A Mouse Model to Test Secondary Gene Rearrangements in the T Cell Receptor α Locus

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von
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aus Düsseldorf

2000

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Tag der letzten mündlichen Prüfung: 15. Januar 2001

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CONTENTS

1 INTRODUCTION

Any living organism, uni- or multicellular, has to defend itself against potential pathogens. Defense mechanisms include physical and chemical barriers, cellular and humoral responses. Cellular and humoral responses against pathogens are mediated by the immune system. A hallmark of the vertebrate immune system is its capability of mounting an adaptive immune response and the aquisition of memory. Adaptive immune responses enable vertebrates to fend off pathogens and toxins which have never been encountered before. Basis for the adaptivity is the generation of receptor and effector molecules through random gene rearrangements. The receptors are clonally expressed and allow recognition of antigen. Two cell types use this type of non-germline encoded receptors: B and T lymphocytes. B lymphocytes express the B cell receptor (BCR), also called antibody or immunoglobulin (Ig). Binding of an antigen to the BCR together with additional secondary signals results in activation and differentiation of B lymphocytes which then can then start to secrete Ig. The secreted Ig is produced in isoforms with different effector functions. T lymphocytes on the other hand express the T cell receptor (TCR), of which two types can be distinguished: αβ TCR and γδ TCR. Triggering of TCR can result in activation and differentiation of T cells, but in contrast to Ig the TCR is never secreted and does not carry out any effector functions by itself.

Unless otherwise stated, T cells in this thesis are defined as cells of the αβ TCR lineage.

1.1 The Role of T Cells in Adaptive Immunity

T lymphocytes cannot recognize native antigen. Most T cells are specialised on recognition of fragments of polypeptides, which are presented to them by other cells. These short peptides are generated by degradation of synthesised or phagocytosed protein and displayed in an exposed groove on molecules of the major histocompatibility complex class I and class II (MHC class I/II). Such peptide/MHC complexes are recognized by the TCR (Zinkernagel and Doherty, 1979; Garcia et al., 1996). A peptide/MHC complex which triggers activation of a specific T cell is referred to as cognate antigen. MHC class I and class II molecules consist of an α- and a β-chain. In the mouse the α-chain of MHC class I is encoded by the genes H-2K, H-2D and H-2L and forms a dimer with the β-microglobulin molecule. The two MHC class II molecules consist of an α-chain and a β-chain which are encoded by the genes H-2I-Aα/β and H-2I-Eα/β. Allelic diversity leads to a complex pattern of MHC expression in each individual. In mouse inbred strains, the MHC haplotype is indicated by use of superscript letters: i.e. H-2^b in C57BL/6 or H-2^d in BALB/c. T cells of any individual are only acit-

vated by foreign peptide complexed in self-MHC, a phenomenon called self restriction. The self restricted T cell repertoire is shaped in selection processes in T cell development after TCR gene rearrangement.

By presenting peptides of different origin MHC class I and class II allow T cells to monitor the body for intracellular and extracellular pathogens, respectively.(for review: Yewdell and Bennink, 1990).

MHC class I is expressed by all nucleated cells and the peptides presented by it are mostly fragments of proteins synthesised by the same cell. This mechanism allows the detection of pathogens which either take over the protein synthesis machinery (e.g. viruses) or secrete proteins into the cytoplasm (e.g. bacteria, unicellular parasites). T lymphocytes which recognize peptide presented by class I molecules express the co-receptor CD8 and are also called cytotoxic T cells (Tc cells) or killer T cells. They can differentiate into cytolytic effector cells which prevent further spread of a pathogen by killing infected cells. The cytolytic activity may result either from release of perforin and granzyme, release of cytokines or triggering of Fas protein.(for review: Kisielow and von Boehmer, 1995).

MHC class II proteins are constitutively expressed on specialised antigen presenting cells (APCs) which comprise macrophages (MΦ), monocytes, dendritic cells (DC), and B cells (for review: Benoist and Mathis, 1990). However, expression of MHC class II is inducible on a large number of other cell types (for review: Benoist and Mathis, 1990). APCs present peptides generated in the lysosomal pathway after phagocytosis of the antigen. MHC classII/peptide complexes are recognized by T cells expressing the coreceptor CD4, called T helper cells (Th cells). They are involved in the regulation of cellular immunity and antibody production. Th cells exert their regulatory function by differentiation into differrent subsets which secret distinct sets of cytokines (for review: Kisielow and von Boehmer, 1995).

1.2 The T Cell Receptor

The αβ T cell receptor consists of two transmembrane polypeptide chains. The two chains form a V-(variable) and a C-(constant) domain. They are connected by a disulfide chain between the C-region and the transmembrane domain. The cytoplasmic tail consists of 4 amino acids.

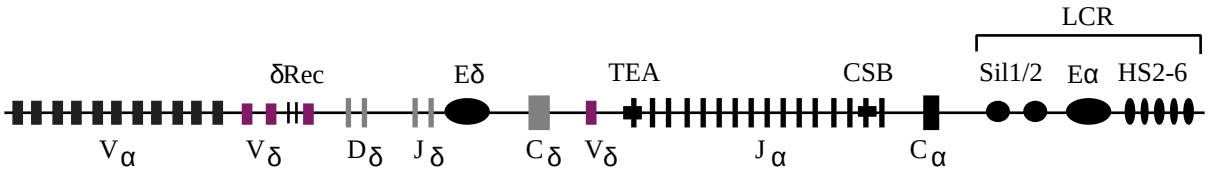
The TCR itself is unable to transduce signals into the cytoplasm. It associates with the CD3γ/ε, and CD3δ/ε heterodimers, and a CD3ζ/ζ(η) homo-(hetero-) dimer to form the signal transducing TCR complex (for review: Cantrell, 1996; Wange and Samelson, 1996; Jacobs, 1997). It is common for antigen receptor complexes to contain immunoreceptor tyrosine-based activation motifs (ITAMs) (Reth, 1989) of which ten are present in a single TCR complex (for review: Love and Shores, 2000). T cell

activation results in phosphorylation of the ITAM tyrosine residues which serve as binding sites for protein tyrosine kinases like Lck, Fyn, ZAP70 and Syk (for review: Cantrell, 1996; Schraven et al., 1999; Kane et al., 2000). These kinases activate a plethora of signal transduction pathways including the mitogen-activated kinase (MAP) pathway, calcium-flux, phospholipase C, the θ-isoform of phoshokinase C (Monks et al., 1997) and others (for review: Damjanovich et al., 1992; Schraven et al., 1999). The TCR complex is organised in special detergent resistant microdomains of the membrane, called rafts, which are enriched in glycosphingolipids and cholesterol (Xavier and Seed, 1999). It was shown that organisation of the TCR complex in rafts is required for successful activation of a T cell (Moran and Miceli, 1998; Xavier et al., 1998). Triggering of the TCR results in a complex reorganisation of the T cell - APC contact zone (Grakoui et al., 1999). Activation of the T cell is then induced by serial triggering of TCR complexes leading to downregulation of surface TCRs (Valitutti et al., 1995). However, T cell activation does not only depend on triggering of the TCR, it is also modulated by other receptors such as the CD28-B7 pair (Viola and Lanzavecchia, 1996).

