration of the *neo*^{R} gene. Selection against HSV-*tk* containing random integrants started at day five after transfection by supplementing the medium with 2 µM gancyclovir (Cymeven, Syntex). 8 to 12 days after transfection, double resistant ES cell colonies were picked and split into EF-containing 96-well tissue culture dishes for expansion.

B 2.2.2 Preparation of cell suspensions from lymphoid organs

Spleens were strained through 70 µm nylon meshes (Becton Dickinson, Discovery Labware) to obtain single cell suspensions. Bones were flushed with medium to extract bone marrow cells and the peritoneal cavity was flushed with 10 ml of medium to recover cells. Erythrocytes were lysed from spleen and bone marrow preparations by incubation in lysis buffer for 3 min on ice (140 mM NH_4Cl, 17 mM Tris-HCl pH7.65). Cells were resuspended in medium (DMEM, 5% FCS, 2 mM L-glutamine) and kept on ice prior to analysis.

B 2.2.3 Flow cytometry, cytoplasmic staining and cell sorting.

Single cell suspensions from bone marrow and spleen were stained with monoclonal antibodies (mAbs) or polyclonal Abs conjugated to fluorescein isothyocyanate (FITC), phycoerythrin (PE), PerCP or biotin. Biotin conjugates were visualized with Streptavidin-allophycocyanin (APC). The IgG1-digoxigenin (DIG) conjugate was detected by fluorescein-coupled anti-DIG Fab fragments (Roche). For additional cytoplasmic stainings, cells were subsequently fixed in PBS/2% formaldehyde for 20 min at RT. Generally, 10^6 cells were stained in 50 µl staining buffer (PBS, 0.5% BSA, 0.05% N_3). Intra-cellular staining was performed with FITC conjugated Ab in staining buffer containing 0.05% saponin. Monoclonal antibodies, listed in Table 3, were either prepared in our laboratory by C. Uthoff-Hachenberg and B. Hampel or purchased from the indicated suppliers. Cells were acquired on FACSCalibur and data were analyzed with CellQuest software, cell sorting was performed on FACS Vantage with the help of C. Goettlinger and N. Barteneva. Instruments and software were from Becton Dickinson (USA). All analyses were restricted to cells within the lymphocyte gate. Dead cells were labelled with propidium iodide or Topro-3 prior to acquisition and excluded from the analysis.

B 2.2.4 Magnetic cell sorting

Specific cell populations were either sorted or depleted from a heterogenous cell suspension by magnetic cell sorting (MACS; Miltenyi Biotec). Cell populations were labelled with antibody-coupled microbeads (50 µl beads, 450 µl staining buffer per 10^7 cells) and separated on LS MACS-columns in a magnetic field (Miltenyi et al., 1990). The purity of isolated populations was subsequently tested by FACS analysis; MACS-isolated lymphocytes were typically ≥ 90% pure.

Specificity	**Clone**	**Reference and supplier (all USA)**
IgM	R33-24.12	lab-made (Gruetzman, 1981)
IgM	(1B4B1)	eBioscience
IgD	1.3-5	lab-made (Roes et al., 1995)
IgG1	X56	gift from Miltenyi
IgG2a^b	G12-47/30	lab-made (Seemann, 1981)
IgG2b	R14-50	lab-made (Gruetzman, 1981)
IgE	95.3	lab-made (Baniyash and Eshhar, 1984)
Ig	R33-18-10	lab-made (Gruetzman, 1981)
Ig	187.1	Pharmingen
Ig	gam Ig	Southern Biotech
Ig1	L22.18.2	gift from S. Weiss
NP	B1-8µ	lab-made (Reth, 1981)
NP	S43-10	lab-made (Wildner, 1982)
NP	S24/63/63	lab-made (Baumhackel et al., 1982)
NP	N1G9	lab-made (Cumano and Rajewsky, 1985)
NP	D3-13 F1	lab-made (Gause-Pfreundschuh, unpublished)
NP	S8	lab-made (Reth, 1981)
NP	233.1.3	lab-made (Siekevitz et al., 1987)
B220/CD45R	RA3-6B2	lab-made (Coffman, 1982)
CD3	145-211	Pharmingen
CD5	53-7.3	Pharmingen
CD19	1D3	Pharmingen
CD21/CD35	7G6	Pharmingen
CD23	B3B4	Pharmingen
CD24/HSA	M1/69	Pharmingen
CD25 (IL2R)	PC61.5	eBioscience
CD43	S7	Pharmingen
HSA	30F1	lab-made (Ledbetter and Herzenberg, 1979)

Table III. List of antibodies used for flow cytometry.

B 2.2.5 Cytospin and cytoplasmic staining on glass slides

Ex vivo isolated bone marrow cells were resuspended in PBS, 0.5% BSA, 0.05% N_3 at 5 x 10^5 cells/ml. 10^5 cells were centrifuged onto a glass slide at 350 rpm for 4 min in a Cytospin3 centrifuge (Shandon). The slides were subsequently dried at RT. To fix the cell spots, slides were incubated in 95% EtOH/5% glacial acetic acid for 20 min at –20°C. Slides were then washed once with PBS, twice with tap water and once with PBS. For cytoplasmic staining, cell spots were incubated for 20 min at RT in a wet chamber with 10-20 µl of fluorochrome-coupled or biotinylated antibody. Biotin conjugates were visualized using streptavidin coupled to Alexa 350 (Molecular Probes). Slides were washed after each staining step as described above. Before adding the cover slip, 1 drop of Gel/Mount (Biomeda) was added onto the cell spot to prevent fluorochrome fading. Slides were analyzed using an Axiovert 200M fluorescent microscope (Zeiss) and Openlab software (Impro*vision*).

Specificity	**Clone and conjugate**	**Reference and supplier**
IgM	R33-24.12; biotin	lab-made
Ig□	gam Ig□; TRITC*	Southern Biotech
Ig□	gam Ig□; FITC	Southern Biotech

Table IV. List of antibodies used for cytoplasmic stainings of glass slides. * Tetramethyl rhodamine isothiocyanate.

B 2.3 Mouse Experiments

B 2.3.1 Mice and general experimental procedures

C57BL/6 mice were obtained from Charles River. CB20 mice were generated and maintained in our animal facility. All animals were kept under standard conditions in conventional mouse facilities. Mouse experimental procedures and surgery essential for the generation of VJ1i knock-in mice were performed by A. Leinhaas and A. Egert. The general handling and marking of mice was done according to Hogan (Hogan et al., 1987) and Silver (Silver, 1995).

B 2.3.2 Immunizations

Primary T dependent antigen responses were induced with alum-precipitated phycoerythrin (PE) (Cyanotech Corporation). The immunogen was prepared by mixing 1 volume of PE (0.1 mg/ml in PBS) with 1 volume of Imject Alum (Pierce). To allow precipitation of PE in alum, the solution was incubated at RT for 30 min and mice were subsequently immunized by intraperitoneal (i.p.) injection of 200 µl of the precipitate.

B 2.3.3 Serum Analysis by ELISA

Ig serum concentrations were determined by C. Uthoff-Hachenberg and S. Willms via enzyme-linked immunosorbent assays (ELISA, (Kendall et al., 1983)) as described in Roes and Rajewsky (Roes and Rajewsky, 1993). Microtiter plates (Costar) were coated in PBS plus reagent at 4°C o/n and subsequently blocked at RT for 30 min in PBS, 0.5 % BSA, 0.05 % N_3, pH 7.2. Next, serially diluted sera samples were added to the wells and incubated at 37°C for 1 hour. Secondary biotinylated antibody was added for 1 hour at 37°C. Detection of the biotinylated reagent was achieved with SA-conjugated alkaline phosphatase (AP, Roche: 30 min at RT) and p-nitrophenylphosphate as substrate (Roche). Following each incubation step, unbound antibodies or SA-conjugated AP were removed by five washes with tap water. To determine the respective Ig serum concentration, the absorption at 405 nm (OD_{405}) of each sample was measured with an ELISA-photometer (SpectraMax, Molecular Devices) and compared to that of a standard of known concentration.

Coating	**Biotin-Conjugate**	**Specificity**	**Standard**
R33-24.12	gam IgM (SBA)	IgM	B1-8
gam IgG1 (Sigma)	gam IgG1 (SBA)	IgG1	N1G9
G12-47/30	G12-47/30	IgG2a[b]	S43-10
gam R14-50	IgG2b (SBA)	IgG2b	D3-13F1
gam 2E.6	IgG3 (SBA)	IgG3	S24/63/63
gam IgA (Sigma)	gam IgA (SBA)	IgA	233.1.3
187.1	R33-18-10.1		S8
L22.18.2	gam (SBA)		N1G9

Table V. Antibody combinations to determine serum antibody isotypes. For references, see Table III.

B 2.3.4 BrdU incorporation analysis

BrdU labeling and analysis was performed using BrdU Flow Kit (BD PharMingen) according to the manufacturer's instructions. In brief, mice were injected with 1 mg BrdU i.p. and analyzed at the indicated time-points thereafter. Bone marrow single cell suspensions were stained for B220, Ig and Ig1, fixed, DNase treated and subsequently stained for BrdU incorporation.

B 3 RESULTS

B 3.1 Expression of an inserted λ1 light chain is developmentally regulated and independent of Igκ rearrangement

B 3.1.1 Targeted insertion of a pre-rearranged VJλ1 gene segment into the Igκ locus

To generate Igκ-transgenic mice in which the expression of the transgenic λ light chain is regulated by its physiological control elements, I inserted a pre-rearranged VJλ1 gene into the Igκ locus via homologous recombination in murine embryonic stem (ES) cells. A targeting vector was designed to replace 18 kb of genomic DNA spanning from Vλ1 to Jλ1 by a pre-rearranged VJλ1 gene (see Figure 2A and B). This region contains the JCλ3 cluster but no apparent cis-regulatory elements according to DNase hypersensitivity assays in various cell lines (Hagman et al., 1990). A 2.8 kb short arm of homology (SAH) located 5' of Jλ1 was generated in two steps: in order to introduce a NotI site at the distal end of the SAH for linearization of the final vector, phage clone KX39 (covering 15 kb upstream of Vλ1, gift from Ursula Storb) was PCR-amplified using the primer pair 5'NXF1 and 3'NXF-1 (see Table I). The PCR product was cut with NotI and EcoRI to obtain the distal SAH fragment. The proximal SAH fragment, which also contains the pre-rearranged VJλ1 gene, was excised from pA8-6κ (gift from Sigfried Weiss) with EcoRI and AccI. PCR was used to introduce a silent GTC→GTG (codon 36) mutation in framework region 2 of VJλ1, which destroys an AvaII restriction site and thus allows the targeted allele to be distinguished form an endogenous rearrangement. A 5.4 kb AccI/EcoRI fragment located 3' of Jλ1 and excised from cosmid cos-2 (gift from Ursula Storb (Miller et al., 1988)) served as long arm of homology (LAH). A *loxP* flanked ACN cassette containing the *cre*-recombinase gene under the control of the sperm-specific ACE promotor and the neo^R gene driven by the ubiquitously expressed Phosphoglycerate kinase promoter (PGK) (excised with EcoRI and XhoI from pACN (Bunting et al., 1999)) was cloned into an intronic AccI site downstream of Jλ1. The ACN-cassette is deleted in chimeric mice during spermatogenesis. To select against random integration, a thymidine kinase (TK) gene (excised with XhoI and SalI from

pBS-TK (Thomas and Capecchi, 1986)) was inserted 3' of the LAH. The targeting construct was linearized with NotI and transfected into Bruce4 C57BL/6 embryonic stem (ES) cells (Kontgen et al., 1993) as described in chapter 2.2.1. G418- and gancyclovir- resistant ES cell clones were screened for homologous recombination by Southern blot analysis using a BamHI digest and external probe 5'V1 (data not shown). To exclude additional random integration of the targeting vector, correctly targeted ES cell clones were digested with EcoRI and hybridized to internal probe 3'C1. Seven out of 480 double-resistant ES cell clones were homologous integrants. Two of these were injected into CB20 blastocysts and the resulting chimeric males were bred to C57BL/6 females for germline transmission. Figure 2 shows a schematic of the targeting strategy and the verification of both a targeted ES cell clone and its germline transmission with concomitant neo^R deletion by Southern blotting. The mutant Igλ allele (referred to as VJλ1i) mimics the WT Igλ allele after Vλ1λ Jλ1 rearrangement. The VJλ1 gene was chosen since it is the predominant Igλ rearrangement in mice (Sanchez et al., 1996).

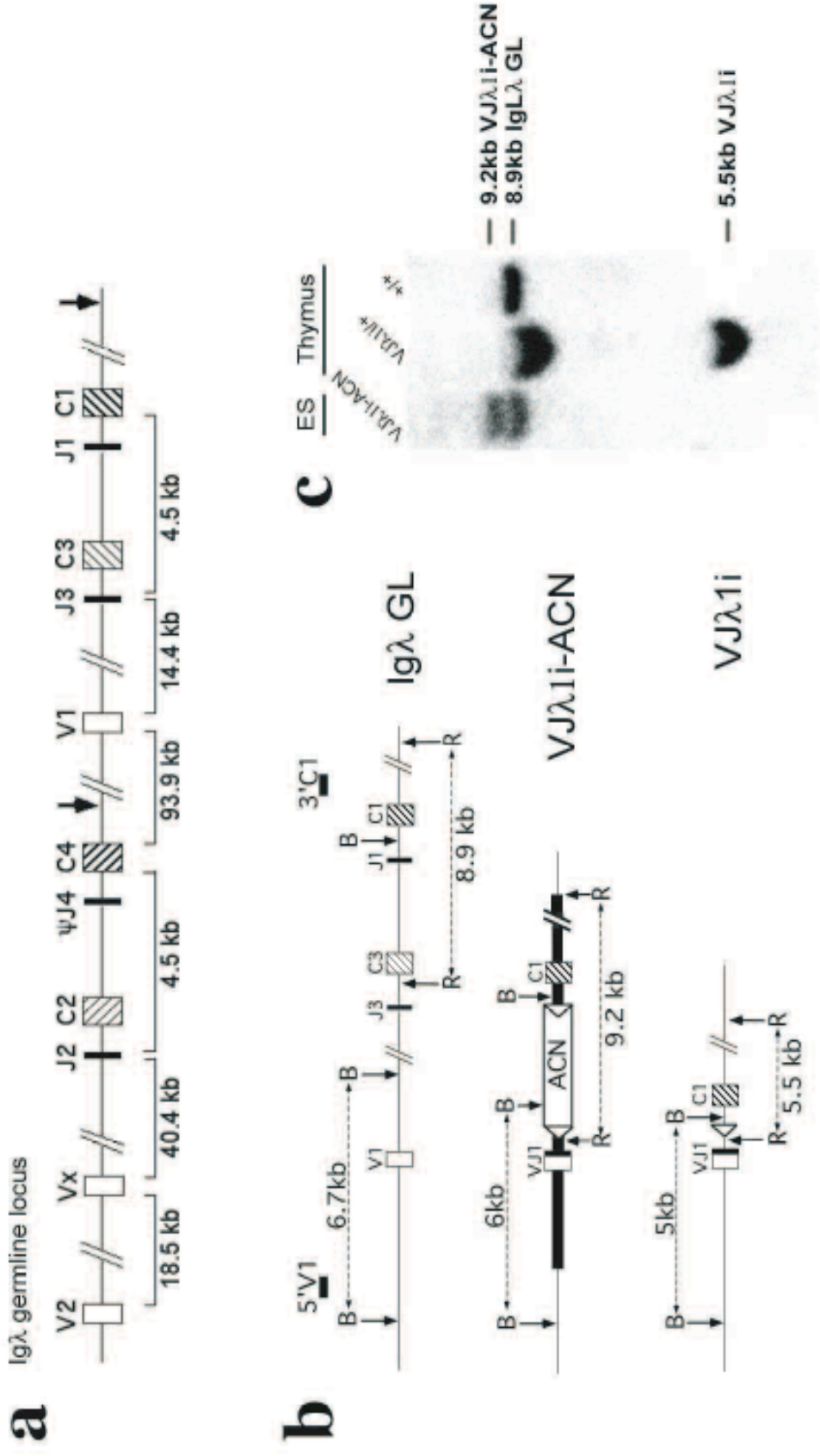

a
Igλ germline locus
V2
Vx
J2 C2
ψJ4 C4
V1
J3 C3
J1 C1
18.5 kb
40.4 kb
4.5 kb
93.9 kb
14.4 kb
4.5 kb
b
5'V1
6.7kb
B
V1
J3
C3
J1
C1
8.9 kb
R
Igλ GL
3'C1
6kb
VJ1
ACN
9.2 kb
VJλ1i-ACN
5kb
5.5 kb
VJλ1i
c
ES
Thymus
VJλ1i-ACN
VJλ1i+
+/+
9.2kb VJλ1i-ACN
8.9kb IgLλ GL
5.5kb VJλ1i

Figure 2: Targeted insertion of a rearranged VJλ1 gene segment into the germline of the Igλ locus. (a) Overview of the genomic organization of the Igλ germline locus. The Igλ locus is composed of three functional Jλ-Cλ clusters (JCλ1-3) and one pseudo Jλ-Cλ cluster (JCλ4). Three Vλ gene segments have been identified; Vλ1, Vλ2 and Vλx (Miller et al., 1988; Storb et al., 1989). Constant region (C) exons are depicted as hatched boxes, V segments as open boxes, J segments as closed boxes. Numbers indicate distances between selected exons in kb (Gerdes and Wabl, 2002). **(b)** Partial restriction endonuclease map of the Igλ germline locus (Igλ GL), the mutated allele after homologous recombination (VJλ1i-ACN) and the mutated allele after Cre-*loxP* mediated deletion of the neo^R containing ACN cassette (VJλ1i). Arms of homology are shown in bold in VJλ1i-ACN. V, J and C region gene segments are indicated as described in (a), *loxP* sites are shown as open triangles. Double headed arrows and associated numbers depict the indicative restriction fragments and their respective sizes as revealed by either an external probe (5'V1) or an internal probe (3'C1). B, BamHI; R, EcoRI. **(c)** Southern blot analysis of one injected ES cell clone (VJλ1-ACN), a heterozygous mouse mutant (VJλ1i/+) and a wild-type littermate (+/+). ES cell or thymic genomic DNA was digested with EcoRI and hybridized with 3'C1.

B 3.1.2 All mature B cells of VJλ1i mice express the inserted λ1 light chain

The inserted λ1 light chain is expressed in all mature B cells of VJλ1i mice (Figure 3) and the distribution of peripheral B cell subsets appeared normal (data not shown). Ig serum titers, except for IgA, were comparable to WT controls (Figure 4A), and the majority of serum Ig is $\lambda1^+$ (Figure 4B) indicating that $\lambda1^+$ B cells contribute normally to serum IgM and serum IgG. Since IgA is predominantly generated in gut-associated lymphoid organs (reviewed in (Fagarasan and Honjo, 2003)), the increase in IgA serum titers may reflect preferential activation of $\lambda1^+$ B cells in response to gut derived pathogens. The total number of splenic B cells in VJλ1i mice was reduced by 35% when compared to WT mice ($2.3 \times 10^7 \pm 0.6 \times 10^7$ and $3.5 \times 10^7 \pm 1.0 \times 10^7$ B cells, respectively). A similar reduction in B cell numbers has been reported for mice that carry an inserted VJλ gene (Pelanda et al., 1996) and may reflect the restricted B cell repertoire in mice that predominantly express one particular light chain. The majority of splenic B cells in VJλ1i mice express λ1 exclusively. However, a substantial fraction of B cells (~30%) express both λ1 and κ on the surface and less than 5% appear to have lost surface expression of λ1 (Figure 3A). Due to the organization of

the Ig locus, the VJ1 gene cannot be deleted by "secondary" V J rearrangements (see Figure 2A). B cells that lack surface 1 expression may represent naive B cells with inefficient heavy/1 light chain pairing or memory B cells that have inactivated the VJ1 coding region through somatic hypermutation (see chapter 3.2.2). A semi-quantitative RT-PCR analysis of sorted $\lambda1^+$, $\kappa/\lambda1^+$ and κ^+ splenic B cells from VJ1i mice confirmed that the inserted gene segment is transcribed at similar levels in both surface 1-positive and -negative subpopulations (Figure 3B).

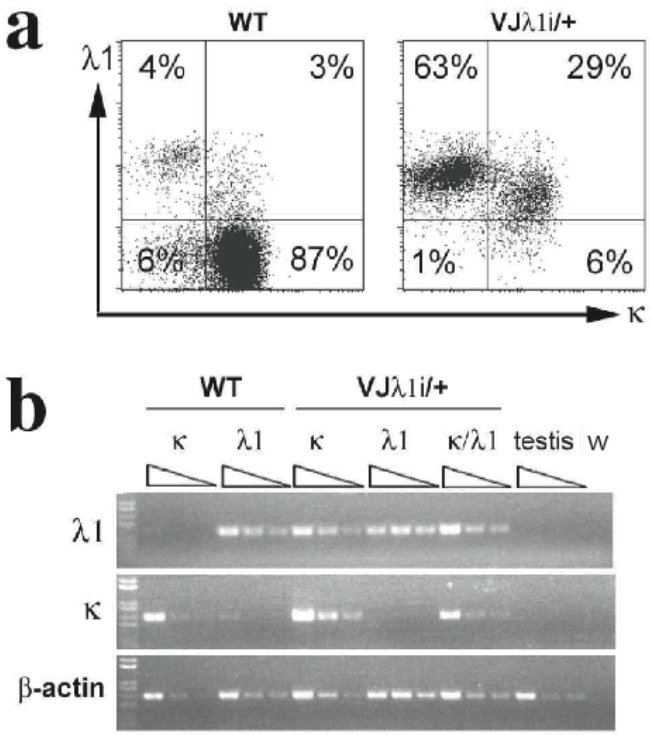


Figure 3: All mature B cells of VJ1i/+ mice express the inserted 1 light chain gene. (a) Representative staining for and 1 on $CD19^+$ splenocytes from VJ1i/+ and WT mice. Numbers indicate the percentage of cells per quadrant. **(b)** Semi-quantitative RT-PCR analysis of sorted κ^+, $\lambda1^+$ and $\kappa/\lambda1^+$ splenic B cells from WT mice and VJ1i/+ mice. Testis RNA from WT mice served as negative control. Serially diluted cDNA was analyzed for reverse-transcribed 1- and -LC message by PCR. A -actin PCR was performed as internal control.