1.3 Organisation and Recombination of TCR Genes

The exons encoding for the V regions of TCRα and TCRβ chains are generated by recombinatorial joining of variable (V), diversity (D), and joining (J) elements mediated by the site specific V(D)J recombinase. Recognition of recombination signal sequences (RSS) flanking each V, D, and J element initiates the recombination process (for review: Fugmann et al., 2000). An essential part of the V(D)J recombinase are products of the genes rag-1 and rag-2 (Schatz, 1989; Oettinger et al., 1990; Mombaerts, 1992a; Shinkai, 1992). Removal or addition of nucleotides at the joints further increases the diversity of the TCR V regions (Schatz, 1992). The highest diversity within a V region is found in the three hypervariable complementary determining regions (CDR). CDR1 and CDR2 determine the MHC restriction, CDR3 is formed by the junction of the combinatorial joining proces and contacts the peptide (Garcia et al., 1996). A specific clonally expressed TCR of a certain specificity is characterized by its particular Vα and Vβ regions and called idiotype (Id).

TCRβ recombination is characterized by joining of Vβ, Dβ, and Jβ elements, TCRα recombination, however, involves direct joining of a Vα with a Jα element.The V, D, and J gene segment families are organised in groups on the chromosomes which most likely have been generated through successive genomic duplication events. The genes for the TCRα and TCRδ chains map to murine chromosome 14 (Dembic et al., 1985). The whole region spans about 1000 kb (Griesser et al., 1988) and its organisation of the locus is peculiar in that Dδ and Jδ elements and the Cδ exons are placed



E	Enhancer	Sil	Silencer
ψ	Pseudogen	HS	DNase-hypersensitive Region
TEA	T Early Alpha		
CSB	Conserved Sequence Block		
LCR	Locus Control Region		

Fig 1: The TCRα/δ-Locus of the mous.The exons of the C regions were not shown. The number of depicted V and J segments is arbitrary.

between the Vα and Jα elements (fig. 1; Chien, 1987). The TCRα and TCRδ genes even share the use of some V elements (Arden et al., 1995). One out of 100–200 Vα segments can be joined to one of the 61 Jα segments (Koop et al., 1992; Koop et al., 1994; Koop and Hood, 1994; Arden et al., 1995) resulting in genomic excision of the exons of the TCRδ constant region (Petrie, 1993).

1.3.1 Control of Recombination and Transcription of the TCRαδ locus

In the last years, the TCRα/δ locus has been extensively used to study the control of transcription and recombination during T lymphocyte development. Ordered rearrangement of the TCRδ and TCRα genes is essential to ensure T cell lineage specificity since a Vα-Jα recombination event in the γδT cell lineage would lead to the deletion of the TCRδ gene (Burtrum et al., 1996).

Transcription prior to acquisition of V(D)J joints is called germline transcription and is associated with gene rearrangement, but its involvement in the recombination process is still unclear (for review: Sleckman et al., 1996). Transcription at the TCRα/δ locus depends highly on two enhancers, because the promotors of Vα/δ gene segments display only weak transcriptional activity (Ho et al., 1989; Winoto and Baltimore, 1989). The TCRα enhancer is located 4.5 kb downstream of Cα (Winoto and Baltimore, 1989) and in mice lacking the TCRα enhancer (Sleckman et al., 1997) absence of germline transcription and of recombination of the TCRα locus is observed This results in an almost complete block in T cell development. Recombination of the TCRδ gene is not impaired in these mice, but a decrease in peripheral transcription of the TCRδ gene is seen (Sleckman et al., 1997).

Two silencers which restrict the expression of the TCRα/δ locus to αβ T cells are located 5' of the TCRα enhancer (Leiden, 1993). Enhancer and silencer elements seem to be part of a larger locus control region (LCR) downstream of Cα (von Boehmer, 1990; Diaz et al., 1994; Hong et al., 1997).

The TCRδ enhancer lies between Jδ2 and Cδ (fig. 1; Redondo et al., 1990; Gill et al., 1991). It is essential for germline transcription of the TCRδ locus and the control

of the VDδ to Dδ recombination step (Lauzurica and Krangel, 1994a; Lauzurica and Krangel, 1994b). Mice with a genomic deletion of the TCRδ enhancer show a severe reduction of peripheral γδ T cells (Monroe et al., 1999). Moreover, recombination at the δ locus recombination is initiated by the joining of a Vδ to a Dδ which takes place independently of the TCRδ enhancer. A switch from TCRδ enhancer to TCRα enhancer function is observed during thymic development of both αβ and γδ T cells (Sleckman et al., 1997; Hernandez-Munain et al., 1999; Monroe et al., 1999). Thus, the TCRδ enhancer is not necessary for expression of mature TCRδ transcripts (Monroe et al., 1999) The change in enhancer activity ensures that rearranged and in-frame δ-genes are not expressed from retained excision circles which are generated by Vα-Jα joining in precursors of αβ T cells (Nakajima et al., 1995; Livak and Schatz, 1996).

Germline transcripts originate not only from V element promotors but also from promotors in the D and J regions (Shimizu et al., 1992). One important and well characterized promotor is the T early alpha promotor (TEA; fig. 1) upstream of the Jα61, the most 5´ Jα element (de Villartay et al., 1988; Hockett, 1988; de Chesseval and de Villartay, 1993). Mice with a targeted mutation of this promoter show diminished rearrangements of the seven upstream Jα gene segments (Villey et al., 1996).

Other elements which were shown to control recombination at the TCRα/δ locus include: the blocking element alpha/delta (BEAD) thought to separate recombinational control of TCRδ and TCRα (Zhong and Krangel, 1997) the δRec sequences, shown to be targets of initial Jα recombinations (Hockett et al., 1988; Hockett et al., 1989; Janowski et al., 1997; Rytkönen-Nissinen et al., 1999) and the highly conserved sequence block (CSB) which influences the Jα segment usage in recombination (fig. 1; Koop and Hood, 1994; Riegert and Gilfillan, 1999).

1.4 T Cell Development

Production of T lymphocytes starts in late embryogenesis and slowly declines after maturation of the mouse (for review: Kisielow and von Boehmer, 1995). T cells develop in the thymus from bone marrow derived precursors which can be separated into distinct developmental stages corresponding to rearrangement of the different TCR loci.

1.4.1 The $CD4^-CD8^-$ Stage

The first thymic precursors of T lymphocytes are the TCR^- cells which do not express CD4 or CD8 and are called double-negative (DN) cells (fig 2 & 4). Following developmental progression DN cells are subdivided into 4 stages (I-IV) with respect to expression of CD25 and CD44 (fig. 2; Ceredig et al., 1985; Raulet, 1985;

Trowbridge et al., 1985; Shimonkevitz et al., 1987; Pearse et al., 1989). The expression of the rag-genes starts in DN II (Ismaili et al., 1996) while rearrangement processes at the β, γ, and δ loci can only be detected in DN III (Pearse et al., 1988; Pearse et al., 1989; Rodewald, 1993; Wilson et al., 1994). TCRβ chain recombination starts at one allele and a subsequently produced β-chain protein pairs with the non-rearranging preTCRα chain (pTα) to form the pre T cell receptor (pTCR; for review: Groettrup and von Boehmer, 1993). Being autonomously recruited to membrane rafts the pTCR inititates signaling (Irving et al., 1998; Saint-Ruf et al., 2000) which terminates further recombination at the TCRβ locus (Wilson et al., 1994) and prevents acquisition of another VDJ joint on the second allele, a process called allelic exclusion (Alt et al., 1981; Aifantis et al., 1997). Because T cell development is blocked at DN III in mice deficient for TCRβ (Mombaerts et al., 1992a) this checkpoint for expression of TCR β-chain is also termed β-selection (for review: Rodewald and Fehling, 1998) The last DN compartment (DNIV) is enriched for in-frame TCRβ genes (Pearse et al., 1989; Mallick, 1993; Dudley et al., 1994; Tourigny et al., 1997) and first germline transcripts of the TCRα locus are detectable (Kishi et al., 1991).

1.4.2 The $CD4^+CD8^+$ Stage

After β-seletion the DN thymocytes progress into the $CD4^+CD8^+$, called double positive (DP) stage (fig. 2 & 4; Fehling et al., 1995), start to proliferate (8-9 times, Falk et al., 1996) and rearrange the genes for the TCRα chain (Mombaerts, 1992b; Wilson et al., 1994). At this stage the fate of the thymocytes depends on the reactivity of the expressed complete TCR. Small DP cells which display a TCR with too low affinity towards self-peptide/self-MHC do not receive a signal for further differentiation and undergo “death by neglect” after 3.5–4 days (Egerton et al., 1990; Huesmann et al.,

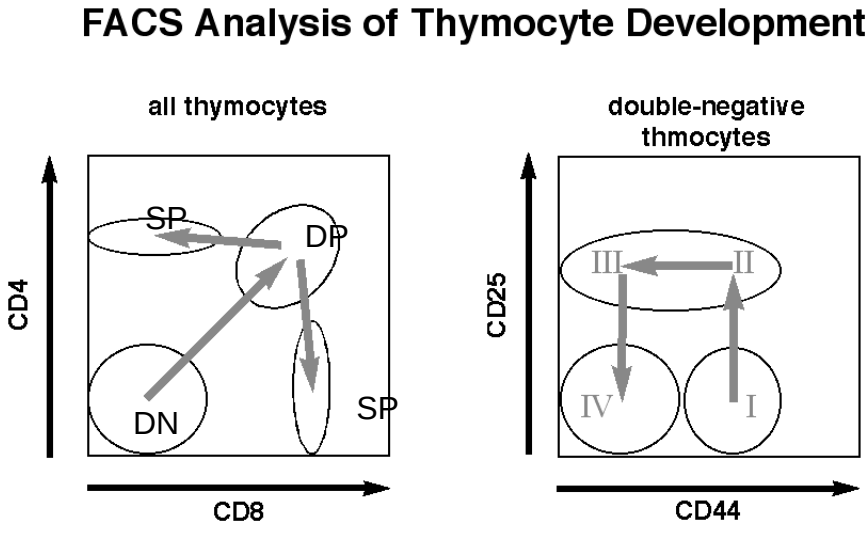


Fig. 2: Surface markers used to subdivide thymic development into different stages. The CD4 and CD8 staining is performed on total thymocytes. The DN compartment is analysed by gating on $CD4^-CD8^-$ cells and subsequent analysis of CD25 and CD44.

1991; Grusby, 1993).

The rescue program from this pre-defined fate is called positive selection (for review: Anderson et al., 1999). It is initiated by an intermediate affinity interaction (Alam et al., 1996) of the newly made TCR with self-peptide/self-MHC (Grusby, 1993) on cortical epithelial cells (Jenkinson et al., 1992; Anderson et al., 1993; Anderson et al., 1994; Lundberg and Shortman, 1994; Merkenschlager et al., 1994; Ernst et al., 1996). Positive selection results in shut-down of recombination of the TCRα loci (Turka et. al., 1991) and upregulation of the TCR (for review: Jameson et al., 1995) Subsequently, the surface expression of CD69 (Swat et al., 1993; Kishimoto and Sprent, 1997) and CD5 (Dutz et al., 1995; Azzam et al., 1998) is increased. The cells differentiate either into $CD4^+$ or into $CD8^+$ thymocytes, called single positive cells (SP) (fig. 2 & 4; Teh et al., 1988b; Cosgrove, 1991; Cosgrove, 1992; Das and Janeway, 1999) and migrate into the thymic medulla (Scollay and Shortman, 1985; Egerton et al., 1990). Maturation of SP cells is indicated by reduced surface expression of heat stable antigen (HSA) (Lucas et al., 1994). Before emigration the cells increase surface levels of the CD62L molecule (Scollay and Godfrey, 1995).

Cells expressing a TCR with high affinity for self-peptide/self-MHC undergo apoptosis (Kisielow et al., 1988a; Murphy et al., 1990; Baldwin et al., 1999). This process is termed negative selection and is one mechanism preventing autoreactivity (for review: Stockinger, 1999). In the thymus, cortical and medullary epithelial cells as well as DCs can mediate negative selection (van Ewijk et al., 1988; Marrack et al., 1988; Jenkinson et al., 1992; Anderson et al., 1993; Burkly et al., 1993; Surh and Sprent, 1994; Ernst et al., 1996; Laufer et al., 1996; Volkmann et al., 1997; Kishimoto and Sprent, 1999). Negative selection can also be induced by some proteins of bacterial or viral origin called superantigens. These superantigens bind the V region of certain TCRβ chains and link them with MHC class II molecules (Fields et al., 1996; Li et al., 1999). This results in strong activation of the thymocytes and henceforth negative selection (Berg, 1989; Pircher, 1989).

Positive and negative selection appear to be mediated by the selective activation of different mitogen-acitvated protein (MAP) kinase pathways.(Alberola-Ila et al., 1995; Swan et al., 1995; Alberola-Ila et al., 1996; O´Shea et al., 1996; Rincon et al., 1998; Sugawara et al., 1998; Pages et al., 1999). The transcription factors Nur77 and Nor1 of the orphan steroid receptor family were also shown to be involved in negative selection (Woronicz et al., 1995; Cheng et al., 1997; Xue et al., 1997).

1.4.3 Fetal Thymic Development

The development of the first T lymphocytes in the fetal thymus follows in most respects the same order as in the adult thymus. Fetal thymi can serve as a model system to analyse waves of developing thymocytes. However, some differences to T cell development in the thymus of a 4-10 week old mouse can be observed.

The first T lymphocytes generated during ontogeny of the thymus are γδ T cells. The development of these early γδ T cells is highly regulated. They appear in successive waves which are characterized by the use of particular Vγ segments, termed canonical TCRs (for review: Cheng et al., 1991; Haas, 1993). The generation of lymphocytes expressing canonical γδ TCRs ceases at around birth. First progenitors of αβ T cells are found on embryonic day E15 (Snodgrass et al., 1985a; Snodgrass et al., 1985b). They start expression of CD8 at E16 and $CD4^+CD8^+$ cells can be found on E17 (Kisielow, 1990). Two days later the first cells develop into $CD4^+$ or $CD8^+$ T cells (Kisielow, 1990).

1.5 Secondary Rearrangements in T and B Cell Development

During thymic selection 95–97% of the newly generated DP thymocytes die.The estimates of the proportion of cells undergoing apoptosis upon negative selection vary broadly from 5% to 50% (Laufer et al., 1996; van Meerwijk et al., 1997). All DP cells have already been selected for the presence of an in-frame VβDβJβ joint (see: 1.4.1). It is difficult to envisage that most of these cells get only a single chance to generate a proper TCRα protein. One possibility to reduce the waste of $TCR\beta^+$ T cell precursors would be a consecutive test of several α-chains. This can either take place by continuous recombination of the TCRα locus in the absence of a positively selecting signal or by reinduction of recombination activity at the TCRα locus upon negative selection. Receptor change supposedly due to reinduction of recombination has been first observed in B cells and termed receptor editing (Tiegs et al., 1993; for review: Radic and Zouali, 1996; Nemazee, 2000). Circumstantial and experimental

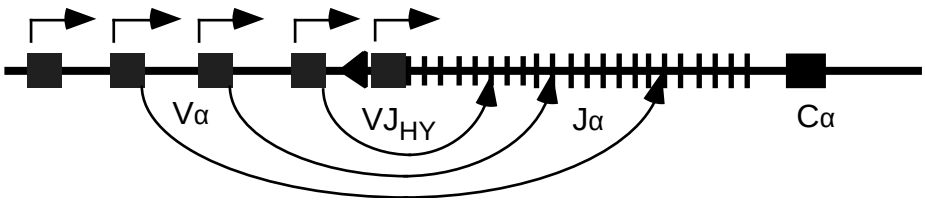


Fig. 3: Editing by successive nested rearrangments can replace a VαJα exon introduce by targeted mutagenesis into the TCRα locus of the mouse. The transcriptional orientation of Vα elements is indicated by arrows on the top. A black triangle shows the remaining loxP site. Possible secondary recombination events are symbolised by bowed arrows.

evidence suggests that also thymocytes use multiple consecutive rearrangements of TCRα genes to increase the efficiency of the thymic selection processes (fig. 3 & 4).