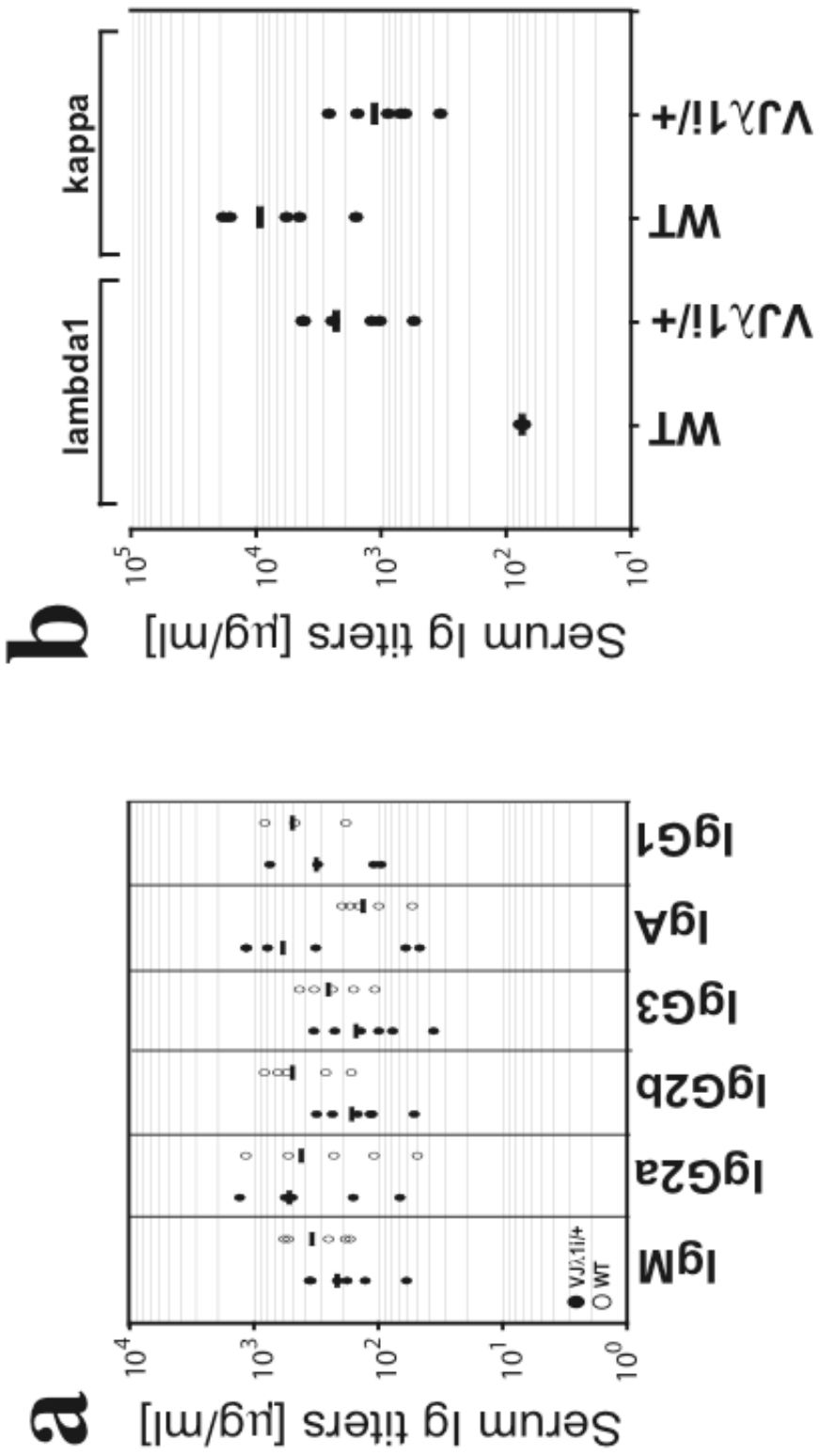


Figure 4. Serum Ig titers in VJ1i/+ mice. (a) Concentrations of indicated Ig isotypes from adult VJ1i/+ mice (closed circles) and WT controls (open circles). **(b)** Concentrations of total Ig and Ig1 in adult VJ1i/+ and WT mice.

B 3.1.3 Expression of a pre-rearranged λ1 light chain is developmentally controlled like that of a pre-rearranged κ light chain

I next assessed whether the pre-rearranged VJλ1 gene is expressed in a developmentally regulated fashion. In WT mice, IgL rearrangements occur mainly in pre B cells and functional λ light chains can be detected in ~20% of pre B cells (Pelanda et al., 1996). However, it has been demonstrated that IgL rearrangements can occur in a small fraction of pro B cells independently of IgH rearrangements (Ehlich et al., 1993; Novobrantseva et al., 1999). To detect light chain expression in WT and IgL insertion mice, I performed intracellular stainings for Igκ or Igλ light chains in pro and pre B cells. The light chain expression pattern in WT mice is in accordance with published results (Figure 4 and (Pelanda et al., 1996)) and shows fourfold less λ^+ pro than pre B cells. VJλ1i mice show a similar, developmentally regulated expression pattern for the inserted light chain with three- to fourfold less λ^+ pro than pre B cells (Figure 5). This expression pattern is comparable to that of an inserted κ light chain in mice that carry a pre-rearranged VJκ gene and either retain (in the case of the D23κi allele (Novobrantseva, 2000)) or lack (in the case of the LN1κ allele (Hochedlinger and Jaenisch, 2002)) the genomic sequence between Vκ and Jκ (Figure 5). The fractions of pro and pre B cells that express the inserted light chain in either VJκi or VJλ1i mice are 4-5 fold increased when compared to λ^+ pro and pre B cells in WT mice. Since only one third of newly formed rearrangements in WT B cell progenitors is expected to be productive, the fractions of pro and pre B cells that undergo IgL rearrangement in WT mice correspond approximately to the fractions of pro and pre B cells that express the pre-rearranged light chain in IgL insertion mice. I therefore conclude that transcription of inserted κ and λ1 light chain genes is regulated similarly and coincides developmentally with the initiation of Igκ rearrangements in WT mice.

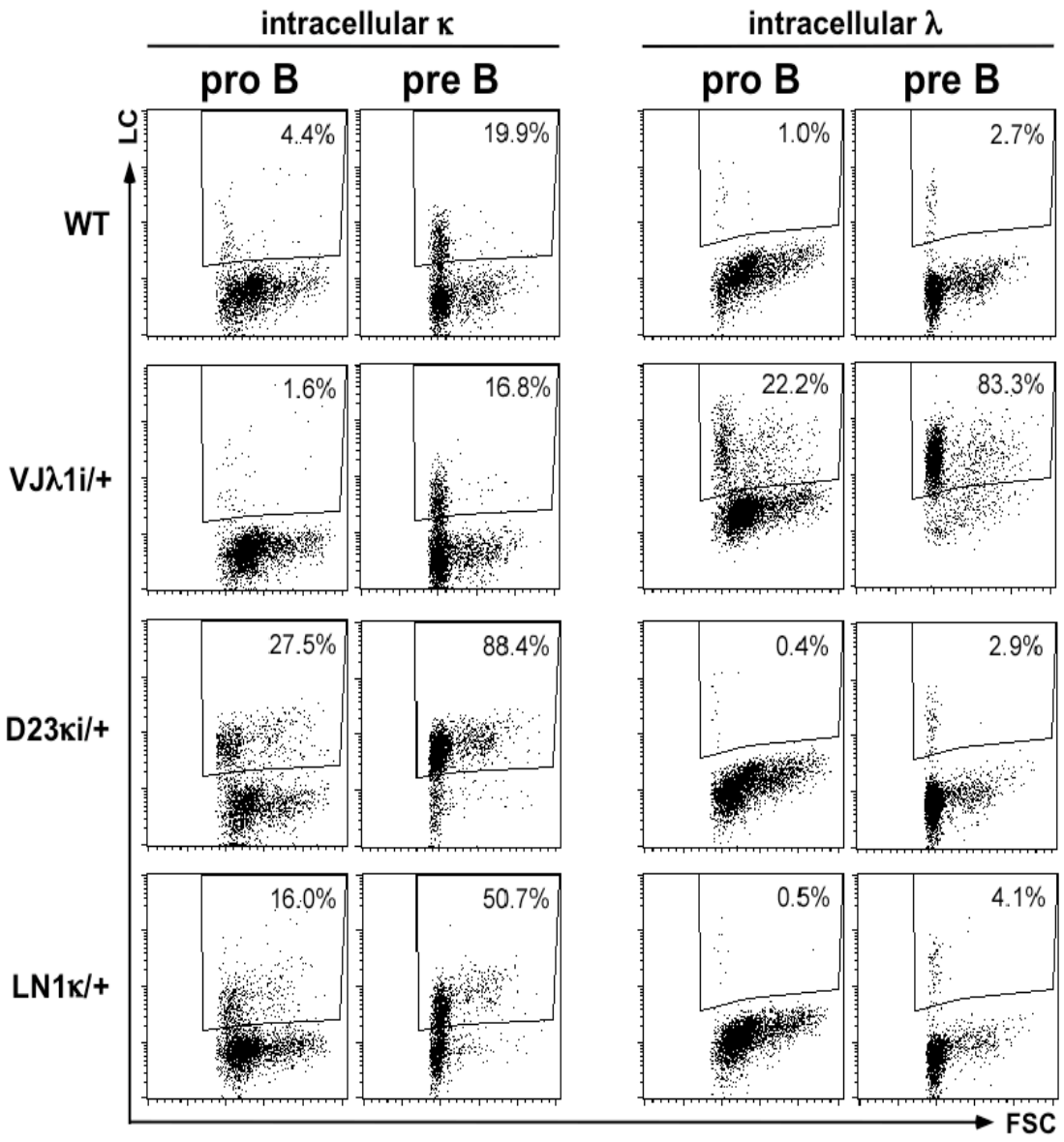


Figure 5: Intracellular light chain expression in CD25$^+$ pro B cells and CD25$^-$ pre B cells of light chain insertion and WT mice. Bone marrow lymphocytes were stained for surface expression of B220, IgM and CD25 and for intracellular light chain expression. Flow cyometric analyses of intracellular κ and λ expression in CD25$^-$, B220$^+$, IgM$^-$ pro B cells and CD25$^+$, B220$^+$, IgM$^-$ pre B cells are shown for WT, VJλ1i, D23κi and LN1κ mice. Light chain expression is plotted against cell size (Forward Scatter, FSC). Numbers indicate the percentage of light chain expressing cells. Similar results were obtained in three or more independent experiments.

B 3.1.4 The majority of mature B cells in VJκ1i mice carries the Igκ locus in germline configuration

It has been shown previously, that a pre-rearranged VJκ gene efficiently drives pre B cell to immature B cell differentiation without allowing Igκ rearrangements to occur (Pelanda et al., 1997). Simultaneous initiation of transcription of pre-rearranged VJκ and VJκ1 genes suggests that the same phenomenon may occur in VJκ1i mice. To determine the extent of recombination at the Igκ locus in B cells from VJκ1i mice, κ/λ1$^+$ and λ1$^+$ B cells were sorted and analyzed for Vκκ Jκ rearrangements and RS recombination by Southern blotting. Thymocytes served as negative control. To detect RS recombination, DNA was digested with EcoRI and hybridized to RS-probe (Durdik et al., 1984) resulting in a 5.8 kb RS-germline fragment which is lost upon RS recombination. To detect Vκκ Jκ rearrangements, DNA was digested with EcoICRI and hybridized to 5'Jκ-probe (Pelanda et al., 1996) giving rise to a 4.5 kb κ-germline fragment. Depending on the orientation of the Vκ segment, Igκ-rearrangements lead to deletion or inversion of the DNA between Vκ and Jκ. In both cases, the characteristic 4.5 kb fragment is lost. To control for DNA loading, blots were stripped and rehybridized with an IL-4 gene specific probe (Kühn et al., 1991) yielding a 10 kb fragment for the EcoRI digest and a 4.8 kb fragment for the EcoICRI digest. Individual samples were assayed for the retention of a germline EcoIRCI fragment spanning the Jκ region and for the retention of a germline EcoRI fragment spanning the RS region (Figure 6).

While the majority of λ1$^+$ B cells from WT mice were shown to carry two rearranged Igκ alleles (Coleclough et al., 1981; Takeda et al., 1993), more than 75% of λ1$^+$ B cells from VJκ1i mice retain the Igκ locus in germline configuration (Figure 6B). RS recombination is not detectable above background in VJκ1i mice. In λ1$^+$ B cells of WT mice, on the other hand, more than 60% of Igκ alleles have undergone RS recombination (Figure 6C), which is in accordance with published results (Coleclough et al., 1981). Together, these data imply that in VJκ1i mice, the majority of pre B cells express the inserted λ1 light chain gene and subsequently enter the immature B cell compartment before endogenous Igκ rearrangements have occurred.

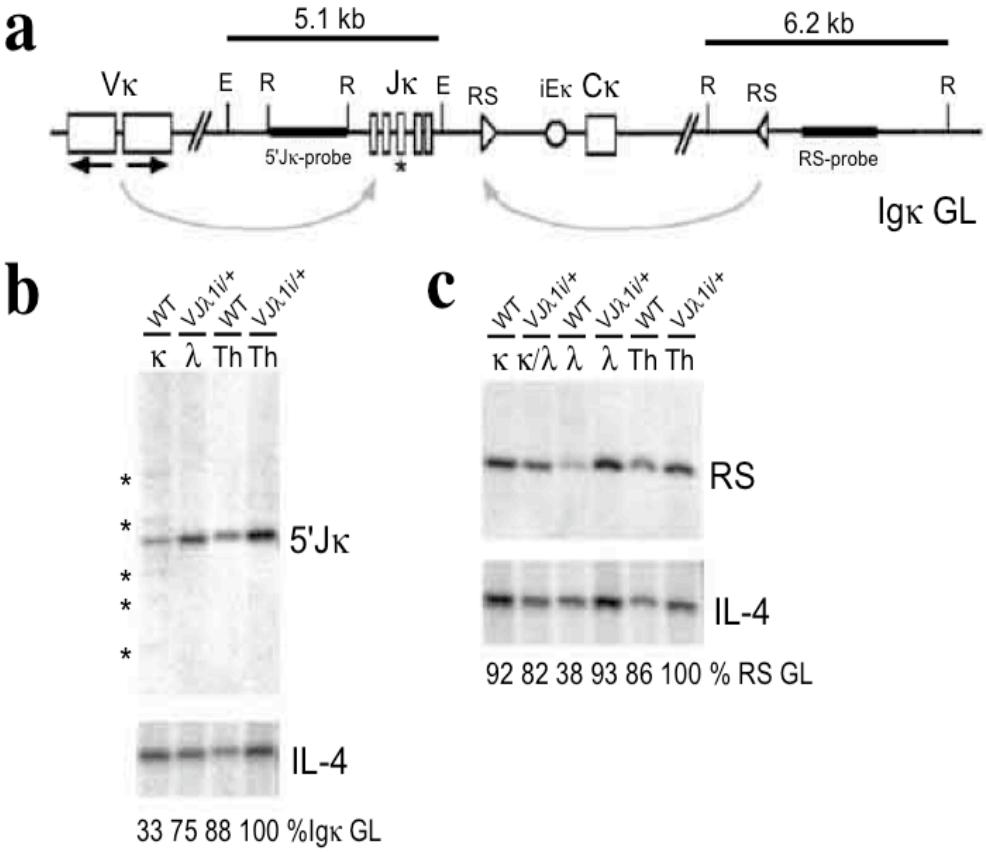


Figure 6: Only low levels of Ig gene rearrangements occur in 1$^+$ B cells of VJ1i/+ mice. (a) Partial restriction endonuclease map of the germline Ig locus (not to scale). V, J and C gene segments, the 5'J probe and the RS-probe are shown as boxes, the asterisk indicates a pseudo-J segment. The internal enhancer (iE) is shown as open circle, triangles depict RS recombination sites. The arrows below the V gene segments indicate their transcriptional orientation. The sizes of the germline EcoIRCI fragment and the germline EcoRI fragment are indicated as revealed by probes 5'J and RS, respectively. E: EcoIRCI; R: EcoRI. **(b)** and **(c)** Genomic DNA of sorted $^+$, /1$^+$ and 1$^+$, CD19$^+$ B cells from VJ1i/+ and WT mice was analyzed by Southern blotting. To detect V J rearrangements, DNA was digested with EcoIRCI and hybridized to 5'J probe (b). RS-rearrangements were analyzed using an EcoRI digest and RS probe (c). Re-hybridization with an IL-4 gene-specific probe served as internal control. The percentage of cells retaining either the Ig germline- or the RS germline-fragment is shown for each lane. Asterisks indicate fragments that originate from VJ recombination by inversion.

B 3.1.5 Co-expression of κ and λ1 light chains in VJλ1i mice is likely to be the consequence of receptor editing

The appearance of κ/λ1$^+$ mature B cells in VJλ1i mice indicates that a fraction of λ1$^+$ B cells has undergone Igκ rearrangements and has thus escaped isotype exclusion. Given the findings described above, this can only be explained by Igκ rearrangements occurring in a subpopulation of pro B cells (Ehlich et al., 1993; Novobrantseva et al., 1999) or by secondary Igκ rearrangements in a fraction of λ1$^+$ pre B cells. In VJκi mice, pre B cells that undergo secondary light chain rearrangements were shown to take longer to exit the pre B cell compartment than pre B cells that express the pre-rearranged light chain gene (Casellas et al., 2001). We thus compared the kinetics of pre B to immature B cell transition for λ1$^+$ and κ/λ1$^+$ B cells from VJλ1i mice. WT pre B cells were analyzed in parallel as a control for cells that undergo IgL rearrangements. Large, cycling pre B cells were pulsed with BrdU *in vivo* and the fraction of BrdU$^+$ immature B cells was determined at different time-points thereafter. Immature B cells were subdivided according to light chain expression (Figure 7A). Figure 7B shows a comparison of BrdU-incorporation kinetics in WT and VJλ1i mice. Two conclusions can be drawn from this analysis. First, in VJλ1i mice, κ/λ1$^+$ B cells exit the pre B cell compartment ~12h later than B cells that express only λ1 and thus appear to be the consequence of secondary Igκ rearrangements. Second, WT B cells exit the pre B cell compartment with kinetics similar to κ/λ1$^+$ B cells from VJλ1i mice. The delay with respect to B cells that carry an inserted light chain is likely to reflect the kinetics of Igκ rearrangements in WT mice. The accelerated pre B cell to immature B cell differentiation of cells expressing an inserted light chain has also been observed in VJκi mice (Casellas et al., 2001) and no major differences were observed in the frequency of BrdU$^+$ immature B cells of either VJκi or Vλ1i mice (Figure 7C). This result further corroborates the conclusion that expression of pre-rearranged κ and λ1 light chain genes is initiated simultaneously in pre B cells.