The high number of Vα and Jα gene segments (>75 elements and 61elements, respectively, Koop and Hood, 1994; Arden et al., 1995) build the basis for multiple rearrangements at the TCRα locus. The deletional mode of recombination guarantees that the TCRδ-locus is excised from the genomic DNA (Takeshita, 1989; Petrie, 1993) and the lack of D elements allows remaining α-elements to take part in additional recombination events.

Replacement of a rearranged and expressed TCRα gene has been observed for the first time in the cell line M14T wich also expresses RAG-1/2 proteins (Marolleau et al., 1988). Accessibility of the TCRα locus in this cell line was indicated by its continued germ line transcription (Fondell et al., 1990; Fondell et al., 1992). As was shown in murine thymocytes the replacement of VαJα joints results in excision circles which contain these joints (Okazaki and Sakano, 1988).

Other, indirect evidence for successive rearrangements at the TCRα locus come from the analysis of Vα and Jα elements used in recombination. Fetal thymocytes recombine Jα elements at the 5´ end of the Jα cluster in contrast to thymocytes from adult mice, which use the whole Jα cluster for rearrangements (Thompson et al., 1990b; Rytkönen et al., 1996; Rytkönen-Nissinen et al., 1999). Cells which have rearranged only one chromosome use Jα elements from the 5´end of the locus (Thompson et al., 1990b). Thus, it seems that thymocytes which have just started recombination of the TCRα loci use 5` Jα elements. In addition, 3´ Vα elements recombine preferentially with 5´ Jα-elements and 5´ Vα elements mainly join 3´ Jα elements (Roth et al., 1991) which could be the result of consecutive nested rearrangements.

The initial Vα - Jα joining may be targeted to the most 5´Jα segments by a strong TCRα locus germline transcript, called TEA (see: 1.3.1), which has been shown to control rearrangement of the seven upstream Jα segments (Villey et al., 1996). Subsequently, secondary recombinations result in a steady state population of thymocytes which have rearranged Jα elements from the whole region.

Additional evidence for regulation of TCRα recombination was found in thymocyte cultures. In these experiments both alleles of a cell used Jα elements from the same sub-region (Hurwitz et al., 1989; Thompson et al., 1990a) which could be the result of coordinated nested recombinations having started on both alleles (zipper-model) or the targing of the V(D)J recombinase to certain elements.

Another mechanism to increase the production of useful TCRs is the recombination and expression of both TCRα alleles, a phenomenon called allelic inclusion. In wildtype mice co-expression of two α-chains was observed in up to 30% of peripheral T cells (Casanova et al., 1991; Padovan, 1993). In TCR transgenic animals expres-

sion of the transgenic α-chain is also frequently accompanied by expression of endogenous α-chains (Malissen et al., 1988; von Boehmer, 1990; Borgulya et al., 1992). However, expression of allelically included α-chainsis often restricted to the cytoplasm (Bluthmann et al., 1988; Petrie, 1993; Alam et al., 1995; Boyd et al., 1998).

Peripheral T cells also seem to be able to escape autoreactivity by secondary recombination as observed in a TCRβ-chain transgenic mouse model (McMahan and

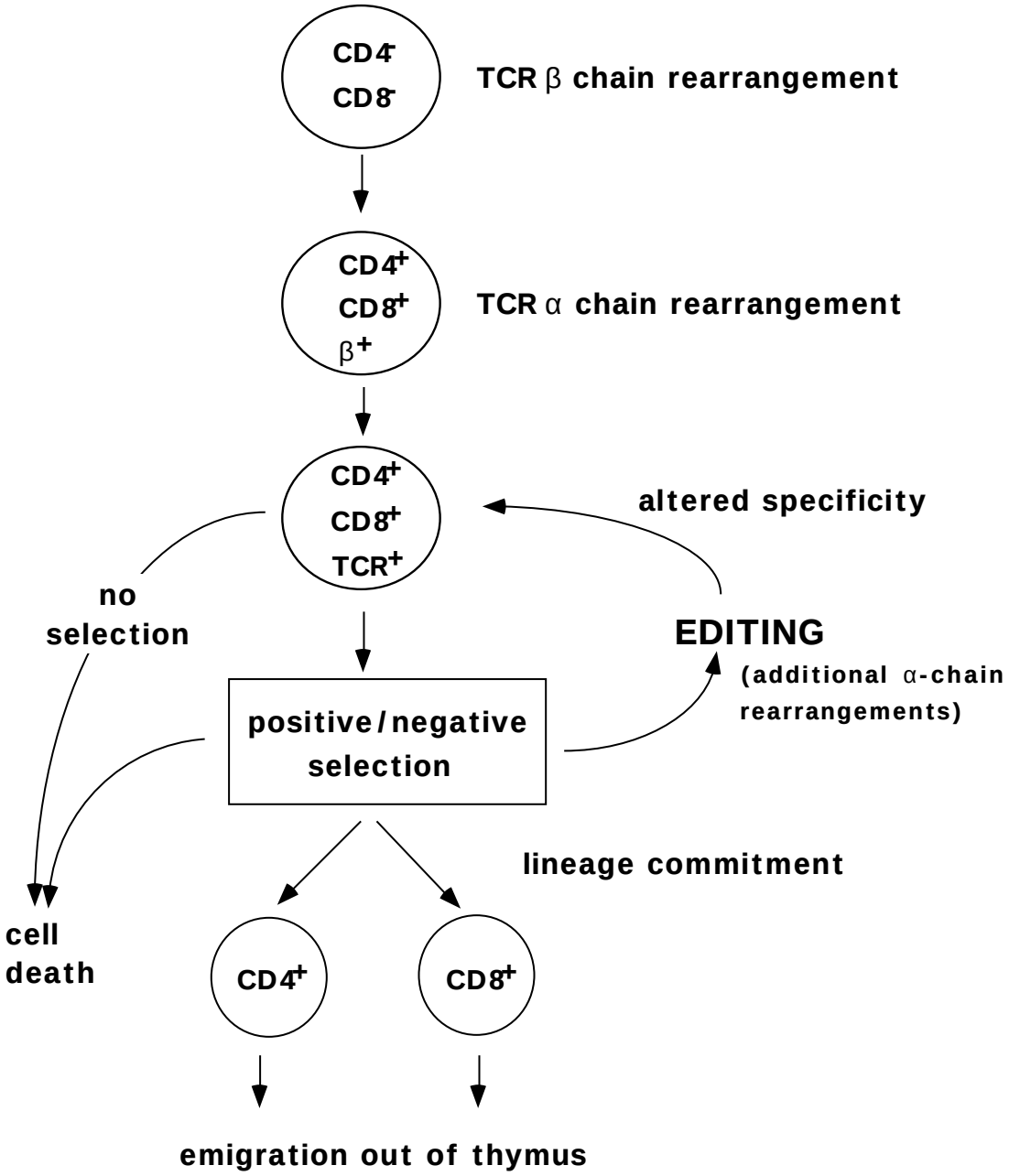


Fig. 4: Editing as a mechanism in thymic development to change receptor specificity in the case of failure during the selection processes.