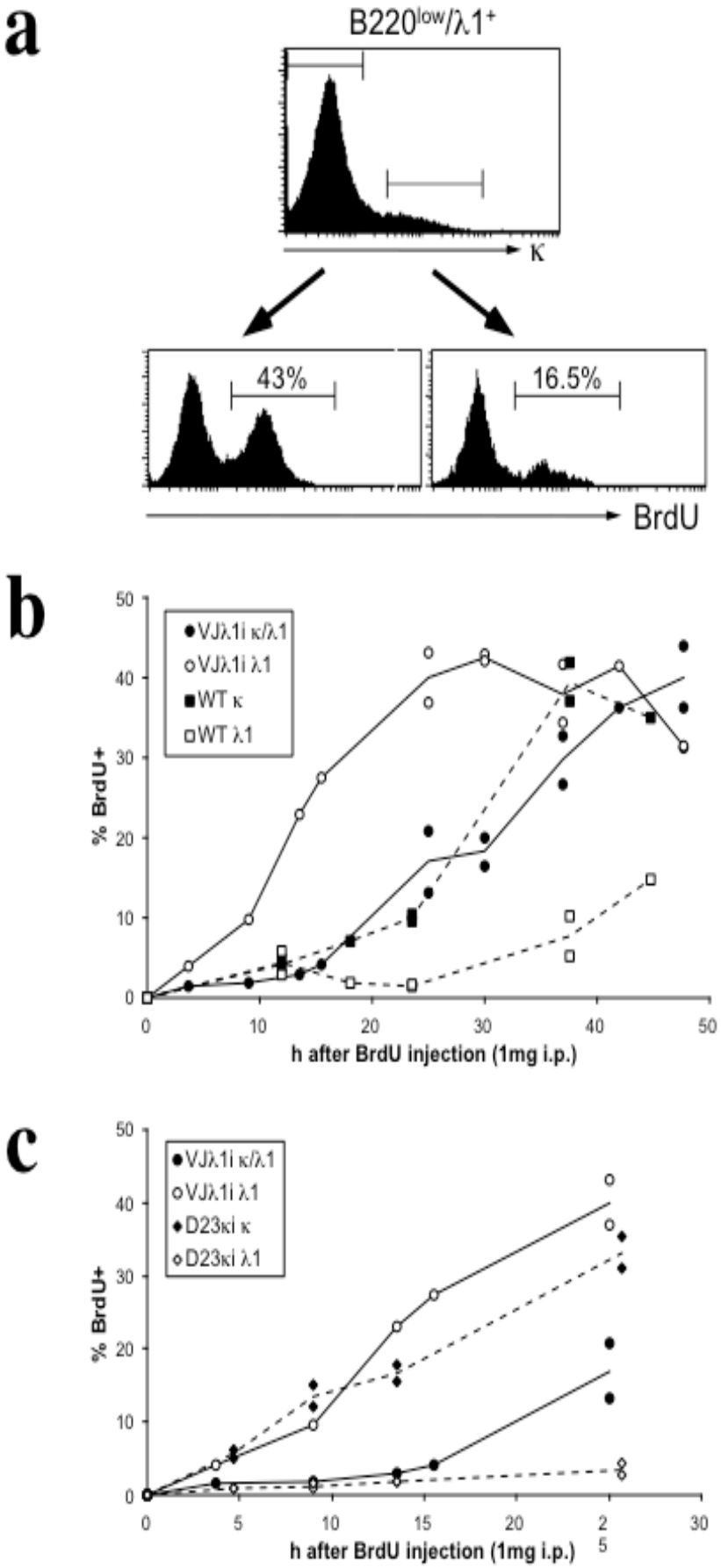
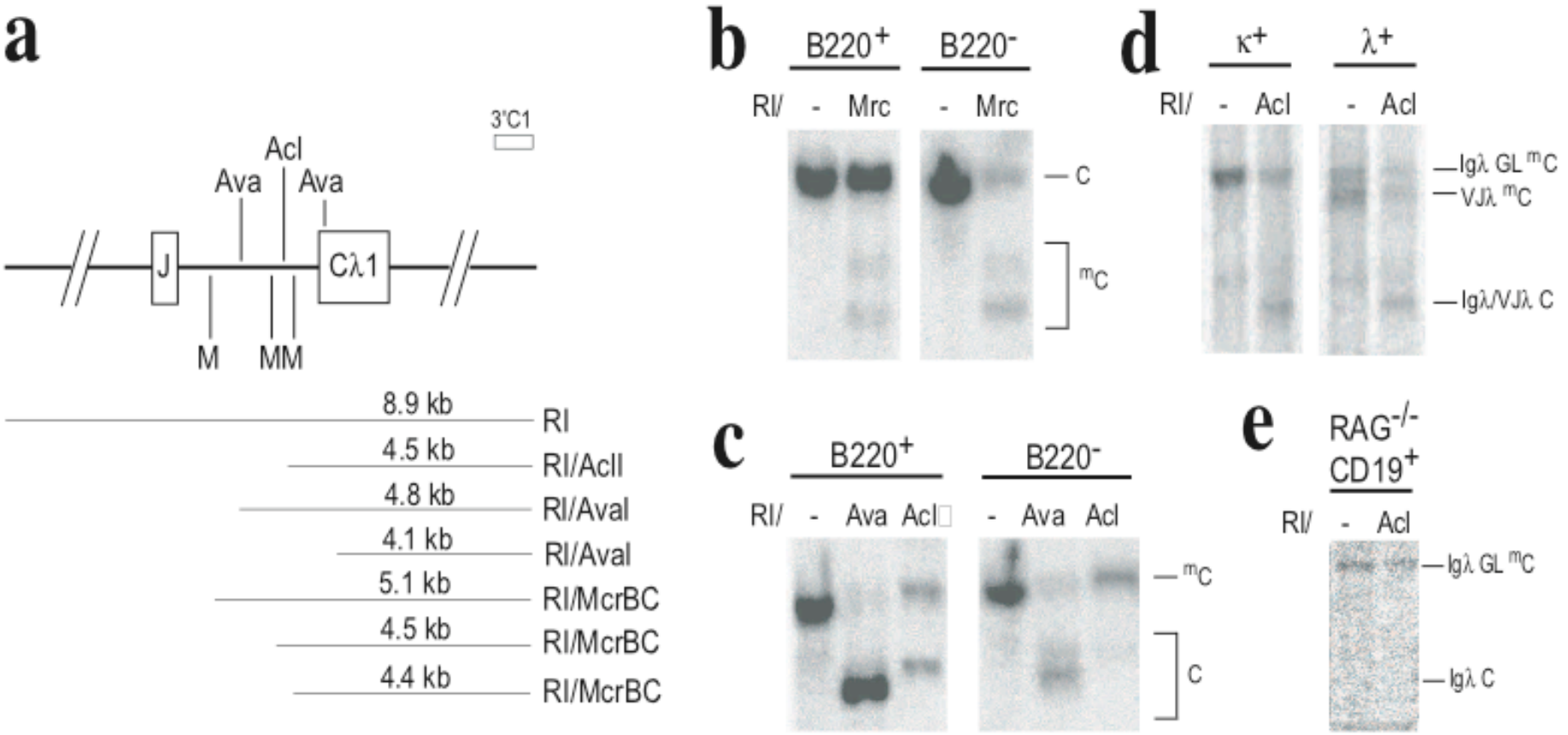

a
B220low/λ1+
κ
43%
16.5%
BrdU
b
VJλ1i κ/λ1
VJλ1i λ1
WT κ
WT λ1
% BrdU+
h after BrdU injection (1mg i.p.)
c
VJλ1i κ/λ1
VJλ1i λ1
D23κi κ
D23κi λ1
% BrdU+
h after BrdU injection (1mg i.p.)

Figure 7. BrdU incorporation in immature B cells. Mice were i.p. injected with BrdU and analyzed at different timepoints thereafter. Immature B cells were defined as $B220^{low}/\lambda1^+$ and were subdivided according to Igλ expression. **(a)** Representative histograms from a mouse analyzed 30h after BrdU injection. The percentage of $BrdU^+$ cells in λ^- (left) and λ^+ (right) B cell subpopulations was determined as shown. **(b)** and **(c).** The percentage of $BrdU^+$ immature B cells is plotted against the time after BrdU injection. VJλ1i/+ mice (circles) were compared to either WT mice (squares) (b) or D23λi/+ mice (diamonds) (c). Open symbols correspond to $\lambda1^+$ immature B cells, closed symbols depict λ^+ immature B cells in WT and D23λi/+ mice or $\lambda/\lambda1^+$ immature B cells in VJλ1i/+ mice. Each pair of symbols represents one animal.

B 3.1.6 DNA methylation in the Jλ1-Cλ1 intron is developmentally regulated but not required for Igλ rearrangement

Despite the developmentally regulated expression of the inserted VJλ1 gene, I cannot rule out that accessibility for transcription and/or rearrangement differs between a pre-rearranged and an unrearranged Igλ locus. One way to address chromatin accessibility of a particular genetic locus is the analysis of its DNA methylation pattern. It has been previously shown that demethylation of the Jλ region developmentally coincides with VλJλ rearrangement, and Igλ rearrangements are confined to the demethylated allele. Demethylation of the Jλ region is acquired during the pre B cell stage and appears to persist in mature B cells (Mostoslavsky et al., 1998). Here, I analyzed the methylation status of the Jλ1 region in the Igλ locus in mature wild-type B cells using three methylation-sensitive restriction enzymes: two of these recognize their DNA target sequence only if it is unmethylated (AvaI, AclI), whereas the third one requires DNA methylation in order to cut (MrcBCI) (Figure 8A). DNA samples from sorted B and T cells were digested with EcoRI in the presence or absence of the methylation-sensitive enzyme and subsequently analyzed by Southern blotting using probe 3'C1 (see Figure 2). The EcoRI digest distinguishes the rearranged VJλ1 allele from the germline Igλ allele. The former yields a 7.5 kb fragment, the latter an 8.9 kb fragment. AvaI, AclI and MrcBCI cut both the germline Jλ1-Cλ1 fragment and the rearranged VJλ1 fragment into sub-fragments that are indistinguishable when hybridized to probe 3'C1. The resulting fragment sizes are shown in Figure 6A. More than 50% of Igλ alleles are demethylated in splenic B cells and considerably less or no detectable demethylation is found in T cells, indicating that demethylation of the

J□1-C□1 region is specific for B cells (Figure 8B, C). However, this process is not necessarily linked to Ig□ rearrangement, since the majority of splenic B cells carry germline Ig□ loci. Indeed, in sorted □1^{+} splenic B cells, AclI-demethylation can be detected to similar extents (~50%) on both the unrearranged and the rearranged Ig□ allele (Figure 8D). Therefore, AclI-demethylation is not required for Ig□ rearrangement. To determine whether AclI-demethylation is developmentally regulated, I analyzed sorted CD19^{+} pro B cells that were derived from the bone marrow of RAG1$^{-/-}$ mice. RAG1$^{-/-}$ mice cannot rearrange their Ig loci and B cell development is arrested at the pro B cell stage resulting in a considerable increase in pro B cell numbers (Mombaerts et al., 1992). No AclI-demethylation could be detected in RAG1$^{-/-}$ pro B cells (Figure 8E). Thus, the J□1 region appears to be demethylated in a developmentally regulated manner only after the pro B cell stage. Taken together, these results imply that J□1-C□1 demethylation reflects a B cell specific change in chromatin accessibility of this part of the Ig□ locus, which is, however, not required for Ig□ rearrangement.


a
3'C1
AclI
Ava
Ava
J
Cλ1
M
MM
8.9 kb
RI
4.5 kb
RI/AclI
4.8 kb
RI/AvaI
4.1 kb
RI/AvaI
5.1 kb
RI/McrBC
4.5 kb
RI/McrBC
4.4 kb
RI/McrBC
b
B220+
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RI/ - Mrc - Mrc
C
mC
c
B220+
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RI/ - Ava AclI - Ava AclI
mC
C
d
κ+
λ+
RI/ - AclI - AclI
Igλ GL mC
VJλ mC
Igλ/VJλ C
e
RAG-/-
CD19+
RI/ - AclI
Igλ GL mC
Igλ C

Figure 8. Demethylation of Igκ locus is B cell specific and developmentally regulated. (a) Partial restriction endonuclease map of the Igκ germline EcoRI fragment spanning the Jκ1-Cκ1 region. Boxes depict exons and Southern probe 3'C1. Acl, AclI; Ava, AvaI; M, MrcBCI. Fragment sizes as detected with probe 3'C1 are indicated for each restriction site. (b) and (c) Southern blot analysis of $B220^{+}$ and $B220^{-}$ splenocytes from adult mice. Genomic DNA was digested with EcoRI (RI) +/- methylation sensitive restriction enzymes MrcBCI (Mrc), AvaI (Ava) or AclI (Acl). C, fragment corresponding to demethylated restriction sites; ^{m}C, fragments corresponding to methylated restriction sites. (d) Southern blot analysis of sorted κ^{+} and $\kappa1^{+}$ splenic B cells from adult mice. Genomic DNA was digested as indicated. Fragments corresponding to the methylated germline Igκ locus (Igκ GL ^{m}C), the methylated Igκ locus upon Vκ1_Jκ1 rearrangement (VJκ ^{m}C) or the unmethylated Igκ locus (Igκ/VJκ C) are indicated. In the latter case, the unrearranged and the rearranged Igκ alleles cannot be distinguished. (e) Southern blot analysis of sorted $CD19^{+}$ bone marrow B cells from a pool of 4 $RAG1^{-/-}$ mice. Genomic DNA was digested as indicated, fragments are labeled as in (d). This result is representative of two independent experiments.

B 3.2 IgL double producers contribute normally to mature B cell subsets and are counter-selected upon BCR-ligand interaction

B 3.2.1 Contribution of κ/λ1$^+$ B cells to peripheral B cell subsets

In WT mice, allelic and isotype exclusion ensure the generation of monospecific B cells and B cells that express two distinct light chains are therefore rarely found. Here, we generated a system, where κ/λ1 double-producing immature B cells are readily selected into the peripheral B cell pool and represent ~30% of mature B cells. To determine the fate of κ/λ1$^+$ B cells in VJλ1i mice, we analyzed the distribution of κ/λ1$^+$ B cells in different B cell subsets. In the bone marrow, IgM$^+$ cells can be subdivided into immature B cells (B220^{low}, IgM$^+$) and mature, recirculating B cells (B220^{high}, IgM$^+$) and the fraction of κ/λ1$^+$ cells is slightly lower in the former (19% and 24%, respectively). The fact that κ/λ1$^+$ B cells represent ~35% of total B cells in the spleen indicates positive selection of κ/λ1-expressing immature B cells (Figure 9A) into the peripheral B cell pool, but preferential homing of mature λ1-single positive B cells back to the bone marrow. While the former phenomenon may reflect selection for a broader BCR repertoire, the reasons for the latter observation are currently unknown.

In the spleen, CD19$^+$ B cells were subdivided according to the expression of CD21 and CD23 into follicular (FO) B cells (CD21^{low}, CD23$^+$) and MZ B cells (CD21^{high}, CD23^{low}). Both subsets harbor a similar fraction of κ/λ1$^+$ B cells (~35%, Figure 9B). In the peritoneum, the fraction of κ/λ1$^+$ cells ranged from 20% to 40% between individual mice but was comparable between B1 cells (CD19^{high}, CD23$^-$) and B2 cells (CD19$^+$, CD23$^+$). Interestingly, λ1 expression levels were greatly reduced in κ/λ1$^+$ peritoneal B cells when compared to λ1$^+$ peritoneal B cells or splenic κ/λ1$^+$ B cells and this reduction could be observed in both B1 and B2 cells (Figure 9C). This result suggests that expression of two distinct BCR specificities interferes with homing and/or selection of B cells into the peritoneal cavity.

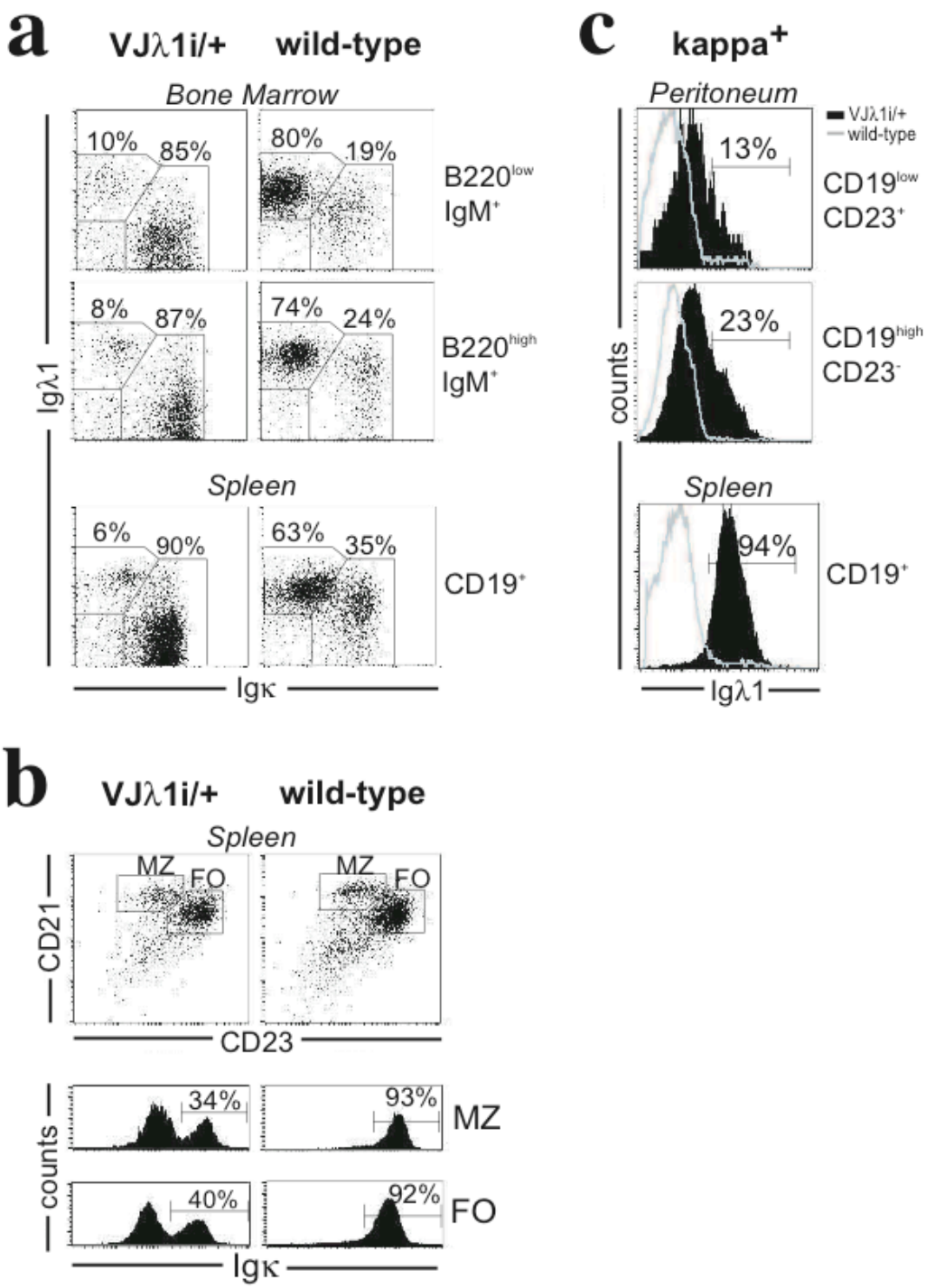


Figure 9. Distribution of □/□1$^+$ cells in peripheral B cell subsets. (a) IgL expression pattern in immature (B220^{low}/IgM$^+$) and mature (B220^{high}/IgM$^+$) bone marrow B cells and CD19$^+$ splenocytes from VJ□1i/+ and WT mice. **(b)** CD19$^+$ splenocytes from VJ□1i/+ and WT mice were subdivided into MZ and FO B cells according to CD21/CD23 expression as indicated. Histograms show the fraction of □$^+$ B cells in each subset. **(c)** Ig□ expression levels in □$^+$ peritoneal B cells. B2 cells were identified as CD19^{low}/CD23$^+$, B1 cells as CD19^{high}/CD23$^-$. Percentages indicate the fraction of cells that express Ig□ at levels comparable to CD19$^+$, □$^+$ splenocytes.

B 3.2.2 Counter-selection of □/□1$^+$ B cells in memory B cells

Given the selective pressure during affinity maturation of memory B cells, B cells with dual receptor specificity are expected to have a selective disadvantage. To test this hypothesis, VJ□1i/+ mice were immunized with phycoerythrin (PE) in adjuvant. PE is predominantly recognized by □$^+$ B cells and therefore allows one to determine the fate of □/□1$^+$ B cells in a TD immune response. IgG1$^+$ memory B cells were sorted 4 weeks after immunization according to their ability to bind PE. The majority of PE$^+$ memory B cells did not or only weakly express □1 on the cell surface whereas PE$^-$, IgG1$^+$ cells were predominantly □1$^+$ (data not shown). Based on this result, I analyzed the VJ□1 gene for mutations that would inactivate □1 expression as a consequence of somatic hypermutation. DNA from sorted cell populations was PCR amplified using the primer pair VJ□1-ext and LAMINFOR (see Table I) and subjected to sequencing. While no deleterious mutations could be detected in PE$^-$, IgG1$^+$ B cells, 8 out of 18 mutated VJ□1 sequences in PE$^+$, IgG1$^+$ B cells carried either frameshift mutations or mutations that introduced pre-mature STOP codons (Table VI). This result suggests that □/□1$^+$ B cells that have inactivated one IgL allele through somatic hypermutation during the germinal center reaction are preferentially selected into the memory compartment.