Fink, 1998). In this mouse strain transgenic T cells are chronically deleted by interaction with a weak superantigen. The T cells loose expression of the transgenic β-chain, transcribe the rag-loci and have double-strand breaks at TCR loci.

1.6 Editing in B Cell Development

Editing as a mechanism to escape apoptosis induced by negative selection during B cell development has been investigated in detail (for review: Radic and Zouali, 1996; Nemazee, 2000). In mice transgenic for Ig with specificity for either DNA or MHC class I peripheral B cells do not express the autoreactive specificity (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993). Increased rag-expression mediates allelic inclusion of endogenous light chains which replace the autoreactive receptors (Tiegs et al., 1993; Hertz and Nemazee, 1997; Melamed and Nemazee 1997; Melamed et al., 1998).

However, these models do not allow replacement of the transgenes by secondary recombinations, since conventional transgenes are randomly integrated into the genome (Palmiter et al., 1982; for review: Palmiter and Brinster, 1986) and expressed independently of endogenous Ig loci (Ritchie et al., 1984; Rusconi and Kohler, 1985; Weaver et al., 1985). Gene targeting technology (Hooper et al., 1987; Capecchi, 1989) allowed the generation of mouse strains which express transgenic Ig from endogenous loci. VκJκ joints introduced into the κ locus were shown to be replaced efficiently by secondary recombination if the specificity of the Ig was autoreactive (Chen et al., 1995; Prak and Weigert, 1995; Pelanda et al., 1997; Qin et al., 1999).

1.7 The HY-TCR Model

One well characterized transgenic mouse strain to investigate thymic selection expresses a TCR (HY-TCR) recognizing a peptide encoded by the Y-chromosomal gene smcy (Agulnik et al., 1994) in the context of the MHC class I molecule H-2D^b (Markiewitz et al., 1998). The expression of the HY-TCR in transgenic mice can be followed by binding of the monoclonal antibody T3.70 to the HYα-chain (Teh et al., 1989).

In female H-2D^b mice thymocytes expressing the transgenic TCR are positively selected and differentiate into CD8$^+$T cells (Teh et al., 1988a). Negative selection is observed in transgenic H-2D^b males. Thymic cellularity in negatively selecting mice

is reduced at least 10 times and the thymus is devoid of DP and SP cells (Kisielow et al., 1988a). Transgenic thymocytes which develop in non-selecting thymic environment (e.g. H-2D^d) proceed in thymic development using allelically included endogenous α-chains (Kisielow et al.,1988b).

However, the transgenes in this conventional HY transgenic mouse strain (HYtg) are integrated randomly into the genome. This strain does not allow the investigation of secondary recombinations in thymic development.

1.8 Aim of this Thesis

Evidence has been presented which suggests that multiple rounds of recombination play a role in thymocytes which express a non-selectable receptor**.** However, the involvement of induced receptor change in tolerance induction (editing) has not been adressed before. The aim of this thesis was to generate a mouse strain which expresses the VαJα of the HY-TCR in the TCRα locus (fig. 8) and allows to investigate whether thymocytes expressing an autoreactive receptor can be rescued by secondary rearrangements.

2 MATERIAL AND METHODS

2.1 CLONING

Cloning of the targeting vectors and other plasmid vectors was performed according to standard protocols from *Molecular Cloning* (Sambrook et al., 1989). The bacteria strain DH5α was used for the amplification of plasmid DNA (Hanahan, 1985).

The restriction enzymes used in cloning and other molecular-biological methods are listed in table.

Asp718I (Boehringer)	HindIII (Gibco/BRL)	SacI (Boehringer)
BamHI (Gibco/BRL)	HincII (Biolabs)	SalI (Biolabs)
BlnI (Boehringer)	NdeI (Biolabs)	SpeI (Biolabs)
BssHI (Biolabs)	NheI (Biolabs)	SphI (Boehringer)
ClaI (Boehringer)	PstI (Gibco/BRL)	Tth111I (Biolabs)
EagI (Biolabs)	PvuII (Boehringer)	XbaI (Boehringer)
EcoRI (Boehringer)	RsaI (Biolabs)	XhoI (Gibco/BRL)
EcoRV (Gibco/BRL)		

Table 1: Restriction enzymes used in experiments described in this thesis.

2.2 VECTORS

2.2.1 Construction of the Targeting Vector pC_1T

To generate the targeting vector pC_1T (fig.5), a BamHI/XbaI fragment containing the Vα6δ3 element was cloned from cosmid 16-15 (a gift of L. Hood) into pBluescriptKS(+). The thymidinkinase gene was introduced into the BamHI and XhoI sites. Subsequently, the neomycin resistance gene which was flanked by loxP sites was inserted into the two BlnI sites.

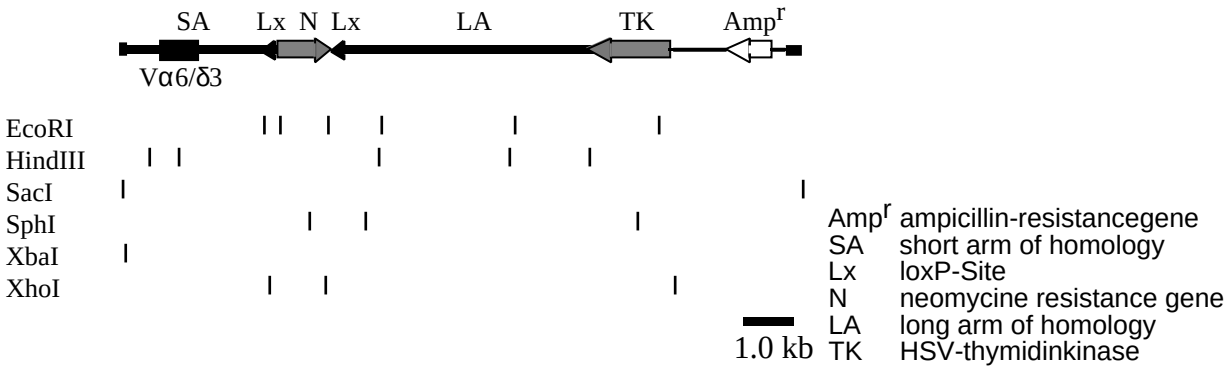


Fig. 5: Restriction map of the targeting vector pC_1T.

2.2.2 construction of the targeting vector pC_3T

The short arm of the targeting vector pC_3T (fig. 6) was generated by PCR using the primers SAForwEco and SARevSal. The product was cloned into the EcoRI and SalI sites of pBluescriptIIKS(+) and shortened by excision of a PstI fragment. The neomycin resistance gene with a 3´ loxP site was cut from pL3Neo (U. Betz) by XhoI and cloned into the SalI and XhoI sites. The short arm of homology together with the Neomycin resistance gene and the loxP site were cut out by NotI and XhoI and introduced into the NotI and SalI sites of pBS-HY which had been generated by introduction of a 11.4 kb SalI fragment containing the VaJa of the HY-TCR from the cosmid CHY-α9.1 (a gift of L. Hood) into pBluescriptIIKS(+). The thymidinkinase gene was introduced blunt end into the Asp718I site.

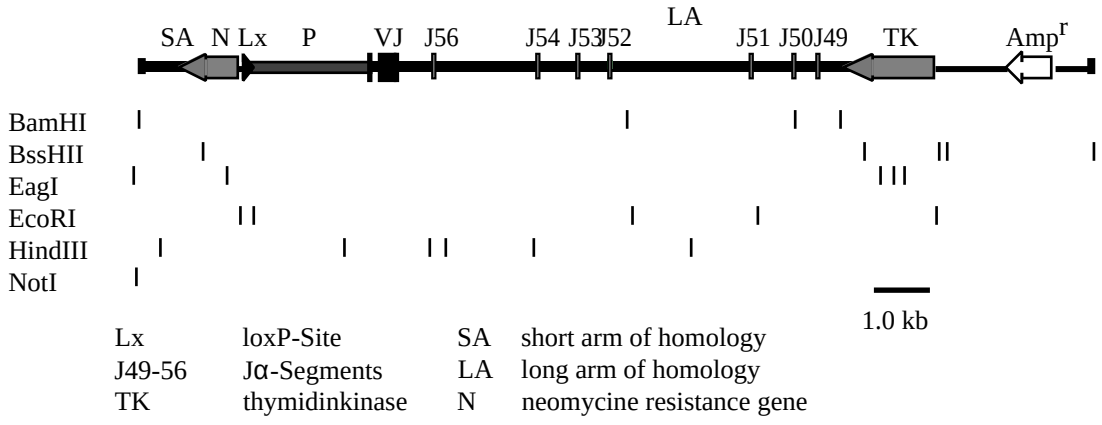


Fig. 6: Restriction map of the targeting vector pC_3T.

2.2.3 pSR65 (F. Rieux-Laucat, unpublished)

The 1.3 kb probe P1 was cut by XbaI digest from the plasmid pSR65 (fig.7) which contained genomic DNA flanking the Vα6/δ3 gene segment.

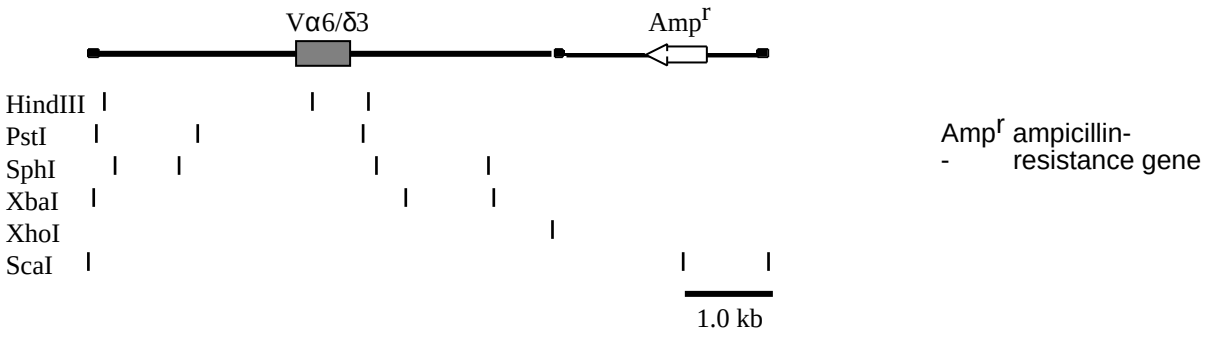


Fig. 7: Restrictionmap of pSR65.

2.3 DNA SEQUENCING

Plasmid DNA, cosmid DNA, and PCR products were sequenced using the *Ready Reaction DyeDeoxyTerminator Cycle Sequencing KIT* (Applied Biosystems) and the automatic sequencers ABI373 and ABI377 with the help of G. Zoebelein and S. Wilms. The method is based on the dideoxy-chain termination reaction with fluorescently labeled dNTPs (Sanger et al., 1977).

2.4 ES CELL CULTURE

ES cell culture was performed according to the protocols found in *Laboratory Protocols for Conditional Gene Targeting* (Torres and Kühn, 1997). The targeted introduction of a loxP site into the Va locus was verified using a Southern blot screening method. 10 days after transfection with the targeting vector pC1T and double-selection with G418 and gancyclovir, ES cell colonies were picked, grown in duplicates in a 96 well plate and then transferred to 24 well plates. The duplicate plates were overlayed with medium containing 10% DMSO and frozen at -80°C. The original plates were grown to confluency, DNA was prepared from the cells, and the clones were analyzed for the presence of the targeted mutation. Mutant clones were then thawn, the cells trypsinised and propagated according to the standard protocol. In the second targeting experiment pC_3T transfected colonies were grown up for 10 days under G418 and gancyclovir selection and then a PCR based screen was performed on half of the cells of each picked colony. This method allowed me to continue the propagation of only PCR positive clones. The cells were frozen down in 10% DMSO containing medium in a densitiy of $2x10^6$ cells in standard freezing vials. Southern blot analysis was

performed on DNA extracted from confluent wells of a 24 well plate.

In vitro deletion by Cre-mediated recombination was performed with the Cre-expression plasmid pGK-creBpA (K. Fellenberg).

Blastocyst injection and morula aggregation of mutated ES cell lines were perfomed according to protocols in *Manipulating the Mouse Embryo* (Hogan et al., 1994).

2.5 PREPARATION OF GENOMIC DNA

ES cells from one well of a 24-well plate or 0.5 cm of a mouse tail biopsy were lysed in 0.5 ml lysis buffer (100 mM Tris/HCl pH 8.5; 5mM EDTA; 0.2% SDS; 200 mM NaCl; 100µg/ml proteinase K; Laird et al., 1991) . After digestion for at least 2 hrs at 55°C (or 37°C if live ES cells remained in the 24-well plate) debris was removed by centrifugation at 14000 rpm in a table top centrifuge. The supernatant was transferred to 0.5 ml 2-propanol, mixed and the DNA was pelleted by centrifugation. After washing with 70% ethanol the DNA was dried and dissolved in 200 µl TE buffer.

2.6 SOUTHERN-BLOT ANALYSIS

For Southern blot analysis (Southern, 1975; Sambrook et al., 1989)10 µg of genomic DNA were digested with 60–100 U of restriction enzyme over night. The fragments were separated in 0.8% agarose gels stained with ethidiumbromide by electrophoresis in TAE buffer, photographed and depurinized with 0.25 M HCl for 20 min. After washing with transfer buffer (0.4 M NaOH, 0.6 M NaCl) the DNA was blotted on a Nylonmembrane (GenescreenPlus, NEN, Boston, USA) by capillary transfer for at least 4 hrs. The membrane was neutralized in 0.5 M Tris pH7.5; 1 M NaCl and prehybridized in hybridization solution (50 mM Tris pH 7.5, 1 M NaCl, 1% SDS, 10% Dextran Sulfat) for 30 min. The probe was boiled, cooled on ice, and labeled by random priming through Bca-Polymerase with 25 µCi aATP32 at 55°C for 30 min–2 hrs.

probe	description	origin	position in Jα locus (Koop et al. 1994)
probe 1	external probe 5`targeting	pSR65 1.3 kb XbaI fragment	
probe 2	eternal probe 3´targeting	pSA3 0.6 bp fragment	22614-23217
probe 3	internal probe large deletion	pBS-HY 0.5 kb SphI fragment	25317-25822
probe 4	Neomycinresistance gene internal probe	pL3Neo 1.3 kb fragment	

Table 2: Probes used for Southern blot analysis.

Unincorporated nucleotides were removed through a S-200HR column (Pharmacia). The probe was added to 400 µl water and 100 µl of salmon sperm solution (1 mg/ml, sheared), boiled, cooled and added to the hybridization solution. Hybridization was performed at 65°C over night. To remove unspecifically bound probe the membrane was washed with 2xSSC, 0.1% SDS at 65°C. The radioactive band was then visualized by autoradiography on Kodak-KAR-5 or Kodak Biomax-MS films.