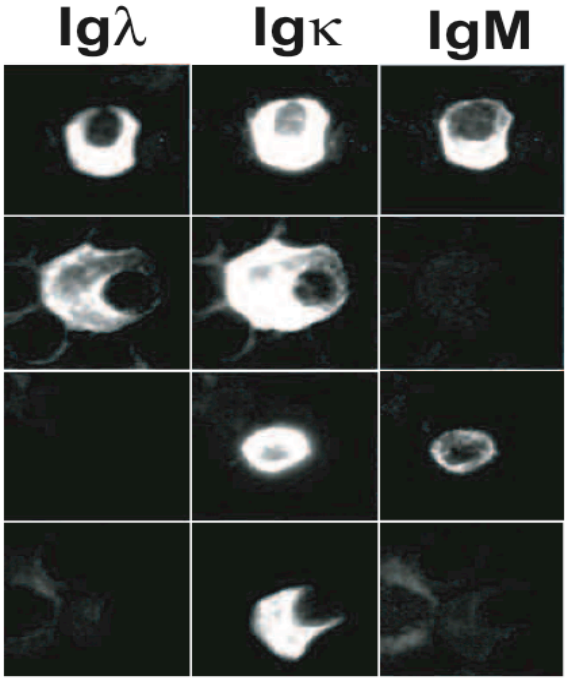
genotype	mutated sequences/ total sequences	mutations/ mutated sequences	STOP codons	Frameshift
PE$^-$/□1$^+$	11/28	18bp/4370bp (0.4%)	0	0
PE$^+$/□1$^-$	18/31	81bp/7315bp (1.1%)	5	3 (1 STOP)

Table VI. Frequency of VJ□1 gene inactivation in hypermutated IgG1$^+$ B cells**.**

B 3.2.3 Reduction of □/□1$^+$ B cells in class-switched bone marrow plasma cells

A subfraction of antigen-selected memory B cells terminally differentiates into long-lived plasma cells and homes to the bone marrow. Counter-selection of □/□1$^+$ memory B cells should therefore extend to this specialized antibody-forming B cell subset. IgM$^+$ and IgM$^-$ bone marrow plasma cells from a VJ□1i/+ mouse were analyzed for their light chain expression pattern by immuno-histochemistry. While class-switched

IgM$^-$ bone marrow plasma cells likely harbor a sizeable fraction of memory B cell derived plasma cells, IgM$^+$ plasma cells are thought to originate mainly from extrafollicular immune responses. The latter can be activated in a polyclonal fashion and are not necessarily antigen-selected. Consistent with this notion, the fraction of $\lambda/\kappa1^+$ IgM$^-$ plasma cells is reduced by approximately one third when compared to mature splenic B cells, whereas only a slight reduction of $\lambda/\kappa1^+$ cells is found in the IgM$^+$ subset (Figure 10). Interestingly, both IgM$^+$ and IgM$^-$ subsets show a dramatic increase in λ^+ plasma cells when compared to splenic mature B cells (30% and ~5%, respectively). This may reflect inactivation of the VJκ1i gene by somatic hypermutation (see above) or inefficient IgH/Igκ1 pairing. Taken together, these results support the idea that B cells with dual receptor specificity are counter-selected upon antigenic selection. However, single BCR specificity is not a requirement for plasma cell generation since $\lambda/\kappa1^+$ plasma cells are readily detected.



	Igλ	Igκ	Igκ/Igλ
IgM$^+$ (n=48)	35%	33%	31%
IgM$^-$ (n=86)	50%	28%	22%

Figure 10. IgL expression in bone marrow plasma cells. Bone marrow plasma cells were transferred onto glass slides by cytospin and subsequently stained for IgM, Igκ and Igλ. The fraction of IgM$^+$ and IgM$^-$ plasma cells that express either one or both IgL chain is shown.

B 4 DISCUSSION

B 4.1 Transcription of an inserted VJ□1 element is developmentally controlled and independent of the VJ□ rearrangements

The predominance of Ig□ over Ig□ rearrangements in mice and humans has been subject of extensive research over the last decades. There is suggestive evidence that in B cell development, Ig□ may become accessible for V(D)J recombination later than Ig□ (Coleclough et al., 1981; Engel et al., 1999; Hieter et al., 1981). More specifically, the analysis of targeted mutations in the Ig□ locus suggested the existence of a negative regulatory signal that originates from an unrearranged Ig□ locus and suppresses Ig□ gene rearrangements (Chen et al., 1993; Zou et al., 1993). Based on evidence that Ig gene rearrangement correlates and is possibly mechanistically connected with transcriptional accessibility of the target genes (Casellas et al., 2002; Sun and Storb, 2001), I sought to test this hypothesis through the analysis of the developmental expression pattern of a VJ□1 rearrangement inserted into its physiological position in the Ig□ locus. The results of this analysis were clear-cut: expression of the inserted VJ□1 element is developmentally controlled and coincides with the developmental stage at which V□ and J□ gene segments are rearranged and VJ□-rearrangements are expressed (Figure 4); and VJ□1 expression does not depend upon Ig□ rearrangement (Figure 5). Thus, at the level of expression of a gene rearrangement in the Ig□ locus there is no evidence for sequential accessibility of Ig□ and Ig□ and a signal originating from a non-rearranged Ig□ locus that interferes with the transcription of a rearranged Ig□ locus can be excluded. This in turn restricts a possible developmental program of successive accessibility of Ig□ and Ig□ loci to the control of the initiation of IgL gene rearrangements. Such a developmental program would further have to assume differential accessibility of rearranged versus non-rearranged Ig□ loci during B cell development, which could be due to juxtaposition of promoter and enhancer elements and/or the loss of cis-regulatory elements upon V□_J□ recombination. Although DNase-hypersensitive sites have not been discovered in the intervening DNA (Hagman et al., 1990), cis-regulatory elements could nevertheless exist in this region.

I therefore analyzed chromatin accessibility of the unrearranged Igκ locus in WT mice. Chromatin accessibility and gene expression can be modulated by DNA methylation, which has been shown to play an important role in the establishment and maintenance of a silent chromatin state (Lee et al., 2001; Makar et al., 2003; Wilson et al., 2002). Demethylation of DNA, on the other hand, is thought to create active chromatin domains and Igκ rearrangement has indeed been correlated with demethylation of the Jκ region in mice (Mostoslavsky et al., 1998; Nakase et al., 2003). Here, I show that parts of the Jκ1-Cκ1 region of the unrearranged Igκ locus are demethylated in a large fraction of mature B cells, but not in T cells, and that this demethylation appears to be developmentally regulated (Figure 7). The fact that demethylated Igκ loci are not restricted to κ^+ B cells suggests that Igκ loci are equally accessible in κ^+ and λ^+ B cells. Whether the establishment of Igκ- and Igλ-demethylation coincides developmentally is, at this point, subject to speculation. Unlike in the Igκ locus, no correlation between rearrangement and recombination could be detected. This implies that demethylation of the Jκ1-Cκ1 region is not functionally required for Igκ rearrangement but may (indirectly) reflect chromatin accessibility. Due to the limited number of methylation-sensitive restriction sites, this analysis does, however, not cover the complete Jκ1-Cκ1 region and a correlation between demethylation and Igκ rearrangement at other Jκ1-proximal sites is still possible. Taken together, my results are in good agreement with models ascribing the predominance of Igκ over Igλ rearrangements to a competition between the two loci, in which the Igκ locus is at an advantage.

Inefficient Igλ rearrangements could be due to differences in the quality of Igκ- and Igλ-specific RSSs with respect to their affinity for the RAG1/2 complex. Indeed, it has been demonstrated earlier that a pair of representative Igκ RSSs rearranges more efficiently than a pair of Vλ1 and Jλ1 RSSs *in vitro* (Ramsden and Wu, 1991). Moreover, RSSs appear to be an important factor in determining the order of V(D)J recombination in T cell receptor α locus (Bassing et al., 2000; Wu et al., 2003). Taken together, these results strongly suggest that different RSSs have different recombination properties. A targeted replacement of Igκ-specific RSSs with Igλ-specific RSSs would address the impact of RSS recognition on Igκ rearrangement.

Alternatively, competition for trans-activating factors might be responsible for different rates of germline transcription at Igκ and Igλ. Interestingly, the rate of

germline transcription has recently been shown to directly influence rearrangement in both the Igκ locus (Casellas et al., 2002) and the Igλ locus (Sun and Storb, 2001). To address potential differences in the efficiency of Igκ and Igλ germline transcription, it will be interesting to analyze how Igκ-specific enhancer elements might influence Igλ germline transcription and rearrangement when inserted into the Igλ locus.

B 4.2 Receptor editing in VJκ1i mice

While most pre B cells in VJκ1i mice exert efficient isotype exclusion and do not undergo Igλ rearrangements, ~20% of immature B cells and more than 30% of mature B cells express both a κ1 and a λ light chain on the surface (Figure 8). In VJκi mice, it has been shown that, depending on the inserted light chain, between 20% and 30% of B cells change their antigen receptor by editing, thereby generating B cells that express an endogenous VJλ gene (Casellas et al., 2001). This process is thought to be a means of revising the specificity of an otherwise self-reactive antigen receptor. The fact that I readily detect small pre B cells that express a λ LC in VJκ1i mice (Figure 4) is consistent with the idea of secondary rearrangements in a fraction of $\kappa1^+$ pre B cells. I further demonstrate that $\lambda/\kappa1^+$ pre B cells take ~12h longer to exit the pre B cell compartment than their $\kappa1^+$ counterparts (Figure 8B). A similar observation has been reported previously to be the consequence of receptor editing in pre B cells (Casellas et al., 2001). Two recent reports have proposed the generation of B cells with dual receptor specificity as a means to "dilute out" the signal strength of a single, self-reactive B cell receptor, thereby circumventing anergy or clonal deletion (Kenny et al., 2000; Li et al., 2002). This suggests that 20-30% of $\kappa1^+$ B cells in VJκ1i mice have undergone receptor editing in order to reduce the surface density of a self-reactive IgH/Igκ1 pair. This fraction is comparable to the fraction of editing λ^+ B cells in WT mice (Casellas et al., 2001). It can be extrapolated from this result that a maximum of two thirds of the IgH repertoire generated in WT mice can be expressed in combination with κ1 light chains.

B 4.3 B cells with dual receptor specificity are counter-selected upon BCR engagement

B cells with dual receptor specificity are a potential threat to the organism since unspecific 'bystander' Ig molecules can be generated in the process of an otherwise antigen-specific immune response and this may ultimately lead to autoimmunity (Li et al., 2002). The low number (~1-2%) of IgL double producers in normal mice (Casellas et al., 2001; Gollahon et al., 1988) suggests that such cells are either inefficiently generated or efficiently counter-selected during B cell development. Inefficient Ig rearrangement and concomitant inactivation of the Ig locus through RS recombination supports the former scenario. Moreover, mice that carry two prearranged IgH genes (Sonoda et al., 1997) as well as VJ1i/+ mice readily generate mature B cells with dual receptor-specificity, indicating that these cells are not counter-selected during the transition form immature to mature B cells. This phenomenon raises the following question: are VJ1i mice able to prevent the generation of unspecific and thus potentially harmful 'bystander' Ig responses? To address this issue, I studied the fate of B cells with dual receptor specificity upon antigen encounter or differentiation into specialized (Ig-producing) B cell subsets. While /1$^+$ B cells contribute equally to marginal zone and follicular B cell subsets in the spleen, the results presented in this thesis suggest that B cells with dual receptor specificity are counter-selected in the peritoneal cavity and in memory B cells; both B cell subsets are known to depend on BCR-ligand interactions.

B 4.3.1 Selection of peritoneal B cells depends on BCR-ligand interaction

The majority of peritoneal B cells assume a B1 phenotype and are responsible for the secretion of natural Igs. Based on results from BCR-transgenic mice, it has been proposed that B1 cells are preferentially generated from B cells that harbor a modestly autoreactive BCR and B1 cells thus appear to be BCR-ligand selected (Hayakawa et al., 1999; Hayakawa et al., 2003). The fact that /1$^+$ peritoneal B cells in VJ1i mice have normal levels of surface expression but 10-fold reduced 1 surface levels is consistent with this idea (Figure 9C). Interestingly, peritoneal B1 cells and peritoneal B2 cells appear to be equally selected, suggesting that BCR-ligand interaction is important for homing and/or selection of B cells into the peritoneal cavity rather than for the generation of B1 cells itself. In particular, signal strength and surface density

of the BCR appear to be crucial factors in this process, putting B cells with two distinct BCR specificities at a competitive disadvantage. A similar observation was made when BCR-transgenic mice that predominantly produce B1 cells were crossed with a second, B2 cell producing BCR-transgenic mouse strain: the majority of double transgenic B cells in the spleen expressed both IgH chains and assumed a B2 phenotype, whereas peritoneal B cells had inactivated the second IgH allele through somatic recombination (Lam and Rajewsky, 1999). Since the VJ□1 gene cannot be inactivated on the genomic level, strongly reduced □1 surface expression in □/□1^{+} cells is likely the consequence of inefficient pairing of IgH and Ig□1. This in turn means that the secretion of unselected, potentially harmful Igs through □$^{+}$/□1^{low} B1 cells cannot be excluded in VJ□1i mice.

Given the functional similarities between B1 cells and MZ B cells as a first line of immune defense to polyreactive antigens, it has been speculated that MZ B cells may also be subject to BCR-mediated selection (Martin and Kearney, 2000b; Martin et al., 2001). My results demonstrate that B cells with equal levels of two distinct BCR specificities can readily form MZ B cells (Figure 9B). This result is consistent with the idea that the generation of MZ B cells and peritoneal B cells may require different levels of BCR-signaling and/or -surface density (reviewed in (Niiro and Clark, 2002)), yet, it does not allow conclusions with respect to antigenic selection. In contrast to the results presented here, it has been reported previously that receptor-edited B cells with dual receptor specificity accumulate in the MZ (Li et al., 2002). However, in this particular case, B cells were transgenic for both IgH and IgL chains and the observed accumulation may not be representative for a broad repertoire of BCR specificities as it is found in □/□1^{+} B cells from VJ□1i mice.

B 4.3.2 Inactivation of the VJ□1 gene in memory B cells via somatic hypermutation

While the necessity of BCR-ligand interaction during the selection of peritoneal B cells is a relatively new concept, its importance in the generation of memory B cells is well established and B cells with dual receptor specificity are expected to have a selective disadvantage in the highly competitive germinal center environment. This selective disadvantage was observed for the first time in mice that carry multiple insertions of the same pre-rearranged Ig□ light chain. Mutations that improve the

antibody affinity of one transgene copy cause antigenic selection to favor cells that downregulate the other copies at multiple levels of gene expression. This includes examples where nonsense mutations correlate with a drop in messenger RNA levels (Lozano et al., 1993). Consistent with this prediction, I find that when VJ□1i mice are immunized with an antigen that is preferentially recognized by □$^+$ B cells, 45% of antigen-specific memory B cells have inactivated the VJ□1 gene through somatic hypermutation (Table VI) and have consequently lost □1 surface expression. Moreover, unimmunized VJ□1i mice show modest counter-selection of □/□1$^+$ double producers in class-switched bone marrow plasma cells, which are likely derived from memory B cells. Taken together, these results suggest that B cells with dual receptor specificity are generally counter-selected during TD immune responses. However, a considerable fraction of bone marrow plasma cells express both IgL chains and it is unclear whether or not these cells have been exposed to stringent antigenic selection. Given that every □$^+$ B cell that is recruited into germinal centers carries an intact VJ□1 allele and that individual germinal centers show varying degrees of affinity selection (Kuppers et al., 1993; Vora et al., 1999), it is conceivable that some germinal centers do not accumulate B cells with inactivated VJ□1 genes. In addition, certain TI antigens can lead to polyclonal B cell activation and class-switch recombination resulting in both IgM$^+$ and IgM$^-$ □/□1$^+$ plasma cells (Litinskiy et al., 2002). These cells have not been exposed to the highly selective environment of a germinal center and enrichment for B cells that have inactivated one of the two light chains is unlikely. However, further analyses are required to settle this issue. Irrespective of their origin, it will be interesting to analyze whether the abundance of □/□1$^+$ plasma cells in VJ□1i mice can lead to the generation of unspecific 'bystander' Ig molecules, which may eventually cause autoimmune disease. The fact that B cells with two distinct receptors can be found in mice (Casellas et al., 2001; Gollahon et al., 1988) and possibly humans (Hieter et al., 1981) raises the possibility that these cells may promote the generation of B cell-mediated autoimmunity. In T cells, coexpression of two different T cell receptors can lead to escape from self-tolerance induction and T cells with dual receptor specificity were shown to cause organ-specific autoimmune disease (Sarukhan et al., 1998).

B 5 SUMMARY

The B cell antigen receptor (BCR) consists of two immunoglobulin heavy (IgH) chains, two immunoglobulin light (IgL) chains and the signaling molecules Ig and Ig. The variable regions of IgH and IgL chains confer antigen specificity and are assembled from various gene segments through a unique series of somatic recombination events. Ig rearrangement is a developmentally regulated process with IgH rearranging prior to IgL. IgL rearrangements can take place on two distinct genomic loci, Ig and Ig, but occur more frequently at the latter. Previous results suggested that the unrearranged Ig locus negatively regulates Ig transcription and/or rearrangement. Here, I demonstrate that expression of a pre-rearranged 1 light chain gene inserted into the Ig locus is independent of Ig rearrangements. Expression of the inserted 1 gene is developmentally regulated like that of a pre-rearranged Ig gene inserted into the Ig locus and coincides developmentally with the occurrence of Ig rearrangements in wild-type mice. I thus conclude that transcription of a gene rearrangement in the Ig locus is developmentally controlled and not negatively regulated by the unrearranged Ig locus.

Mice carrying an inserted 1 light chain gene undergo secondary IgL rearrangements at a frequency that is comparable to WT mice. This process is presumably a consequence of receptor editing in B cells that expressed an auto-reactive IgH/Ig1 pair. Unlike Ig rearrangements, Ig rearrangements cannot be deleted from the genome and the resulting 'receptor-edited' 1^+ B cells express both the inserted 1 light chain and an endogenous Ig molecule on the cell surface. While allelic and isotype exclusion generally ensure that B cells express a unique receptor specificity, my results indicate that dual receptor specificity poses no problem for the generation of mature B cells. However, B cells with two distinct receptor specificities are counterselected upon BCR-ligand interaction, which is a crucial event during immune responses and possibly in the generation of B1 cells. Counterselection of Ig/Ig double positive B cells can occur on the protein level and on the genomic level and ensures the generation of an antigen-specific immune response.

B 6 ZUSAMMENFASSUNG

Der B-Zellrezeptor besteht aus zwei schweren und zwei leichten Immunglobulin-Ketten (IgH und IgL) und den für die Signalvermittlung verantwortlichen Molekülen Igα und Igβ. Die variablen Domänen der schweren und leichten Ketten sind für die Antigen-Erkennung verantwortlich und setzen sich aus mehreren Gensegmenten zusammen, die durch somatische Genumlagerungen in zeitlich regulierter Abfolge zusammengesetzt werden. Genumlagerungen der IgH loci finden vor denen der IgL loci statt. IgL-Umlagerungen können auf zwei unterschiedlichen Genloci initiiert werden (Igκ und Igλ), wobei Igκ bevorzugt wird. Es gibt Hinweise in der Literatur, dass der nicht umgelagerte Igκ Locus ein negatives Signal übermittelt, welches Igλ Genumlagerungen verhindert. In der vorliegenden Arbeit zeige ich, dass eine bereits umgelagerte Igλ1-Kette, die durch homologe Rekombination in den Igκ Genlocus integriert wurde, unabhängig von Igκ-Genumlagerungen ausgeprägt wird. Die Ausprägung dieser leichten Kette ist ähnlich reguliert wie die einer bereits umgelagerten Igκ-Kette, die in den Igκ Genlocus integriert wurde, und findet zur selben Zeit statt wie Igκ-Genumlagerungen in WT Mäusen. Daraus folgt, dass die Ausprägung einer Genumlagerung im Igκ Locus zwar vom Entwicklungsstadium der B-Zelle abhängig ist, aber nicht von Igκ-Genumlagerungen beeinflusst wird.

Ähnlich wie in WT Mäusen findet man auch in Mäusen mit umgelagerter Igλ1-Kette 20-30% sekundäre IgL-Genumlagerungen. Die Ursache dafür ist vermutlich eine selbstreaktive IgH/Igλ1 Kombination. Da Igλ-Genumlagerungen im Gegensatz zu Igκ-Genumlagerungen nicht deletiert werden können, prägen $\lambda1^+$ B-Zellen mit sekundären IgL Genumlagerungen zwei unterschiedliche B-Zellrezeptoren aus. In WT Mäusen garantieren allele sowie isotypische Ausschliessung, dass B-Zellen normalerweise nur einen einzigen B-Zellrezeptor ausprägen. Hier zeige ich, dass B-Zellen mit zwei unterschiedlichen B-Zellrezeptoren problemlos reifen und in sekundäre Lymphorgane gelangen können. Allerdings findet eine Gegenselektion dieser Zellen statt, sobald der B-Zellrezeptor mit Antigen interagiert. Diese Interkation spielt eine wichtige Rolle bei Immunreaktionen und wahrscheinlich auch bei der Entstehung von B1-Zellen. Gegenselektion von Igκ/Igλ1 Doppelproduzenten wurde sowohl auf der Ebene der Genausprägung als auch durch Geninaktivierung erreicht und stellt eine ausschliesslich antigenspezifische Immunantwort sicher.

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C Part II) Novel approaches for Cre-mediated inducible gene alteration in mice

C 1 INTRODUCTION

C 1.1 Manipulating gene expression in mice

The study of gene function in mice encompasses two complementary approaches: gain of function and loss of function analyses. Gain of function is generally achieved through overexpression of a certain gene product and thus directly addresses its functional properties. Loss of function, on the other hand, represents an indirect assessment of gene function as it involves gene inactivation. Since the severity of a loss of function phenotype often reflects the importance of the respective gene, loss of function studies are widely applied in mouse genetics. A classical way to generate loss of function mutants is the inactivation of genes through targeted disruption or 'knock-out' of the gene of interest by homologous recombination in embryonic stem (ES) cells (reviewed in (Capecchi, 1989)). An alternative way to achieve loss of function mutants employs the recently discovered cell-intrinsic RNA interference (RNAi) pathway (see chapter 1.2). This approach acts post-transcriptionally through sequence-specific degradation or 'knock-down' of messenger RNA. While classical gene inactivation generally requires targeted disruption of two alleles, RNAi-mediated loss of function can be achieved using a single transgene. Moreover, RNAi allows the simultaneous knock-down of several members of a highly homologous gene family. A limitation of RNAi as a means to study loss of function phenotypes is incomplete suppression of target mRNA. RNAi and gene targeting must therefore be considered complementary approaches to study gene function *in vivo*.