2.7 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) (Saiki et al., 1985; Saiki et al., 1988) was used for the cloning of the short arm of homology of pC_3T, for the screening for homologous recombinants and for the typing of transgenic mouse strains. The primers were either synthesised by G. Zoebelein in a DNA synthesizer (Applied Biosystems 380A) and cleared from unincorporated nucleotides through a NEP-25 column (Pharmacia) or bought from ARK. Taq-Polymerase and Taq buffer were prepared by C. Uthoff-Hachenberg. Only for the PCR using the Primers VαLoxP-F and VαLoxP-R Klentherm Taq was used. The reaction mix (1 µl genomic DNA, 2 µl of each primer 10 µM, 5 µl dNTP-mix 10 mM, various amount of 50 mM $MgCl_2$, 0.5 µl Taq-Polymerase, and water up to 50 µl) underwent repeated heating-cooling cycles either in Biometra-Triblock or Biometra 96 well plate thermocyclers. All primers used for PCRs are listed in table 3.

2.8 MOUSE MAINTENANCE

2.8.1 General Maintenance

Generation of the transgenic mouse strains $HY\alpha^{IF}$ and $HY\alpha^{I\Delta}$ was performed in a conventional barrier mouse facility in our instute. HY-I mice had been generated through breeding of homozygous $HY\alpha^{I\Delta/I\Delta}HY\beta^{+}$ mice with TCRα deficient animals in the same facility. Maintenance breedings of $HY\alpha^{I\Delta/I\Delta}$ $HY\beta^{+}$ (H-2D^{b}), $TCR\alpha^{-/-}$ (H-2Dβ and H-2D^{d}), $HY\alpha/\beta^{+/+}$, however, were performed in the specific pathogen free (SPF) facility in our institute. The mice were maintained in microisolater cages (Thorensen) according to the guidline of the *Tierschutzgesetz*.

$HY\alpha\beta^{+/+}$ mice were otained from the Basel Institue of Immunology and were a kind gift of J. Fehling.

Nr.	Name	Sequence (5´ –> 3´)	Target	Position	Orien-tation	Product	T Ann. °C
	Universal	gtaaaacgacggccagt	pBS	617	AS	Seq.	50
	Reverse	aacagctatgaccatg	pBS	809	S	Seq.	50
1585	SA Forw Eco	tgcctggaattcagatactgaccagcaac	E14.1 gen.	22573	S	SA	60
1584	SA Rev Sal	atactgtcgacattcttctgctagccgatag	E14.1 gen.	24095	AS	SA	60
1649	Del Neo Sense =Vα Sense (Fred)	acatctatctccatcaactg	pC_1T	5772	S	Screen	58
1650	DelNeoAS =Vα AS (Fred)	ttatgtctatgaatgaaactg	pC_1T	3671	AS	Screen	58
2126	5´ins Neo	ggagactcaccggggctctggctc	pC_3T	1496	S	Seq.	50
2125	5´ins VJ vorm Leader	ccttgcagctctcagaagtgcagttg	pC_3T	5644	S	Seq.	50
2124	3´ins VJ	acttggaatatataagtagttaagacttg	pC_3T	6387	AS	Seq.	50
2123	5´ins VJ nach Leader	cctagtcagaatctacacaagggac	pC_3T	5893	S	Seq.	50
2122	3´in Prom	gagctagaaatgctaccgtcctcg	pC_3T	5411	AS	Seq.	50
2121	5´loxP	ccacactgctcgacctgcagcca	pC_3T	2673	S	Seq.	65
1992	Screen SA Forw	cctaattcccacagaaccaca	Jα Loc.	23161	S	Screen	65
2688	Neo Sense for Screen	tcgtgctttacggtatcgcc	pC_3T	1723	AS	Screen	65
2636	V alpha Sense f. gr. Deletion	caggcaccacagaccatccttgc	pC_1T	5959	AS	Screen	60
2637	Promotor HY VJ 5´Ende AS	ccagacagttcctactacaccacgttc	pC_3T		AS	Screen	60
	HYβ1s	cacatggaggctgcagtcac	tail DNA		S	Typing	62
	HYβ2s	gtttctgcactgttatcacc	tail DNA		AS	Typing	62
	5´ Deleter	gaaagtcgagtaggcgtgtacg	tail DNA		S	Typing	58
	Mx-Cre R	cgcataaccagtgaaacagcat	tail DNA		AS	Typing	58
	5´ of Cα E1-F	ccaaatcaatgtgccgaaaac	tail DNA		S	Typing	58
	pGK Prom-R	tacccgcttccattgctcag	tail DNA		AS	Typing	58
	TCRδ-F:	caaatgttgcttgtctggtgaaag	tail DNA		S	Typing	62
	TCRδ-R1:	caaaagttatccaccccgagttc	tail DNA		AS	Typing	62
	NDS3	gagtgcctcatctatacttacag	embryo DNA		S	sexing	57
	NDS4	tctagttcattgttgattagttgc	embryo DNA		AS	sexing	57
	ZFY11	gtaggaagaatctttctcatgctgg	embryo DNA		S	sexing	57
	ZFY12	tttttgagtgctgaggggtgacgg	embryo DNA		AS	sexing	57

Table 3: List of primers used for PCR and Sequencing in the described experiments.

2.8.2 Typing

Initially, the presence of a transgene in newly generated mouse strains was verified by Southern blot analysis. The initial typing for the presence of the HYb transgene was performed by FACS. Mice were bled by cutting the tail vain after 5 min of heating under red light. 200 µl of blood were collected and mixed with 1 drop of Heparin (Liquemin, LaRoche). After addition of 200 µl PBS the blood leucocytes were separated from erythrocytes on 800 µl of 8% Ficoll by centrifugation at 2500 rpm for 10 min without brake. The cells were then stained with the Vβ8.1,8.2, 8.3 specific antibody F23.1 FITC and CD3 PE. Transgenic mice expressed Vβ8 on almost all $CD3^+$ cells.

Similarly, TCRα deficient and $HY\alpha^{I\Delta}HY\beta^+$ mice were typed for absence of $H\text{-}2^b$ and the presence of $H\text{-}2^d$ after breeding to an $H\text{-}2D^d$ expressing mouse strain (BALB/c).

Later, typing for the presence of the HYβ transgene, the TCRα deficient allele, and all other transgenes was performed by PCR. These PCR reactions are listed in table 4.

primer pair	mouse strain/transgene	product
HYβs1/HYβs2	HYβ-transgene	ca. 300 bp
5´of CαE1-F/ pGK Prom-R	TCRα deficient allele	420 bp
5´deleter/ Mx-CreR	Deleter-cre	600 bp
VαSense f. gr. Deletion/ Promotor HYVJ5´EndeAS	$HY\alpha^{I\Delta}$-Allele	950 bp
VαSense (Fred)/ VαAS (Fred)	$HY\alpha^{IF}$-Allele	926 bp wildtype 837 bp inserted
TCRδF/TCRδ-R1	homozyosity $HY\alpha^{I\Delta}$-Allele	absence of 480 bp product
ZFY11/ZFY12	zfy-1/zfy-2 genes	217 or 199 bp
NDS3/NDS4	dxnds-1 gene	244 bp

Table 4: PCRs used for typing of transgenic mouse strains or of the sex of embryos.

2.9 CYTOFLUOROMETRIC ANALYSIS AND SORTING (FACS)

2.9.1 FACS Analysis

Single cell suspension were stained with FITC-, PE-, APC- and biotin-conjugated antibodies. Staining with these fluorescently labeled antibodies was performed as described elsewhere (Förster and Rajewsky, 1987) . Briefly, $2x10^6$ cells were stained

with antibodies diluted in PBS/1% BSA/0.01% NaN_3 (PBA) for 15 min at 4°C. All antibodies were titrated in separate experiments before use. After washing with PBA either a second staining step including Streptaviding-Cychrome, -APC, -PercP, -Cy7-PE or analysis was performed immediately after resuspending of the cells in 200–400 µl of PBA. 3 color analysis was done on a FACScan, 4-color analysis either on a FACSsort or FACScalibur (BecktonDickinson). In some analysis, dead cells were excluded either by adding Propidiumiodide (PI, 0.2 µg/ml) or Topro-3 (1 nM)**.** The antibodies used in the experiments are shown in table 5.