C 1.2 RNA interference

RNAi is a highly coordinated mechanism involved in post-transcriptional gene regulation and was first observed in the nemoatode worm Caenorhabditis elegans as a response to double-stranded (ds) RNA (Fire et al., 1998). dsRNAs generally originate from replicating RNA viruses or transposable and retroviral elements in the genome

and RNAi is thought to have evolved as an innate antiviral host defense mechanism (reviewed in (Hannon, 2002; Zamore, 2002)). During the initial steps of RNAi, the ribonuclease (RNase) III-like enzyme Dicer processes long dsRNAs and complex hairpin RNAs into small interfering RNAs (siRNAs) (Bernstein et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). siRNAs are generally 21 to 23 bp RNA duplexes with characteristic dinucleotide overhangs (Zamore et al., 2000). These duplexes were shown to be unwound in a reaction which is likely catalyzed by an RNA helicase (Schwarz et al., 2003). Single-stranded (ss) siRNAs can then be incorporated into the so-called RNA-induced silencing complex (RISC), which functions as an siRNA-induced endonuclease. RISC cleaves target RNA with perfect complementarity to the siRNA and thereby amplifies target RNA degradation (Hammond et al., 2000; Nykanen et al., 2001). The sequence specificity of this process is illustrated by the fact that even single point mutations in either the target RNA or the siRNA itself can completely abolish silencing (Elbashir et al, 2001a).

Recent reports show the successful application of RNA interference (RNAi) as a tool for post-transcriptional gene silencing in mammalian cells both *in vitro* and *in vivo* (reviewed in Dykxhoorn et al., 2003). Induction of transient RNAi was accomplished by direct application of siRNA duplexes (Elbashir et al., 2001; Song et al., 2003) or by transfection with siRNA expression vectors (reviewed in (Tuschl, 2002)). These vectors employ RNA polymerase III (polIII) to transcribe short hairpin RNAs (shRNAs) (Brummelkamp et al., 2002; Sui et al., 2002), which are subsequently processed into siRNAs by Dicer (Paddison et al., 2002). RNA polymerase III is normally dedicated to the transcription of genes that encode structural or catalytic RNAs such as tRNAs and components of the splicing machinery. The elongation properties of RNA polIII are unique in that it recognizes a run of 4-5 T residues as a termination signal. This allows the generation of short RNA molecules and RNA polIII transcripts are generally no longer than 400 bp. RNA polIII promoters are divided into three main types, two of which are gene-internal and commonly TATA-less, one is gene-external and contains a TATA box. In the latter case, transcription is initiated at a defined distance from the TATA box (25-32 bp) and thereby ensures the precise generation of RNA polIII transcripts (reviewed in (Schramm and Hernandez, 2002)). These properties make TATA-containing RNA polIII promoters an ideal choice to promote shRNAs transcription. To date, two RNA polIII promoters have been successfully used to transcribe shRNAs, which then efficiently mediated RNAi.

One of these promoters is derived from the human H1 gene (Brummelkamp et al., 2002), the other from the human or mouse U6 genes (Paul et al., 2002; Sui et al., 2002). In cell culture and in mice, stable expression of shRNAs was achieved using shRNA expression cassettes that were integrated into the genome by either retroviral or lentiviral gene transfer (Novina et al., 2002; Rubinson et al., 2003; Tiscornia et al., 2003). However, lentiviral integration into the mouse genome occurs randomly and hence yields unpredictable shRNA expression patterns. Targeted integration of an shRNA expression cassette into a well-defined genetic locus will help to solve this problem.

C 1.3 Conditional gene targeting – the Cre/*loxP* system

A major disadvantage of loss of function analyses is early lethality of the mouse mutant if the target gene is crucial for embryogenesis. In the case of targeted gene disruption, this limitation can be overcome by systems that allow cell-type specific or developmentally regulated inactivation of genes *in vivo*. Such conditional gene inactivation generally relies on prokaryotic recombinases that recognize specific DNA target sequences and excise or invert the intervening DNA, which contains the gene (segment) of interest. Recombinase expression can be regulated and genetic manipulation can thereby be limited to the cell type or developmental time point of choice. The most commonly used recombinase sytem is the Cre/*loxP* system (reviewed in (Rajewsky et al., 1996; Tronche et al., 2002)).

Cre is a bacteriophage P1 derived 38 kDa protein that recognizes a 34 bp DNA target termed *loxP* (locus of X-over of P1), which consists of two 13 bp inverted repeats that serve as Cre-monomer binding sites and an 8 bp spacer region. The spacer region is non-palindromic and provides the *loxP* site with an orientation; recombination can occur between two *loxP* sites of either identical or opposing orientation. The former results in excision of the intervening DNA, the latter in its inversion. Excision is accompanied by the deletion of one *loxP* site from the genome and is therefore essentially unidirectional. Inversion, on the other hand, yields two *loxP* sites that are indistinguishable from the original *loxP* pair due to the conservative nature of the recombination event (Guo et al., 1997), and recombination continues as long as Cre is present. While the full reversibility of Cre-mediated gene inversion limits its applicability as a genetic tool, Cre-mediated deletion is widely used to manipulate the

mouse genome. As mentioned above, it allows cell-type specific and/or developmentally regulated gene inactivation. In addition, deletion of *loxP*-flanked transcriptional STOP cassettes can be applied to induce gene expression in a Cre-dependent manner. Transcriptional STOP cassettes are located downstream of the promoter of the gene of interest and contain several transcriptional and/or translational termination elements (Lakso et al., 1992). Current technology limits this approach to the induction of polII-mediated transcription and hence does not allow Cre-mediated control of RNAi.

C 1.4 Site directed mutagenesis of *loxP* sites

Site-directed mutagenesis of *loxP* sites has led to the generation of mutant *loxP* pairs that extend the applicability of Cre/*loxP* mediated gene alteration. Mutational analyses involved both the spacer region and the inverted repeats. Mutations in the spacer region were shown to interfere with recombination between non-identical mutant *loxP* sites (Hoess et al., 1986). In the case of mutants *lox5171* and *lox2171*, recombination was demonstrated to occur exclusively within identical pairs (Lee and Saito, 1998). This allows the use of two or more distinct, mutant *loxP* pairs at the same time without the potential problem of cross-recombination between different *loxP*-flanked alleles. Mutations in the inverted repeats are thought to interfere with Cre-monomer recruitment and were shown to impair recombination efficiency depending on the extent and site of the mutation (Albert et al., 1995). *loxP* sites in which both inverted repeats are mutated display strongly reduced recombination efficiency even in combination with WT *loxP* sites. These findings have been utilized to generate *loxP* pairs that display a favorable forward reaction equilibrium. One such *loxP* pair consists of two asymmetric, mutant *loxP* sites that carry a 5 bp-mutation in the distal end of either the left (*lox71*) or the right (*lox66*) inverted repeat. Cre-mediated recombination of *lox66*/*lox71* yields one *loxP* site with two mutated inverted repeats (termed *lox72*) and one WT *loxP* site (Figure 1). While *lox66* and *lox71* mediate recombination with an efficiency comparable to WT *loxP* sites *in vivo* in plants, recombination efficiency between *lox72* and WT *loxP* was up to 8 fold reduced (Albert et al., 1995). More recently, two reports showed the successful application of *lox66*/*lox71*-mediated recombination in mouse embryonic stem (ES) cells (Araki et al., 1997; Zhang and Lutz, 2002). Although inversion appeared

irreversible in ES cell colonies (Zhang and Lutz, 2002), a characterization of *lox66*/*lox71*-mediated recombination in *cre*-transgenic mouse strains *in vivo* is still missing.

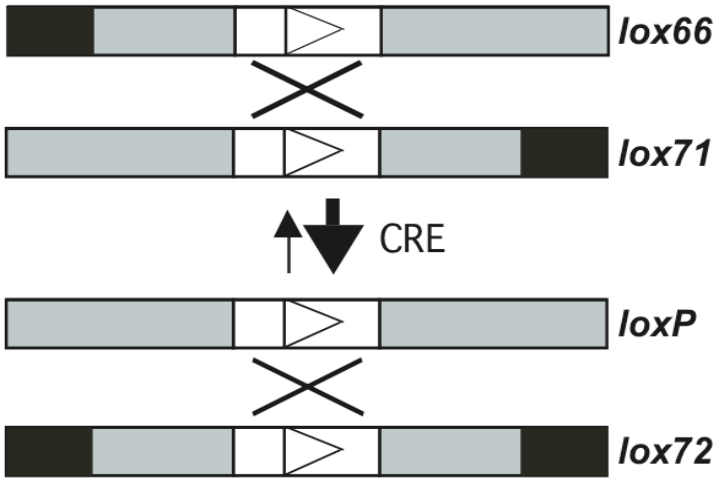

Figure 1. Schematic representing the Cre-mediated recombination of *lox66* and *lox71* resulting in *lox72* and *loxP*. Inverted repeats are shown in grey, black boxes symbolize 5 bp mutations, the open box depicts the spacer region and the triangle represents its orientation. The difference in arrow sizes reflects the unbalanced equilibrium between forward and reverse reaction.

C 1.5 Objectives of this study

The broad applicability of the Cre/*loxP* recombination system and the existing variety of mice that express Cre under inducible and cell-type specific promoters make this system an ideal choice for conditional mutagenesis. The aims of my thesis were to establish irreversible Cre-mediated genetic inversion *in vivo* and to combine Cre/*loxP* technology with RNAi to allow for inducible knock-down of one or multiple, homologous genes in mice. Both methods are expected to provide useful additions to the toolbox of gene manipulation.

C 2 MATERIALS AND METHODS

Only methods different from those described in part I are listed.

C 2.1 Molecular Biology

C 2.1.1 Purification of RNA and reverse transcription

Total RNA was isolated from 10^5-10^7 cells or approximately 100 mg of tissue using TRIzol (Invitrogen) following the manufacturer's protocol. All RNA work was done using DEPC-treated H_2O. cDNA was synthesized from 10^5-10^6 cells using oligo-dT primer and Thermoscript RT-PCR System (Invitrogen) according to the manufacturer's instructions. 1/10 of the cDNA template was subjected to PCR (see chapter 2.1.8).

C 2.1.2 Primers for polymerase chain reaction (PCR)

Name	Sequence (5'-3')	Location	
LAH53	GGACCTCCATCTGCTCTTATTT	5' of DQ52	s*
CDR3-PE	GGTCTATTACTGTGCAAGTTGG	CDR3 of VPE	as
U6termRI	TGTGAATTCGTTCCTCAGAGGAACTGA	3' of U6 gene	s
U6term1-B	TGTGGATCCCCCGGGCGTGGCTTGGTGGTACACCTC	3' of U6 gene	as
XbaI-U6	GACTCTAGATCCGACGCCGCCATCTCTAG	U6 promoter	s
U6loxT-RI	TGCGAATTCAAAAATCGCAAAAACGTAATAACTTCGTATA AGTATGCTATACGAAGTTATAGTCTCAAAACACACAATTA CTTAC	U6 promoter	as
A1d-X	TGCTCGAGATGTCTGAGTACGAGTTCATGCATATC	A1d cDNA	s
mutA1-B	CTGGATCCTTATTTCAGCAGGAACAGCATCTCCCATATCTG	A1d cDNA	as
A1d-B	CTGGATCCTTACTTGAGGAGAAAGAGCATTTC	A1d cDNA	as
HPRT-SAH	TTCCTAATAACCCAGCCTTTG	pMP-10 SAH	s
hHPRT-pro	GTGATGGCAGGAGATTTGTAA	hHPRT promoter	as

Table I. Complete list of PCR primers. * abbreviations: s, sense strand; as, antisense strand

C 2.1.3 Real time PCR analysis

Genomic DNA was subjected to quantitative PCR analysis using SYBR Green PCR core reagents (Applied Biosystems) as described by the manufacturer and the iCycler iQ Real-Time PCR Detection System (Bio-Rad). SYBR Green is an intercalating dye, which binds all double-stranded DNA, regardless of the sequence, and will emit a fluorescent signal. The SYBR Green dye binds amplified products during the annealing and extension steps of each PCR cycle. The accumulation of amplified DNA is measured by the increase in fluorescence over time and the specificity of the amplification product can be evaluated by combining PCR with melt curve analysis. Two serial 1:5 dilutions were included for each sample and served to determine mean and standard deviation.

C 2.1.4 Southern blot probes and quantification

RH probe: RH probe is located downstream of the Eμ enhancer and was used as an external probe to identify homolgous integration of the VNP-VPEinv targeting construct. RH probe was excised from plasmid pRH (Sonoda et al., 1997) using EcoRI and HindIII.

probe B: probe B is located upstream of the DH element DQ52 in the IgH locus and served as an internal probe to verify homologous recombination and identify random integration of the VNP-VPEinv targeting vector. Moreover, probe B was used to analyze Cre-mediated inversion of the VNP-VPEinv switch cassette. Probe B was excised from plasmid pDSP-Qi (Taki et al., 1993) using XhoI and HindIII.

RSA probe: RSA probe is located between exons 3 and 4 of the mouse HPRT locus and served as internal probe to detect homologous recombination of the U6lox-shA1 targeting vector. RSA probe was excised from pRSA (Bronson et al., 1996) using BamHI and HindIII.

Quantification of signal intensities was performed using a Storm 860 Molecular Dynamics scanner and ImageQuant software (Amersham Biosciences).

C 2.1.5 Northern blot analysis

10-20 μg of total RNA in DEPC H_2O were diluted in 1 Vol RNA sample buffer (50% Formamide, 6% Formaldehyde, 10 μg/ml ethidium bromide in 1x RNA Gel Buffer [20 mM MOPS/NaOH, 5 mM Sodium acetate, 1 mM EDTA, pH 8.0]) and denatured for 10 min at 65°C. 0.1 Vol of RNA Dye (50% Saccharose, Bromphenolblue) was

added and the sample was subsequently separated via gel electrophoresis in 1x RNA Running Buffer (20 mM MOPS/NaOH, 5 mM Sodium acetate, 1 mM EDTA, pH 7.0) at 40-60 V in a 1.2% RNA agarose gel (1x RNA Gel Buffer, 2 M formaldehyde). The gel was equilibrated in 10x SSC for 30 min and subsequently transferred onto a Hybond N^+ nylon membrane (Amersham) in 2x SSC by neutral capillary transfer as described (Sambrook et al., 1989). The membrane was baked at 80°C for 40 min. Hybridization and probe labeling was performed as described for Southern blot analysis in part I.

The following probes were used for Northern Blot analyses:

GFP probe: GFP probe comprises the cDNA of EGFP and was excised with XhoI and NotI from pIRES2-EGFP (Clontech).

GAPDH probe: GAPDH probe comprises part of the mouse GAPDH cDNA and was excised from pTRI-GAPDH-mouse (Ambion) using SacI.

C 2.1.6 Northern blot analysis of small RNAs

In order to detect RNAs smaller than 100 bp in size, high resolution polyacrylamide gel electrophoresis (PAGE) was performed under denaturing conditions as described (Sambrook et al., 1989). In brief, 10-20 µg of total RNA in DEPC H_2O were diluted in 1 Vol RNA Loading Buffer (BD Pharmingen) and denatured for 3 min at 95°C. Samples were run at 10-15 W on a 10% PA/8M Urea gel (10% PA (BioRad), 0.5x TBE (Sambrook), 8M Urea, 300µl 10% APS, 40µl TEMED (BioRad)) for 1-2 h. The gel was stained in 0.5x TBE containing 0.5 µg/ml Ethidium Bromide to detect tRNAs, which served as loading control. RNA was then transferred onto a Hybond N^+ nylon membrane in 0.5x TBE in a wet transfer chamber. Setup of the transfer was done as described for Western blots (Sambrook et al., 1989). Transfer was performed at 230 mA for 6-16 h at 4°C. The membrane was subsequently baked at 80°C for 40 min and hybridized as described for Southern blotting in part I.

Detection of siA1 employed an oligonucleotide-probe specific for the siA1 antisense strand. The probe was labeled by phosphorylation of the 5' nucleotide using γ-^{32}P-ATP and T4 Polynucleotide Kinase (NEB) according to the protocol of (Sambrook et al., 1989). Free nucleotides were removed using a Micro-Spin S-25 column (Amersham).

C 2.2 Cell Biology

C 2.2.1 Embryonic stem cell culture

The VNP-VPEinv allele was generated through homologous recombination in IB-10 ES cells (Torres and Kühn, 1997). ES cell culture and transfection was performed as described in chapter 2.2.1 of part I. Removal of the *loxP*-flanked neo^R selection marker from targeted ES cells was achieved by transient transfection with 20 µg of circular Cre-encoding plasmid pIC-Cre (Gu et al., 1993). Two days after transfection, ES cells were split and replated at a density of 2 x 10^3 cells per EF-containing 10 cm tissue culture dish. Colonies were grown in ES cell medium w/o G418 and picked after 8-10 days. Cells were trypsinized and split into a master and a duplicate EF-containing 96-well plate. Cells in the duplicate plate were placed under G418 selection (300-400 µg/ml) and G418 sensitive clones were detectable by cell death. The sensitive clones were then recovered and expanded from the master plate.

The U6lox-shA1 allele was generated through homologous recombination in HM-1 ES cells (Selfridge et al., 1992). ES cell culture and transfection was essentially done as described. Selection of targeted clones was achieved by addition of Hypoxanthine-Aminopterin-Thymidine (HAT, Sigma) to ES cell medium starting 2 days after transfection. HAT resistant ES cell colonies were picked after 8-10 days and expanded as described in chapter 2.2.1 of part I. Due to its cytotoxic effects, aminopterin is thought to interfere with proper blastocyst development. HAT-resistent ES cells were therefore cultured in HAT-free ES cell medium prior to injection. Transition from HAT^+ to HAT^- medium required an intermediate passage in medium that contains Hypoxanthin and Thymidin, but lacks aminopterin (HT, Sigma).

C 2.2.2 Transduction of ES cells with Cre-expressing adenovirus

ES cells were trypsinized as described in chapter 2.2.1 of part I and resuspended at 10^5 cells/ml in ES cell medium. 5 x 10^5 cells were plated in one well of a EF-containing 6 well-plate. 10^7-10^8 infectious units of Cre-expressing adenovirus (Bassing et al., 2002a) were added per well and cells were incubated for 16 h at 37°C. Cells were washed twice with ES cell medium to eliminate residual virus and expanded for freezing and analysis.

C 2.2.3 Flow cytometry and cell sorting

Single cell suspensions were stained with monoclonal antibodies (mAbs) conjugated to fluorescein isothyocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC). Monoclonal antibodies are listed in Table 3 and were either prepared in our laboratory by C. Uthoff-Hachenberg and B. Hampel or purchased from the indicated suppliers. Staining for surface expression of NP-binding Ig was performed in two steps: cells were incubated with NP_{14}-BSA (0.2 ng/ml), washed once with PBS/0.5% BSA and subsequently stained with S43-APC. Cells were analysed as described in part I.

Specificity	**Clone**	**Reference and supplier**
anti-CD4	GK1.5-4	Pharmingen
anti-CD5	53-7.3	Parmingen
anti-CD8	53-6.7	Pharmingen
anti-CD19	1D3	Pharmingen
FcBlock	2.4G2	Pharmingen
anti-IgM	1B4B1	eBioscience
anti-NP	S43	REF lab-made
B220/ CD45R	RA3-6B2	(Coffman, 1982), lab-made

Table II. List of antibodies used for flow cytometry.

C 2.3 Mouse Experiments

C 2.3.1 Protein transduction of splenic B cells with TAT-NLS-Cre

Single cell suspensions of splenocytes were purified by MACS depletion using anti-CD43 beads as described by the manufacturer (Miltenyi). The purity of $B220^+$ cells was greater than 95%. TAT-NLS-Cre transduction was essentially done as described (Peitz et al., 2002). In brief, B cells were washed twice in serum-free medium (Hyclone) and incubated at $5x10^6$ cells/ml with the indicated dose of TAT-NLS-Cre (Peitz et al., 2002) in serum-free medium (Hyclone) for 1 hour at 37 ºC. Cells were then washed twice in complete medium (Peitz et al., 2002) and cultured at a density of $2x10^6$ cells/ml at 37 ºC and 10% CO_2 for 5 days.

C 2.3.2 Induction of *Mx-cre* transgene expression *in vivo*

Mx-cre mice were given 3 doses of 400 µg poly(I)*poly(C) (Amersham Biosciences) via intra-peritoneal injection. Injections were performed on days 0, 3, and 6 and mice were analyzed 3 days or 6 to 8 weeks thereafter.

C 3 RESULTS

C 3.1 Unidirectional Cre-mediated genetic inversion in mice using the mutant *loxP* pair *lox66*/*lox71*

C 3.1.1 Generation of mice carrying a *lox66*/*lox71* flanked VDJ-switch-cassette in the IgH locus

lox66/*lox71*-mediated recombination has been demonstrated to be essentially unidirectional *in vitro*. In order to test whether this approach can be used to irreversibly alter gene expression in mice, I generated a *lox66*/*lox71*-mediated genetic switch system that allows the inducible change of the B cell antigen receptor (BCR) specificity in mice. Change of BCR specificity was chosen as a read-out, since it allows for the detection of Cre-mediated recombination both on the genomic level by Southern blot analysis and on the protein level using flow-cytometry (FACS). The BCR consists of two identical immunoglobulin heavy (IgH) chains, two identical Ig light (IgL) chains and the signal transducing Igα/Igβ heterodimer. The paired variable domains of IgH and IgL chains (termed VDJ and VJ, respectively) form the antigen binding sites of the BCR (for review see (Bassing et al., 2002b)) and a change in either the VDJ or the VJ domain generally results in a change of antigen specificity. I therefore engineered a *lox66*/*lox71*-flanked 'VDJ-switch cassette', which consists of two distinct VDJ gene segments, VNP and VPE (Maruyama et al., 2000) in opposing transcriptional orientations, and inserted it into the mouse IgH locus by homologous recombination in ES cells (Figure 2A). Depending on Cre-mediated recombination, transgenic B cells will express either the VNP or the VPE heavy chain. In combination with a λ1 light chain, the two heavy chains can be distinguished by surface staining: VNP/Igλ1 recognizes 4-hydroxy-3-nitrophenylacetyl-(NP)-haptenated carrier proteins while VPE/Igλ1 binds phycoerythrin (PE). This permits the detection of *lox66*/*lox71*-mediated recombination in single $\lambda 1^+$ B cells (~6% of all splenic B cells).

To generate the targeting vector, the VPE gene segment was PCR amplified from hybridoma 80A3 (Maruyama et al., 2000) and linked to a 0.5 kb EcoRV/SalI-fragment containing the V_H promoter of the V_H3-83 gene (Pelanda et al., 1997). A 2.1 kb BamHI/XhoI-fragment containing the VNP gene segment and its VH promoter (Sonoda et al., 1997) and an inverted *lox71* site 3' of the VNP segment was ligated to

the V_H3-83-promoter-VPE fragment such that the promoters face away from each other. This cassette was subsequently fused to the 3' end of a *lox66*-flanked TK-*neo*^{R} gene (Ferradini et al., 1996) with the downstream *lox66* site facing the inverted VPE segment. An 11 kb XhoI-fragment containing 9 kb of genomic sequence upstream of DQ52 and the HSV-*tk* gene (Taki et al., 1993) served as long arm of homology, and a 0.8 kb EcoRI fragment containing part of the J_H-Cμ intron (Sonoda et al., 1997) was used as short arm of homology. The NotI-linearized targeting construct was transfected into IB10 ES cells (Torres and Kühn, 1997) as described in chapter B 2.2.1. G418 and Gancyclovir double-resistant ES cell colonies were screened for homologous recombination by Southern blotting using internal probe B (Figure 2B) and external RH probe (data not shown). The *lox66*-flanked *neo*^{R} gene was deleted as described in chapter 2.2.1 and G418-sensitive clones were verified by Southern blot analysis (Figure 2B). Two independent ES cell clones that carry the *lox66*/*lox71*-flanked VDJ-switch-cassette without the *neo*^{R} gene, were injected into CB20 blastocysts. Resulting chimeric male mice were bred to CB20 females for germline transmission. In order to obtain a second mouse strain that carries the VNPinv-VPE allele in the germline, VPEinv-VNP/+ mouse mutants were crossed to *deleter* mice (Schwenk et al., 1995), which express Cre during early embryogenesis. Two out of six mice that carried the *deleter* allele also carried the VNPinv-VPE allele.

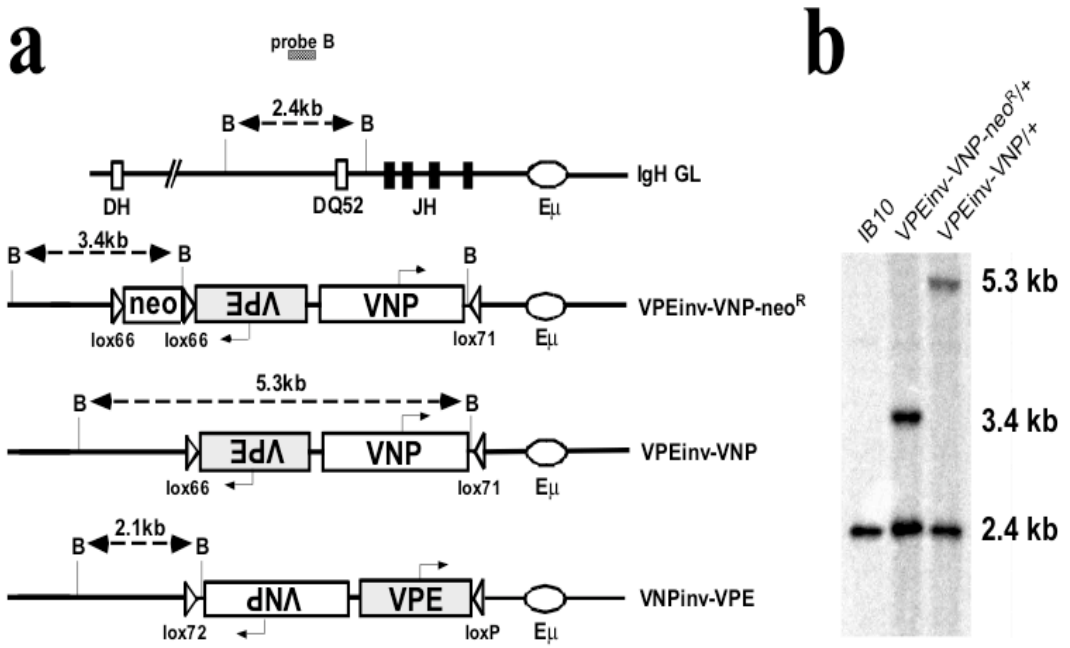

Figure 2. Strategy to switch the IgH variable region exon employing *lox66*/*lox71*. (a) Targeted integration of a VDJ-switch cassette into the IgH locus. BamHI (B) restriction endonuclease map of the IgH germline JH region (IgH GL), the targeted VDJ switch cassette before *neo*^{R} deletion (VPEinv-VNP-neo^R) and before (VPEinv-VNP) and after (VNPinv-VPE) Cre-mediated inversion. Boxes depict D and J exons and the promoter-VDJ exons VPE and VNP as indicated, arrows symbolize the transcriptional orientation of VNP and VPE. The oval represents the Eμ enhancer, triangles depict (mutant) *loxP* sites. Double-headed arrows indicate BamHI-fragments detected with probe B, fragment sizes are indicated. **(b)** Southern blot analysis of IB10 ES cells and a targeted ES cell clone before (VPEinv-VNP-*neo*^{R}) and after *neo*^{R} deletion (VPEinv-VNP). Genomic DNA was digested with BamHI and hybridized to probe B.

C 3.1.2 Dose-response analysis of *lox66*/*lox71*-mediated inversion in splenic B cells *in vitro*

I first determined the efficiency of *lox66*/*lox71*-mediated recombination at different Cre-concentrations in *ex vivo* isolated splenic B cells from VPEinv-VNP/+ mice. B cells that carry a WT *loxP*-flanked VNP gene segment (termed B1-8f, (Lam et al., 1997)) in the same genomic position served as positive control for recombination. In the B1-8f allele, the *loxP* sites are in identical orientation and the VNP segment is deleted in the presence of Cre. To prevent cell death of primary B cells in culture, B cells were transgenic for a cDNA encoding the anti-apoptotic Bcl-2 protein (*bcl-2tg*) (Strasser et al., 1991). Splenic B cells were incubated for 1h with TAT-NLS-Cre at different concentrations (Peitz et al., 2002). TAT-NLS-Cre is a fusion protein of a basic 11-aa peptide derived from HIV-TAT, a nuclear localization signal (NLS) and Cre. The hydrophobic TAT peptide has been reported to facilitate cellular uptake of recombinant proteins *in vitro* and *in vivo* (Schwarze et al., 1999; Schwarze et al., 2000). TAT-NLS-Cre-mediated inversion was analyzed five days after Cre transduction by FACS and by Southern blotting. For FACS analysis, B cells were stained for NP- and PE-binding (Figure 3A) and inversion frequency was calculated as the percentage of PE^+ cells over the sum of PE^+ and NP^+ cells (Figure 3C). For Southern blotting, genomic DNA was digested with BamHI and hybridized to probe B (see Figure 1). Inversion frequency was calculated as the ratio of VNPinv-VPE intensity over the sum of VNPinv-VPE- and VPEinv-VNP-intensities (Figure 3D). Both FACS analysis and Southern blotting yielded comparable inversion frequencies. To determine the efficiency of WT *loxP*-mediated deletion, I analyzed B1-8f/+ B cells for loss of surface IgM expression upon TAT-NLS-Cre transduction (Figure 3B). The comparison of WT and mutant *loxP* sites shows a clear difference in recombination frequencies (Figure 3C). While low doses of TAT-NLS-Cre (≤50µg/ml) induce recombination in up to ~1/4 of all B cells carrying WT *loxP* sites, no recombination is observed with *lox66*/*lox71*. To obtain recombination frequencies similar to those of WT *loxP* sites, mutant *loxP* sites have to be exposed to approximately threefold higher concentrations of TAT-NLS-Cre. To address whether reverse recombination affects the overall recombination frequency in our system, I incubated B cells that carry the pre-inverted VNPinv-VPE allele with 200 µg/ml TAT-NLS-Cre (the highest concentration used in the dose response assay) and analyzed them three days after transduction. No reverse recombination could be detected by Southern blot analysis

(Figure 3D). Given the limited sensitivity of this assay, I conclude that <10% of cells have undergone reverse recombination (see chapter 3.1.3).

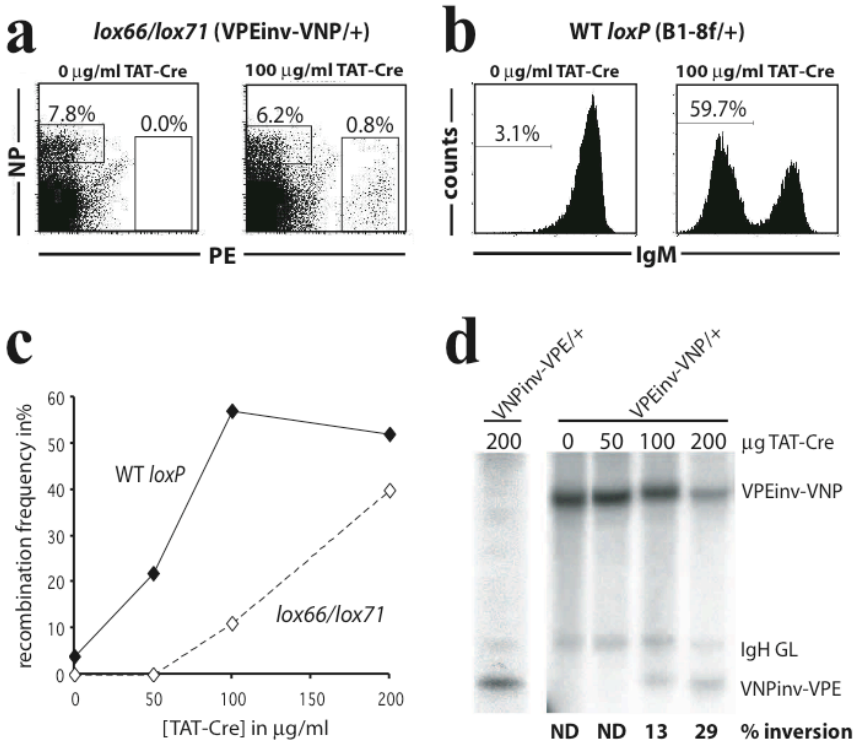

Figure 3. Dose response of *ex vivo* isolated splenic *bcl-2tg* B cells from VPEinv-VNP/+ or B1-8f/+ mice to TAT-NLS-Cre. (a) FACS analysis of splenic B cells from VPEinv-VNP/+ *bcl-2tg* mice to determine *lox66/lox71*-mediated recombination frequency *in vitro*. CD43$^-$ MACS-purified B cells were incubated with 0 µg/ml and 100 µg/ml TAT-NLS-Cre and analyzed five days thereafter. Cells were analyzed for PE- and NP-binding. **(b)** FACS analysis of splenic B cells from B1-8f/+ *bcl-2tg* mice to determine WT *loxP* mediated recombination frequency *in vitro*. Cells were prepared and cultured as described in (a) and analyzed for IgM expression. **(c)** Dose response curve of *lox66*/*lox71* and WT *loxP* mediated recombination to increasing amounts of TAT-NLS-Cre. Recombination frequencies for VPEinv-VNP inversion (*lox66*/*lox71*, hatched line) and B1-8f deletion (WT *loxP*, solid line) were calculated based on FACS analyses performed as in (a) and (b), for details see text. **(d)** Southern blot analysis of VNPinv-VPE/+ and VPEinv-VNP/+ *bcl-2tg* B cells after TAT-NLS-Cre transduction. Total B cell DNA was digested with BamHI and hybridized with probe B. Fragments representing VPEinv-VNP, VNPinv-VPE and IgH germline (GL) alleles are indicated. No *lox72*/WT *loxP*-mediated reverse recombination (VPEinv-VNP signal) could be detected in B cells from VNPinv-VPE/+ mice (left panel). The percentage of cells that carry the VNPinv-VPE allele as a result of *lox66/lox71*-mediated recombination is shown for each lane (right panel); ND, not detected.

C 3.1.3 *lox66*/*lox71* mediates unidirectional inversion in adult *Mx-cre* mice

I next analyzed the efficiency of *lox66*/*lox71*-mediated recombination *in vivo* in combination with the inducible *Mx-cre* transgene. In *Mx-cre* mice, Cre expression is under the control of the Type I Interferon (IFN) inducible Mx1 promoter (Kühn et al., 1995). Injection of Type I IFN or poly(I)*poly(C), a ds RNA homologue that induces an endogenous anti-viral Type I IFN response, leads to efficient Cre expression and subsequent Cre-mediated recombination (Kühn et al., 1995). As potential (counter-) selection of B cells after Cre-induced change of BCR specificity might obscure the actual recombination frequency, the analysis was restricted to organs that harbor few or no BCR^+ B cells, such as liver, thymus and bone marrow. I tested *lox66*/*lox71*-mediated recombination in adult VPEinv-VNP/+ *Mx-cre* mice both three days and eight weeks after Cre induction by poly(I)*poly(C). To compare the recombination efficiencies of mutant and WT *loxP* sites, I also analyzed mice carrying WT *loxP*-flanked gene insertions in the same genomic position (Maruyama et al., 2000). Inversion efficiencies in lymphoid organs, liver and tail were analyzed by Southern blotting and calculated as the ratio of VNPinv-VPE over IgH GL intensity. In the liver, ~80% of VPEinv-VNP/+ cells show *lox66*/*lox71*-mediated recombination both three days and eight weeks after Cre induction while no inversion could be detected in tail tissue at either of the two time points (Figure 4A). These results closely resemble deletion efficiencies observed with WT *loxP* sites ((Kühn et al., 1995; Maruyama et al., 2000) and data not shown). Unlike in liver and tail, recombination frequencies in thymus and bone marrow increased over time (Figure 4A). Three days after induction, *lox66*/*lox71*-mediated recombination in thymus and bone marrow was three to six times less efficient than WT *loxP*-mediated deletion in the same organs (Kühn et al., 1995; Maruyama et al., 2000) and data not shown). Eight weeks after induction, however, ~75% of thymocytes and up to 90% of bone marrow cells carried the inverted allele. This indicates inefficient inversion in B and T lineage cells and bone marrow macrophages but efficient recombination in hematopoietic stem cells, which reconstitute bone marrow and thymus within eight weeks after Cre induction. To determine the extent of *lox72*/WT *loxP*-mediated reverse recombination, I analyzed *Mx-cre* mice that carry the pre-inverted VNPinv-VPE allele. No reverse recombination could be detected eight weeks after injection of poly(I)*poly(C) by Southern blotting in any of the tissues tested (Figure 4B). Due to the limited sensitivity of this analysis, I performed quantitative PCR on thymic and liver DNA of

the same animals using light cycler analysis (Figure 4C). The VPEinv-VNP allele was amplified using the primer pair LAH-53 and CDR3-PE (see Table I) and TNFR-1 was amplified as described (Pfeffer et al., 1993) to normalize the individual DNA samples. No reverse recombination could be detected in the liver of poly(I)*poly(C)-injected VNPinv-VPE/+ *Mx-cre* animals, whereas up to 5% of thymocytes have undergone Cre-mediated reverse recombination. Given that no VPEinv-VNP band could be detected by Southern blotting, the detection limit of recombination by Southern blotting was estimated to be between 5 and 10%. Taken together, I conclude that *lox66*/*lox71*-mediated recombination can be efficiently induced in adult *Mx-cre* mice and reverse recombination appears to occur only in a minor fraction of hematopoietic stem cells.

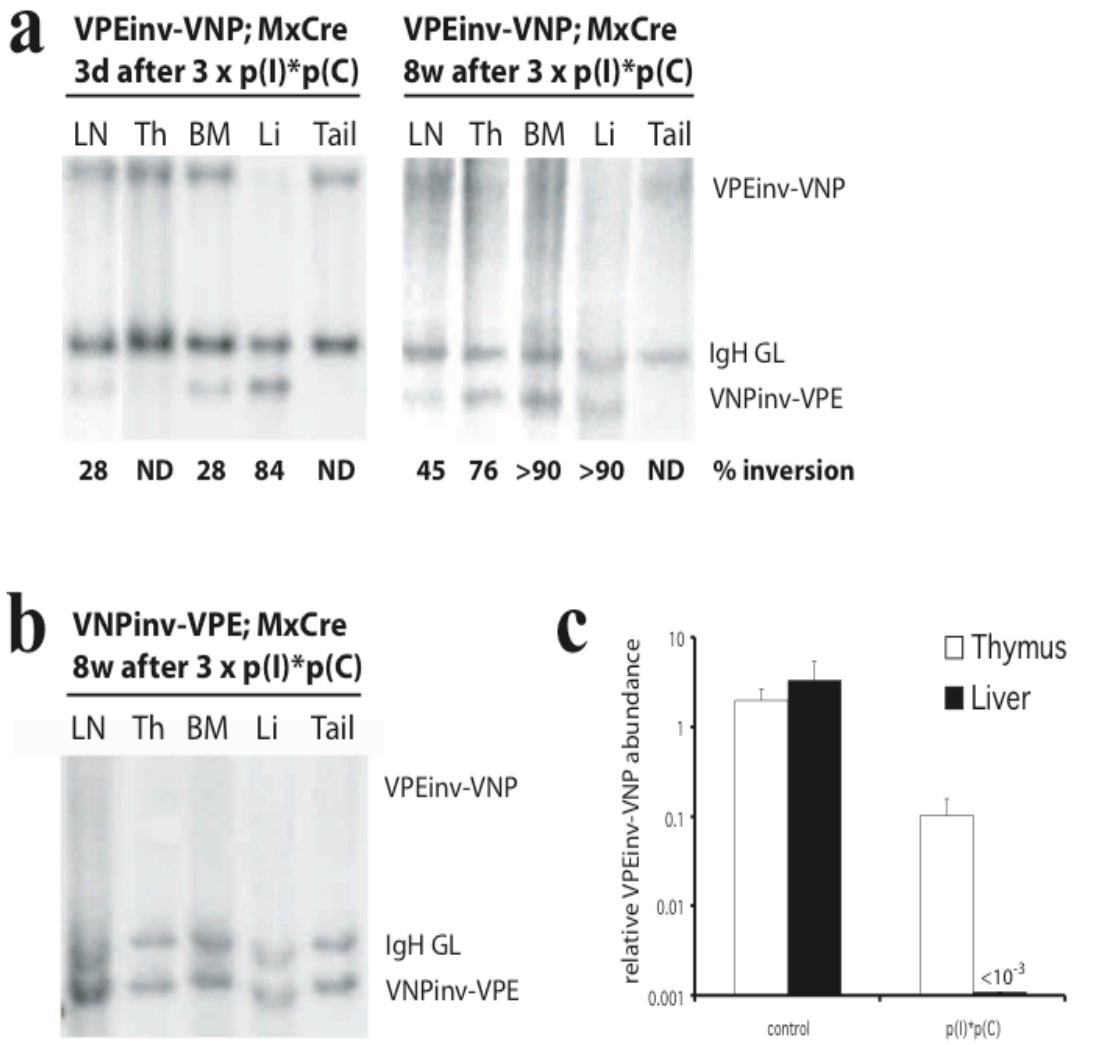

Figure 4. *lox66*/*lox71*-mediated recombination efficiency in *Mx-cre* mice. (a) Adult VPEinv-VNP/+ *Mx-cre* mice were injected with poly(I)*poly(C) and analyzed three days or eight weeks thereafter. Genomic DNA from lymph nodes (LN), Thymus (Th), bone marrow (BM), liver (Li) and tail was digested with BamHI and hybridized to probe B. The percentage of cells that carry the VPEinv-VNP allele in its inverted configuration (VNPinv-VPE) is shown for each lane. These results are representative for at least two independent experiments. ND, not detected. **(b)** Adult VNPinv-VPE/+ *Mx-cre* mice were injected with poly(I)*poly(C) and analyzed eight weeks thereafter as described in (a). **(c)** Quantitative PCR analysis to detect *lox72*/WT *loxP*-mediated reverse recombination in VNPinv-VPE/+ *Mx-cre* mice. Thymic and liver DNA was PCR-amplified with primers detecting either the VPEinv-VNP allele or the TNFR-1 gene to normalize DNA content. The relative abundance of the VPEinv-VNP PCR product is shown for poly(I)*poly(C)-injected animals and VPEinv-VNP/+ control mice.

C 3.1.4 *lox66*/*lox71* mediates cell type-specific unidirectional inversion in *CD4-cre* mice

To determine *lox66*/*lox71*-mediated recombination in mice that express Cre in a cell type-specific manner, I analyzed VPEinv-VNP/+ mice that carry the *CD4-cre* transgene (Wolfer et al., 2001). *CD4-cre* mice show efficient deletion of WT *loxP* flanked gene segments in $CD4^+/CD8^+$ double positive (DP) thymocytes and almost complete deletion in mature T cells (Schmidt-Supprian et al., 2003; Wolfer et al., 2001). Moreover, the VPEinv-VNP allele is not expressed in T cells and is thus not subject to potential (counter-) selection upon its inversion. DP thymocytes and splenic T cells ($CD5^+/CD19^-$) from VPEinv-VNP/+ *CD4-cre* mice were sorted using flow-cytometry and inversion efficiencies were determined by Southern blotting. $CD19^+$ splenic B cells served as negative control. Recombination was observed in 40% of DP thymocytes and ~95% of splenic T cells (Figure 5). The higher recombination efficiency in splenic T cells may be due to the fact that, on average, mature T cells have been exposed to Cre longer than DP thymocytes, in which *CD4-Cre* expression is initiated. The observation that 5% of splenic T cells carry the original VPEinv-VNP allele could reflect either incomplete inversion or the occurrence of reverse recombination in maximally 5% of mature T cells.

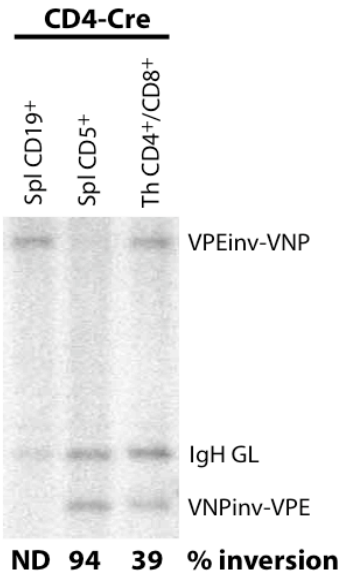

Figure 5. VPEinv-VNP-inversion in 5 week old VPEinv-VNP/+ *CD4-cre* mice. Genomic DNA from FACS-sorted splenic B cells ($CD19^+$), T cells ($CD5^+$) and sorted $CD4^+/CD8^+$ thymocytes was digested with BamHI and hybridized to probe B. Inversion frequencies are indicated for each sample; ND, not detected.

C 3.2 Inducible RNAi in ES cells

C 3.2.1 Generation of an inducible shRNA expression cassette

Stable gene knock-down by RNAi is dependent on RNA polIII-driven transcription of shRNAs, which are then processed into functional siRNAs (reviewed in (Tuschl, 2002)). In order to generate a system that allows the inducible expression of shRNAs and thereby conditional mutagenesis through RNAi, it is therefore imperative to be able to control RNA polIII-mediated transcription. It has been demonstrated previously that transcription via RNA polII can be regulated by Cre-mediated recombination. This approach employs a *loxP*-flanked transcription STOP cassette, which is inserted downstream of the RNA polII promoter and efficiently terminates transcription (Lakso et al., 1992). Upon Cre induction, the STOP cassette is removed and transcription is resumed. In order to be able to regulate RNA polIII-mediated transcription in a similar manner, I designed a RNA polIII-specific transcription termination cassette with the help of C. Kanellopoulou and C. Päper. Termination of RNA polIII-mediated transcription generally occurs when RNA polIII encounters four to five consecutive thymidines (referred to as T-stretch) and transcription is aborted after the second thymidine. Mutational analysis of RNA polIII-mediated transcription showed that the untranscribed sequence immediately downstream of the T-stretch may also be involved in transcriptional control (Das et al., 1988). We therefore generated a U6 STOP sequence that comprises the T-stretch and 190 bp of genomic DNA downstream of it. An additional T-stretch was inserted next to the endogenous T-stretch to enhance the efficiency of transcriptional termination. Insertion of the *loxP*-flanked STOP cassette between the U6 promoter and the shRNA gene required several adjustments in order to ensure proper shRNA transcription upon Cre-mediated deletion of the STOP sequence. Transcriptional initiation at (+1) is crucial for the precise generation of short RNAs by RNA polIII. Deletion of the STOP cassette will leave one *loxP* site at the site of its integration. If the STOP cassette were inserted after the (+1), this would result in a *loxP*-shRNA fusion transcript, which may interfere with proper shRNA processing and siRNA generation. To avoid transcription of the *loxP* site, it had to be integrated into the U6 promoter between the TATA box and (+1). Mutational analyses of the RNA polIII promoter suggest that this sequence can be altered without affecting the efficiency of RNA polIII-mediated transcription (Myslinski et al., 2001). However, (+1) is located 26 bp downstream of

the U6 TATA box and one *loxP* site comprises 34 bp. Accommodation of a *loxP* site in the U6 promoter was achieved as follows: the first 3 bp of the *loxP* site (ATA) were integrated into the TATA box and the last 5 bp of the shRNA-proximal *loxP* site were exchanged for the first 5 bp of the shRNA coding sequence (Figure 6). Based on previous results, a 5 bp mutation at the distal end on the inverted repeat is not expected to dramatically decrease recombination efficiency ((Albert et al., 1995) and chapter 3.1). Indeed, deletion efficiency of the STOP cassette *in vitro* was comparable to that of a WT *loxP* flanked control DNA segment (Päper, 2002).

The inducible shRNA expression cassette employs the shRNA shA1, which has been tested previously (Päper, 2002) and is directed against the bcl-2 family members A1a, A1b and A1d (Hatakeyama et al., 1998; Lin et al., 1993). The cassette is referred to as U6lox-shA1 and was cloned with the help of C. Päper in three steps: the modified U6 promoter was PCR amplified from the U6 promoter containing plasmid pU6 (Sui et al., 2002) using primers XbaI-U6 and U6lox-T-RI (see Table I). The 5' primer introduces an XbaI site 5' of the U6 promoter, the 3' primer replaces the sequence 3' of the TATA box with a *loxP* site, two T-stretches and an EcoRI site. The RNA polIII STOP sequence was PCR amplified from C57BL/6 genomic DNA using primers U6termRI and U6term1B (see Table I), which introduced a 5'EcoRI site and a 3' BamHI site. A third fragment consisting of a mutant *loxP* site fused to shA1 was generated by oligonucleotide synthesis of two complimentary oligomers, lox-shA1-s and lox-shA1-as (see Figure 6 for sequence information). The annealed oligomer contained a 5' BamHI site and a 3' HindIII site. The three subfragments were cloned into a modified pBS-polylinker resulting in an AscI-flanked U6lox-shA1 cassette.

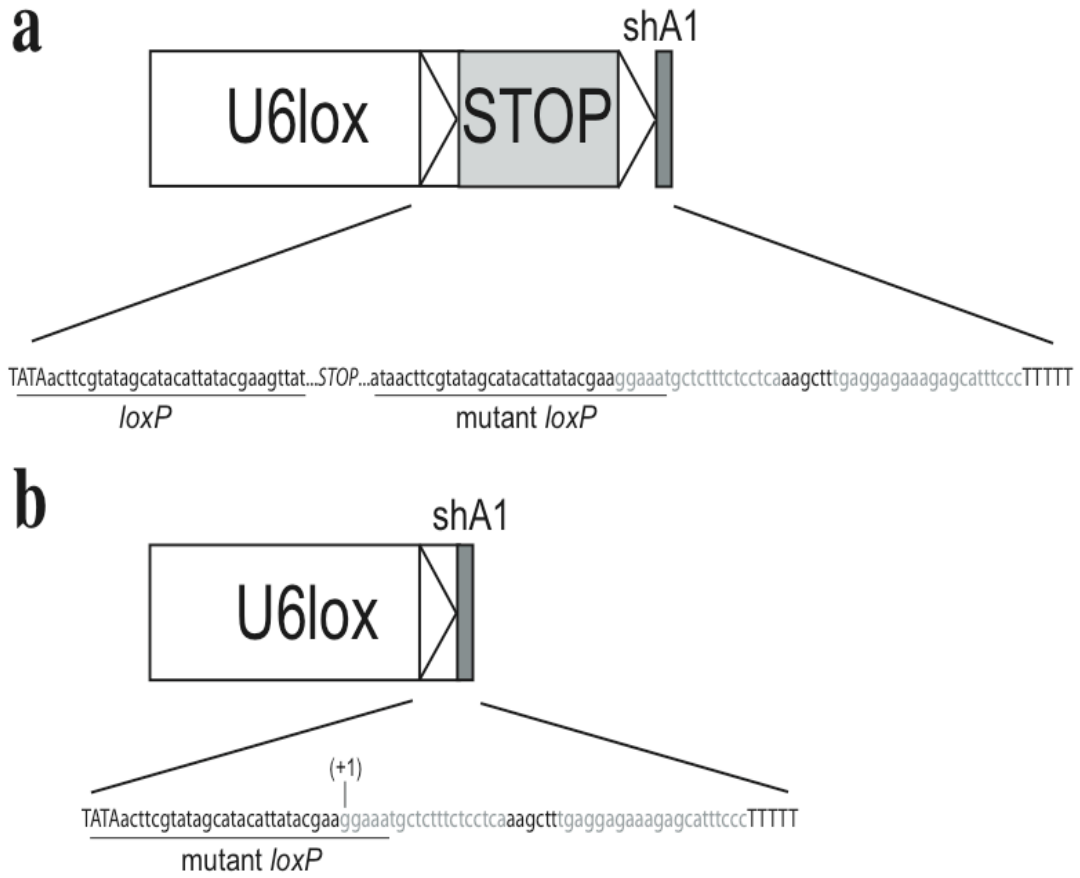

Figure 6. Inducible shA1 expression construct. Schematic of U6lox-shA1 cassette before **(a)** and after **(b)** Cre-mediated deletion of the STOP sequence. Triangles depict *loxP* sites, STOP represents RNA polIII transcription termination sequence including 2 T-stretches, U6lox stands for the modified, *loxP* containing U6 promoter. The sequence between the U6 TATA box and the T-stretch is shown in detail for both constructs. *loxP* sites are underlined, the shRNA coding sequence is shown in gray. TATA box and T-stretch are depicted in capital letters. The distance from the 3' end of the TATA box to the shRNA transcription initiation site (+1) is 26 bp. Note the overlap between TATA and the 5' *loxP* site and the 5 bp mutation in the shA1-proximal inverted repeat of the 3' *loxP* site.

C 3.2.2 Targeted insertion of U6lox-shA1 into HPRT deficient HM1 ES cells

To generate a mouse strain, which allows ubiquitous induction of shA1-mediated RNAi upon Cre-mediated recombination in a defined genetic locus, the U6lox-shA1 cassette was targeted into the X-linked hypoxanthine phosphoribosyltransferase (HPRT) locus by homologous recombination in ES cells. This approach takes advantage of HPRT-deficient HM-1 ES cells (Selfridge et al., 1992), which permit extremely efficient selection of transgenes inserted into the HPRT locus (Thompson

et al., 1989). HM-1 ES cells lack the HPRT promoter and exons 1 and 2. Only reconstitution of the disrupted HPRT locus by gene targeting confers resistance to HAT selection (Littlefield, 1964). Hence, virtually every HAT-resistant ES cell colony carries the targeted HPRT allele. A targeting vector that allows the insertion of transgenes into HM-1 ES cells has been described previously (pMP-8SKB, (Bronson et al., 1996)). S. Casola in our laboratory generated a modified version of this vector termed pMP-10, which can be linearized with SwaI, SbfI or SgfI and harbors two additional unique restriction sites (AscI and PmeI) to insert the transgene of choice. The U6lox-shA1 cassette was inserted into the AscI restriction site of pMP-10 in the same transcriptional orientation as the HPRT gene. The targeting vector was linearized with SwaI and transfected into HM-1 ES cells as described in chapter 2.2.1. The targeting strategy is shown in Figure 7A. The integrity of HAT-resistant colonies was confirmed by Southern blotting using a StuI digest and probe RSA (Figure 7B). Two independent ES cell clones were injected into C57BL/6 blastocysts. Chimeric mice are currently being bred to multiple *cre*-transgenic mice for germline transmission.

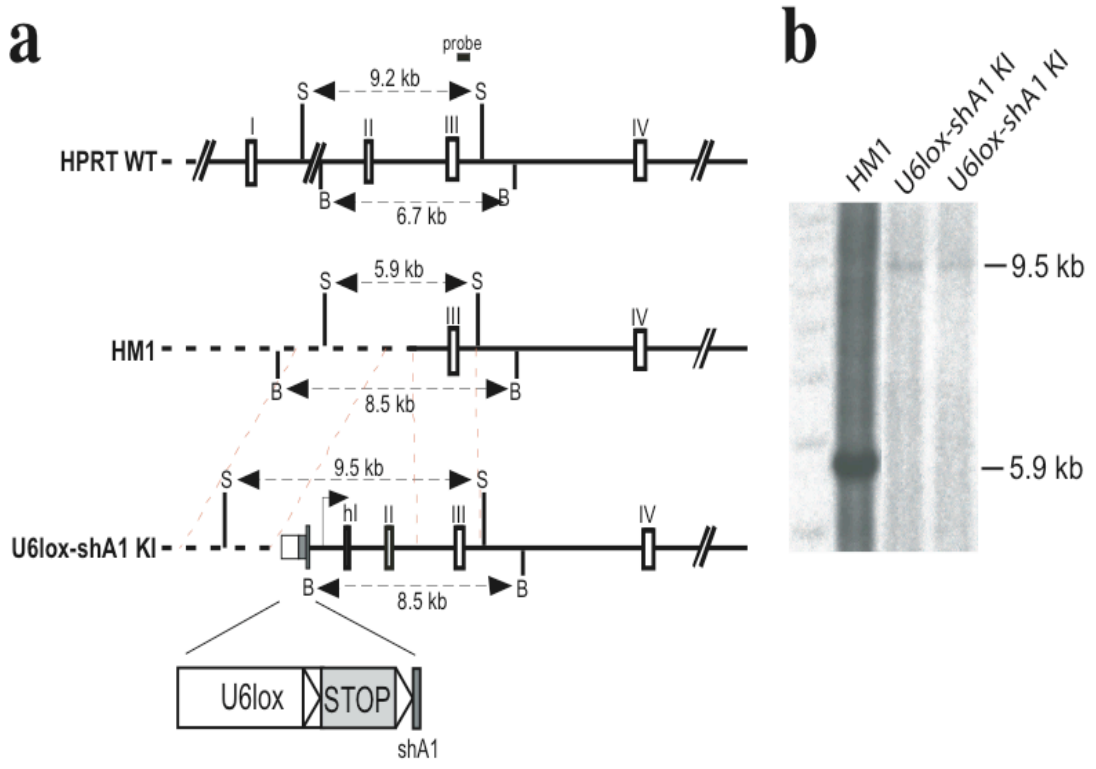

Figure 7. Targeting strategy for U6lox-shA1 insertion into the HPRT locus. (a) Partial restriction endonuclease maps of the HPRT WT locus, the mutant HM1 locus lacking exons 1 and 2 and the targeted U6lox-shA1 locus; KI, knock in. Roman numbers indicate exons, hI, human exon 1. Dashed arrows depict fragment sizes as revealed with RSA probe. B, BamHI; S, StuI. **(b)** Southern blot analysis to verify homologous recombination. Genomic DNA from two targeted clones and HM1 ES cells was digested with StuI and hybridized to RSA probe. Expected fragments before and after homologous recombination are indicated.

C 3.2.3 Generation of (A1)IRES-GFP expression vectors

In order to test whether the U6lox-shA1 cassette is functional, I sought to analyze Cre-mediated induction of shRNA expression and subsequent knock down of A1 in ES cells. Since endogenous A1 expression is barely detectable in ES cells, an A1-encoding transgene was introduced into targeted ES cells. To be able to detect changes in A1 protein levels by FACS analysis, A1 cDNA was fused to an internal ribosomal entry site (IRES, ((Jackson et al., 1990; Jang and Wimmer, 1990)) followed by EGFP cDNA. Expression of this fusion construct results in a bicistronic mRNA encoding A1 and EGFP, and siA1-mediated mRNA degradation will result in loss of both A1 and EGFP expression. The coding sequence of the mouse A1d gene was PCR-amplified from splenic cDNA using primers A1d-X and A1d-B (see Table I), which introduced a 5' XhoI site and a 3' BamHI site. The PCR fragment was then subcloned into BamHI/XhoI-digested pIRES2-EGFP (Clontech) to generate the A1-IRES-GFP fusion construct. In order to address sequence specificity of siA1, a second, mutated A1 expression construct (mutA1-IRES-GFP) was cloned into pIRES2-EGFP. The mutA1 cDNA contains 6 conservative mutations at the siA1 target site (see Figure 8) and was generated by PCR amplification using primers A1d-X and mutA1d-B (see Table I). (A1)IRES-GFP constructs were subcloned into the *neo*^{R} selectable marker containing expression vector pCXN2 (Niwa et al., 1991). A1-IRES-GFP, mutA1-IRES-GFP and IRES-GFP fragments were excised from the respective pIRES2-EGFP vectors using XhoI and NotI and inserted into an XhoI site 3' of the chicken β-actin promoter of pCXN2. Expression vectors were SalI-linearized and transfected into U6lox-shA1 ES cells. Stable integrants were selected with G418 starting 2 days after transfection. Single G418-resistant ES cell colonies were analyzed for GFP expression in order to confirm expression of the reporter transgene.

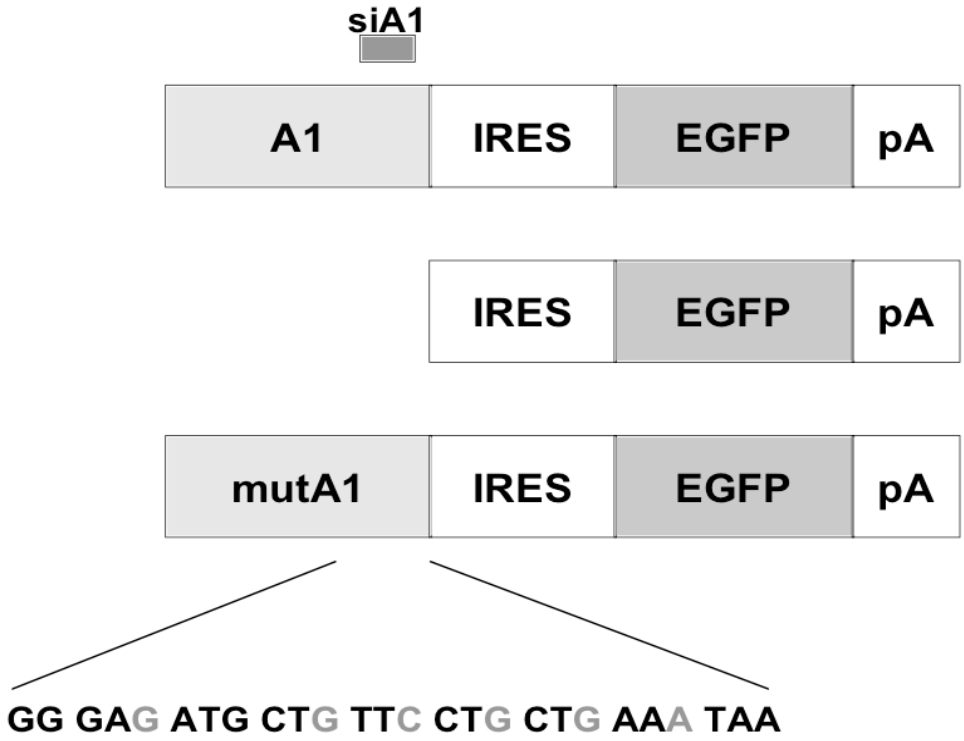

Figure 8. Schematic of (A1)IRES-GFP expression constructs. Constructs are not drawn to scale, The mutated siA1 target sequence of construct mutA1-IRES-GFP is shown, gray letters indicate mutated bases. pA, rabbit β-globin polyA site of the pCXN2 expression vector.

C 3.2.4 Cre-mediated induction of RNAi in ES cells

GFP^+ clones of each (A1-)IRES-GFP transgenic ES cell line were transduced with a Cre expressing adenovirus (Akamatsu et al., 2003; Bassing et al., 2002a; Bassing et al., 2003) in order to delete the *loxP*-flanked STOP cassette and induce shRNA expression. Untransduced cells served as negative control. Seven days after transduction, ES cells were analyzed for GFP expression by FACS analysis. Only ES cell clones that were exposed to Cre and carried the perfectly complementary A1-IRES-GFP transgene showed downregulation of GFP expression, demonstrating sequence specific and inducible RNAi in U6lox-shA1 ES cells (Figure 9A). The fact that GFP downregulation occurred only in ~60% of cells likely reflects incomplete deletion of the STOP cassette. This was confirmed by PCR analysis of genomic DNA isolated from total cell lysate or subpopulations that were sorted according to GFP expression levels. Deletion of the STOP cassette was incomplete in the bulk sample and was exclusively detected in GFP^{low} cells (Figure 9B). Importantly, similar levels

of Cre-mediated deletion and concomitant siRNA generation were detected in all Cre-treated (A1)IRES-GFP ES cell lines, emphasizing the specificity of siA1 for A1-IRES-GFP mRNA (Figure 9B and C). To determine the extent of mRNA degradation, (A1)IRES-GFP mRNA levels were analyzed by Northern blotting using a probe specific for EGFP. The size of the detected mRNA differed depending on the presence or absence of the A1 cDNA. Detection of GAPDH mRNA served to normalize for loading differences. GFP mRNA levels were strongly reduced in total cell lysate and the remaining mRNA is likely to originate from cells that have not undergone deletion of the STOP cassette. Indeed, when cells were sorted according to GFP expression, A1-IRES-GFP mRNA was barely detectable in GFP^{low} cells and image quantification showed a >10 fold reduction of mRNA when compared to GFP^{high} cells. No mRNA reduction could be observed in untransduced A1-IRES-GFP transgenic ES cells or in IRES-GFP control samples. These data demonstrate that a single copy of the U6lox-shA1 cassette mediates efficient, sequence-specific and tightly regulated suppression of A1 *in vitro*.

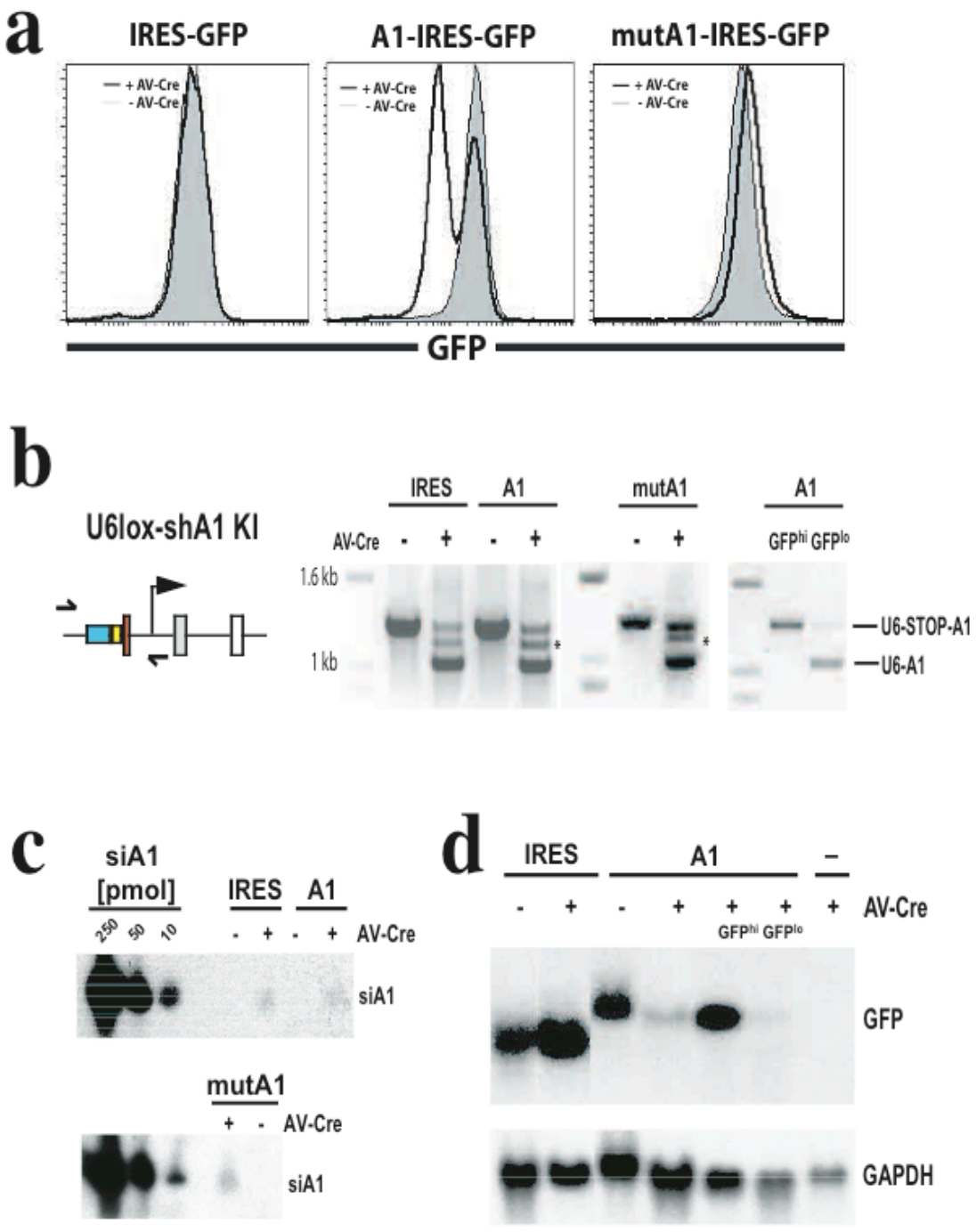
a
IRES-GFP
A1-IRES-GFP
mutA1-IRES-GFP
+ AV-Cre
- AV-Cre
GFP
b
U6lox-shA1 KI
IRES
A1
mutA1
A1
AV-Cre
GFPhi GFPlo
1.6 kb
1 kb
U6-STOP-A1
U6-A1
c
siA1 [pmol]
250
50
10
IRES
A1
AV-Cre
siA1
mutA1
siA1
d
IRES
A1
–
AV-Cre
GFPhi GFPlo
GFP
GAPDH

Figure 9. Sequence-specific Cre-mediated induction of RNAi in ES cells. (A1)IRES-GFP transgenic U6lox-shA1 KI ES cells were transduced with Cre-expressing adenovirus and analyzed 7 days thereafter. **(a)** FACS analysis of GFP expression in transduced (open histograms) or untransduced ES cells (shaded histograms). The respective (A1)IRES-GFP transgene is indicated. AV, adenovirus. **(b)** PCR analysis to detect Cre-mediated deletion of the polIII STOP cassette. A schematic of the targeted HPRT locus is shown, half-arrows depict primers hHPRT-pro and HPRT-SAH (see Table I) flanking the inserted U6lox-shA1 cassette. The arrow represents the human HPRT promoter, the gray box depicts human exon 1, the white box mouse exon 2; map is not drawn to scale. PCR results are shown for transduced and untransduced ES cells transgenic for IRES-GFP (IRES), A1-IRES-GFP (A1) or mutA1-IRES-GFP (mutA1). A1-IRES-GFP transgenic ES cells were sorted according to GFP expression levels. DNA from GFP^{high} cells and GFP^{low} cells was subjected to PCR. The expected sizes for PCR fragments before (U6-STOP-A1) and after deletion of the polIII STOP cassette (U6-A1) are indicated. The asterisks indicate a fragment resulting from a DNA hybrid of one U6-STOP-A1 strand and one U6-A1 strand. **(c)** Northern blot analysis of siA1 expression in transduced and untransduced ES cells carrying the indicated transgene. 20 μg of total RNA were loaded per lane. Synthetic ds siRNA of identical sequence was loaded as indicated to estimate siRNA expression levels. **(d)** Northern blot analysis of (A1)IRES-GFP mRNA expression levels before and after transduction. 20 μg of total RNA were loaded per lane. Cre-transduced A1-IRES-GFP transgenic ES cells were sorted according to GFP expression levels and total RNA from 10^6 cells was loaded for GFP^{high} and GFP^{low} samples. Targeted ES cells without IRES-GFP transgene served as negative control (–). Blots were hybridized to GFP probe. To account for loading differences, blots were stripped and rehybridized to GAPDH probe. The efficiency of siA1 mediated A1-IRES-GFP knock down was determined using phosphoimager.

C 4 DISCUSSION

Both Cre/*loxP*-mediated conditional mutagenesis and RNA interference provide powerful means to study gene function *in vitro* and *in vivo*. Here, I describe an extension of the current applicability of Cre/*loxP* technology as well as the generation of a novel approach to manipulate the mouse genome, which combines conditional gene targeting with RNA interference in order to achieve cell-type specific and/or developmentally regulated gene knock-down in mice.

C 4.1 Unidirectional Cre-mediated gene inversion

Cre/*loxP*-mediated inversion of genomic DNA segments represents a useful tool to alter the mouse genome. However, its applicability is hampered by the fact that this reaction is fully reversible. The mutant *loxP* pair *lox66*/*lox71* has been shown to display a favorable forward reaction equilibrium *in vitro* (Albert et al., 1995; Araki et al., 1997; Zhang and Lutz, 2002). In order to generate a system that allows unidirectional gene inversion in mice, I generated a *lox66*/*lox71*-flanked switch VDJ-switch substrate and analyzed its recombination properties both *in vitro* and *in vivo*. In an *in vitro* dose response analysis, the *lox66/lox71* pair has a higher threshold for the initiation of Cre-mediated recombination and requires two- to threefold higher Cre concentrations to recombine with the same efficiency as WT *loxP* sites (Figure 2). No reverse recombination could be detected *in vitro* by Southern blotting. Given that the forward reaction occurred in less than 40% of cells at the maximal dose of TAT-NLS-Cre and is known to be considerably more efficient than the reverse reaction (Albert et al., 1995), this is not a surprising result. Sensitivity of Southern blot analysis is, however, limited and low levels of reverse recombination cannot be excluded. Toxicity of the TAT-NLS-Cre fusion protein at doses higher than 200 µg/ml made further increase of the TAT-NLS-Cre concentration impossible.

Since Cre/*loxP*-mediated recombination is predominantly used for conditional mutagenesis *in vivo* and different *cre*-transgenic mouse strains are likely to display varying levels of Cre expression, it was important to address the efficiencies of *lox66*/*lox71*-mediated forward and *lox72*/WT *loxP*-mediated reverse recombination *in vivo*. In mice that carry Cre under the control of either a cell-type specific or an inducible promoter, *lox66*/*lox71*-mediated inversion is highly favored over the

lox72/WT *loxP*-mediated reverse reaction. In both cases, the overall recombination efficiency of mutant *loxP* sites is slightly reduced when compared to WT *loxP* sites. Nevertheless, *lox66*/*lox71*-mediated inversion is almost complete in liver and hematopoietic stem cells of poly(I)*poly(C)-treated *Mx-cre* animals (Figure 3) and in mature T cells from *CD4-cre* mice (Figure 4). Up to 5% of *Mx-cre*-transgenic hematopoietic stem cells undergo *lox72*/WT *loxP*-mediated reverse recombination upon poly(I)*poly(C) treatment (Figure 3C) and a similar reversion frequency might account for the incomplete inversion observed in mature *CD4-cre* T cells (Figure 4). This indicates that reverse recombination can occur at low levels and – based on previous work in plants (Albert et al., 1995) – appears to correlate with the concentration of Cre. The difference in WT *loxP*- and *lox66*/*lox71*-mediated recombination frequencies observed *in vitro* and *in vivo* is likely to reflect intrinsic differences between WT and mutant *loxP* sites. In the case of the latter, Cre/*loxP* complex formation might be impaired due to inefficient recruitment of the Cre-monomer to mutated inverted repeats. Potential differences in accessibility of *loxP*-flanked alleles can be ruled out since both WT and mutant *loxP* sites analyzed in this study were targeted into the same genomic positions.

Taken together, *lox66*/*lox71*-mediated recombination represents an easily applicable tool for the inducible and essentially unidirectional alteration of the mouse genome by Cre-mediated gene inversion *in vitro* and *in vivo*. Recently, Schnuetgen et al. (Schnutgen et al., 2003) described a different approach to achieve unidirectional Cre-mediated gene inversion employing a combination of WT and mutant *loxP* sites (*lox511*), which efficiently recombine with themselves but very inefficiently with each other (Hoess et al., 1986). This system has been reported to yield a high inversion frequency *in vivo* with undetectable levels of reverse recombination. However, low levels of *loxP/lox511* mediated reverse recombination could not be excluded by the authors, since the detection limit of the PCR approach used to quantify this event was not determined.

C 4.2 Cre-mediated induction of RNAi in ES cells

Cre-mediated induction of gene expression generally relies on the removal of a *loxP*-flanked STOP cassette, which contains several transcriptional and/or translational termination elements (Lakso et al., 1992). In the case of RNA polII promoters, the STOP cassette can be integrated almost anywhere between the promoter and the first coding exon of the gene of interest. Cre-mediated removal of the STOP cassette results in one remaining *loxP* site, which is ideally located in an intron and will therefore not contribute to the mature mRNA. However, induction of RNAi *in vivo* is generally achieved using RNA polIII-mediated shRNA transcription. This complicates the use of a *loxP*-flanked STOP cassette for two reasons. First, no transcription termination cassette has been described for RNA polIII-mediated transcription. Second, RNA polIII drives transcription of a very specialized set of genes, which commonly encode structural or catalytic small RNAs and do not contain intronic or 'non-coding' sequence (reviewed in (Schramm and Hernandez, 2002)). As a consequence thereof, incorporation of a *loxP* site into RNA polIII transcripts is expected to interfere with the catalytic function of small RNAs. In the case of shRNA transcripts, the presence of a *loxP* site may have unpredictable and possibly harmful effects on shRNA processing into siRNAs and subsequent induction of RNAi.

Here, I describe the generation of a *loxP*-flanked RNA polIII-specific STOP cassette that efficiently inhibits shRNA transcription. The STOP cassette was inserted such that, upon Cre-mediated recombination, the remaining *loxP* site is located in the RNA polIII promoter between the TATA box and the transcription initiation site (+1) and hence is not incorporated into the shRNA transcript. Cre-mediated deletion of this STOP cassette initiates gene-specific knock down of a target gene in ES cells by RNAi. The knock-down efficiency obtained with one copy of the U6lox-shA1 transgene is comparable to knock-down efficiencies that have been observed previously for both stable and transient RNAi (Brummelkamp et al., 2002; Elbashir et al., 2001; Song et al., 2003; Sui et al., 2002).

Accommodation of the remaining *loxP* site upstream of the shRNA transcription initiation site required the mutation of the last 5 bp of the shRNA-proximal *loxP* site (Figure 6). This might limit the general applicability of U6lox-shRNA constructs, since a similar *loxP* mutation showed reduced Cre-mediated recombination efficiency *in vivo* (see chapter B 3.1). This effect is thought to be due to inefficient recruitment

of the Cre-monomer to mutated inverted repeats. Given that only one of the two *loxP* sites in the U6lox-shA1 cassette is mutated, the reduction in recombination efficiency is expected to be less than what has been described in chapter B 3.1. Consistent with this notion, recombination of U6lox-shA1 occurred at levels comparable to WT *loxP*-flanked control segments *in vitro* ((Päper, 2002) and Figure 9). Since different shRNA sequences will result in different mutations of the shRNA-proximal *loxP* site and distinct mutations can result in slightly different recombination properties (Albert et al., 1995), it is advisable to test the recombination efficiency of every newly generated U6lox-shRNA cassette *in vitro*. With this limitation in mind, the system presented here likely provides a useful tool to induce cell-type specific and/or developmentally controlled RNAi *in vivo*.

Recently, Czauderna et al. described an alternative approach to achieve controlled induction of RNAi *in vivo* (Czauderna et al., 2003). This approach employs the tetracylin-regulated bacterial transcriptional repressor tetR, which binds to a specific sequence element (termed tetO) inserted into the promoter region of the gene of interest and thereby inhibits its transcription. Release of the repressor can be triggered by administration of tetracyclin, which binds tetR and leads to a conformational change incompatible with tetR-tetO interaction (reviewed in (Corbel and Rossi, 2002)). Insertion of a tetR-binding element into the U6 promoter has been demonstrated to allow tetracyclin-mediated control of shRNA transcription (Czauderna et al., 2003). While deletion of a transcriptional STOP cassette is an irreversible event and induction of shRNA transcription is permanent, the tetR system allows reversible induction of shRNA transcription. The two systems therefore represent complementary approaches to control RNAi in mice.

Given the vast number of mouse mutants that carry distinct cell-type specific *cre* transgenes, Cre-mediated induction of shRNA expression is expected to provide a useful and widely applicable tool for conditional RNAi *in vivo*. A list of published Cre strains can be found on http://www.mshri.on.ca/nagy/cre.htm.

C 5 SUMMARY

For the last decade, Cre/*loxP*-mediated conditional mutagenesis has been instrumental to the study of gene function in mice. Both Cre-mediated deletion and inversion of *loxP*-flanked gene segments are widely used to manipulate the mouse genome. While Cre-mediated deletion is essentially unidirectional, inversion is reversible and therefore does not support the stable alteration of gene function in cells that constitutively express Cre. Site-directed mutagenesis yielded a pair of asymmetric *loxP* sites (*lox66* and *lox71*) that displays a favorable forward reaction equilibrium *in vitro*. Here, I demonstrate that *lox66*/*lox71* mediates efficient and predominantly unidirectional inversion of a switch substrate that has been targeted into the mouse genome. *lox66*/*lox71*-mediated recombination was two to three times less efficient than that of a WT *loxP* pair both *in vitro* and *in vivo*. Inversion efficiency was generally dependent on Cre expression levels and near complete *lox66*/*lox71*-mediated inversion could be observed *in vivo* using both an inducible and a cell-type specific *cre* transgene. Reverse recombination was undetectable in most tissues. Taken together, *lox66*/*lox71* allows essentially unidirectional gene inversion. Its efficiency is, however, dependent on the expression level of the *cre* transgene of choice.

Over the past few years, RNAi has been introduced as an additional means to manipulate gene expression. RNAi allows the simultaneous knock-down of several highly homologous gene products using a single short hairpin RNA (shRNA) encoding transgene. Here, I combined RNAi with Cre/*loxP* technology to create a system that will allow the cell type-specific and/or developmentally regulated induction of RNAi in mice. In order to control shRNA expression by Cre, I designed a *loxP*-flanked transcription termination cassette, which can be removed by Cre-mediated recombination. A transgene encoding this inducible expression vector has been integrated into the mouse HPRT locus. Efficient and sequence-specific knock-down of a target gene was observed with a single copy of this transgene in embryonic stem cells and this process was strictly dependent on Cre.

C 6 ZUSAMMENFASSUNG

Konditionale Mutagenese spielt eine zentrale Rolle bei Gen-Funktionsanalysen in Mäusen. Das Cre/*loxP* Rekombinationssytem ist das am weitesten verbreitete System, um Gene zelltyp-spezifisch oder zeitlich abgestimmt zu inaktivieren. Cre Rekombinase erkennt eine spezielle DNA Sequenz, die als *loxP* Element bezeichnet wird. Gen-Segmente, die von *loxP* Elementen flankiert sind, können, je nach Orientierung der *loxP* Elemente, entweder aus dem Genom enfernt oder invertiert werden. Cre-vermittelte Genentfernung ist nahezu irreversibel. Gen-Inversion, hingegen, ist umkehrbar solange Cre ausgeprägt wird, und lässt sich nur für bestimmte Fragestellungen anwenden. Anhand gezielter Mutagenese wurden zwei mutierte *loxP* Elemente indetifiziert, die Rekombination vornehmlich in eine Richtung erlauben (*lox66* und *lox71*). Die vorliegende Arbeit zeigt, dass ein *lox66*/*lox71*-flankiertes, ins Maus Genom integriertes Gen-Segment effizient und nahzu irreversibel invertiert werden kann. Obwohl die Rekombinatinseffizient von *lox66*/*lox71* zwei- bis dreifach geringer ist als die unmutierter *loxP* Elemente, wurde in Kombination mit einem gewebespezifischen oder einem induzierbaren Cre-Transgen nahezu komplette Gen-Inversion erreicht. Rückrekombination war in der Mehrzahl der Gewebe nicht detektierbar. Die Effizienz *lox66*/*lox71*-vermittelter Rekombination hängt allerdings vom Expressionsgrad des gewählten Cre-Transgens ab.

Während der letzten Jahre hat sich RNA Interferenz (RNAi) als eine Alternative zur klassischen Genmanipulation etabliert. RNAi ermöglicht die spezifische Degradation von mRNA durch die Transkription kurzer doppelstrangiger RNA-Moleküle (shRNAs). In dieser Arbeit wurde RNAi mit dem Cre/*loxP* Rekombinationssystem kombiniert, um eine Methode zur induzierbaren RNAi in Mäusen zu etablieren. Um die Ausprägung von shRNA mittels Cre kontrollieren zu können, wurde ein *loxP*-flankiertes DNA-Element entwickelt, das zur vorzeitigen Terminierung von shRNA-Transkription führt. Dieses Element kann durch Cre-vermittelte Rekombination entfernt werden. Die induzierbare shRNA Expressionskassette wurde in den HPRT Genlokus der Maus integriert und vermittelte effiziente, Cre-abhängige RNAi in embryonalen Stammzellen.

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D Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die verwendeten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschliesslich Tabellen, Karten und Abbildungen –, die anderen Werken in Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner Fakultät oder Universität zur Prüfung vorgelegt hat; dass sie abgesehen von den unten angegebenen Teilpublikation noch nicht veröffentlicht worden ist, sowie dass ich eine solche Veröffentlichung vor dem Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Klaus Rajewsky betreut worden.

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