AnnexinV staining was performed using FITC-Annexin V reagent (Pharmingen). Cells were stained for 20 min at room temperature in 50 µl Annexin V buffer (10x Annexin V buffer: NaCl 1.5 M, KCl 50 mM, $CaCl_2$ 18 mM, $MgCl_2$ 10 mM, NaOH 100mM, HEPES 0.1M, pH to 7.4) containing AnnexinV-FITC reagent (Pharmingen)**,** buffer was added and cells were immediately analysed by the cytometer (Koopman et al., 1994).

CD3ε	145-2C11	Leo et al. 1987
CD4	GK1.5/4	Dialynas et al. 1983
CD5	53-7.3	Ledbetter and Herzenberg, 1979
CD8α	53-6.7	Ledbetter and Herzenberg, 1979
CD24/HSA	M1/69	Springer et al. 1978
CD25	7D4	Malek et al. 1983
CD44	KM114	Myake et al. 1990
CD69	H1.2F3	Yokoyama et al. 1988
CD90/Thy-1	CGFO-1	Opitz et al. 1982
TCRβ	H57-597	Kubo et al. 1989
HYα-chain	T3.70	Teh et al. 1988
Mac-1	M1/70	Springer et al. 1978
CD11c	HL3	Huleatt et al. 1995
H-2K^b	AF6-88.5	Loken et al. 1992
H-2K^d	SF1-1.1	Abastado et al. 1993
H-2 I-A/I-E	M5/114.15.2	Bhattacharya et al. 1981
Vα2	B20.1	Pircher et al. 1992

Table 5: Antibodies which were used in FACS analysis and sorting.

2.9.2 FACS Sorting

For sorting on a FACSstar (BecktonDickinson) cells were stained in a different staining buffer (RPMI 1640 w/o phenolred, riboflavin/biotin (Gibco 074-90246) in 1l water, 10 ml Hepes buffer 1mM, 10 ml Glutamin, 10 ml Penicillin/Streptomycin (Gibco), 30 ml FCS decomplemented). Experience had shown that cell death was reduced by use of this buffer. Sorting was performed with the help of C. Goettlinger.

2.10 MAGNETIC CELL SORTING

Enrichment and depletion of cellular fractions by magnetic cell sorting was done by use of beads-conjugated antibodies (CD4, CD8, CD90), magnets, and columns from Milenyi Biotec according to the manufacturer´s instructions. However, a different buffer was used (see 2.9.2). Sorting efficiency was monitored by fluorocytometry.

2.11 FETAL THYMIC ORGAN CULTURE

Female TCRα deficient mice were bred to male $HY\beta^{+}HY\alpha^{I\Delta/I\Delta}$ mice and tested for vaginal plug the next morning (d 0.5). Pregnant mice were killed on d14.5–d17.5, embryos placed in PBS, and thymi prepared from the embryos under sterile conditions. It is essential that preparation of the thymi is performed in medium which does not contain HEPES since HEPES was found to inhibit the activity of the HY-peptide. Genotyping of the embryos was performed on tail DNA by use of the primers HYβs-1 and HYβs-2 (tables 3 & 4) for the HYβ transgene on the same day. For sex determination an X-chromosomal and a Y-chromosomal gene were detected by PCR with the primer pairs Nds3 and Nds4 , and Zfy11 and Zfy12, respectively (table 3 & 4, fig. 23; Kunieda et al., 1992). The thymic lobes were placed on Millicell^{TM} culture plate inserts (Millipore) on DMEM-10 medium (DMEM, Lifesciences, 10%FCS**,** 6 ml sodium pyruvate, 6 ml non-essential amino acids, 6 ml glutamin, 50 µM β-mercaptoethanol) HY-peptide (Markiewitz et al., 1998) was added in indicated concentrations, and the lobes were cultured at 37°C 7.5% CO_2 100% relative humidity. Single cell suspensions for analysis were prepared by squashing the lobes between 30 µm nylon mesh.

2.12 THYMIC REAGGREGATION CULTURE

Reaggregation cultures were performed according to a modified version of the protocol of Jenkinson et al., 1992. To obtain thymic stroma, thymi of three C57BL/6 females were digested with collagenaseD and DNase at 37°C for 15 min and 1x trypsin/EDTA (Gibco) at 37°C for another 15 min on a shaker with the help of glas beads. Thymocytes were depleted by two steps of magnetic cell sorting (see 2.10) using first anti-CD4 and anti-CD8 beads and second anti-Thy1.2 beads (MiltenyiBiotec). Thymocytes of female HY-I H-2D^b mice were stained with antibodies against CD4, CD8 and HYα-chain (see 2.9.1). HYα$^+$CD8$^+$ thymocytes were sorted with a FACSstar (see 2.9.2), labeled with CFSE (see 2.13), and mixed at a ration of 3:1 with stroma cells, pelleted and transferred by mouth pipetting with a drawn-out glas capillary into Millicell^{TM} inserts (Milipore) which were placed on DMEM-10 medium (see 2.11). The reaggregates were cultured at 37°C 7.5% CO_2 100% relative humidity.

2.13CFSE LABELING

After sorted T cells had been washed with 37°C warm DMEM without FCS, they were incubated with 1 ml per 10^7 cells DMEM containing 5 µM CFSE (5 mM stock in DMSO, Molecular Probes) at 37°C for 10 min (Lyons & Parish, 1994). The labeling reaction was stopped by addition of 10 ml ice-cold DMEM-10 medium (see 2.11). The cells were washed once with DMEM-10 and then used for proliferation experiments.

2.14 T CELL ACTIVATION AND PROLIFERATION ASSAY

Single cell suspensions were prepared from LNs and spleens of female HY-I mice. The cells were stained with antibodies of the following specificities: CD4, CD8 , and HYa-chain. After sorting of HYα$^+$CD8$^+$ T cells, these cells were labeled with CFSE to allow monitoring of cell division. Subsequently, 10^5 sorted T cells were mixed with 10^5 splenocytes from C57BL/6 or BALB/c mice which had been incubated with 50 µg/ml mitomycin C for 20 minutes. The cells were then cultured with varying amounts of HY peptide in 96 well round bottom plates at 37°C 7.5% CO_2 100% relative humidity. After 3 days of culture the cells were harvested and analyzed by FACS.

2.15 DATA ANALYSIS

Data analysis and processing was performed on Macintosh computers using the following programs:

Microsoft Excel98	QuarkExpress3.0	CellQuest3.3
Microsoft Word5.1	AdobePhotoshop3.0	Oligo4.0
Canvas 3.1	Ofoto2.0	

3 RESULTS

3.1 Generation of the HY-TCRα Chain Insertion Model

Secondary recombination events at the TCRα locus replace an already rearranged VαJα (fig. 3 & 4). To investigate this process it was decided to introduce a recombined VαJα element into its physiological position between remaining Vα and Jα elements. Due to the unique structure of the TCRα/δ locus (fig. 1, Chien et al., 1987) the first TCRα-rearrangement leads to the deletion of the intervening TCRδ locus. To mimic this TCRδ locus deletion I therefore had not only to introduce a recombined VαJα segment but also to remove a 263 kb large chromosomal fragment (fig. 8) utilising the Cre-loxP system (Sauer and Henderson, 1988; Gu et al., 1994) in vivo. In a first targeting experiment in murine ES cells a neomycin gene flanked by loxP sites was integrated into the Vα region and subsequently removed in vitro by Cre mediated recombination. In a second targeting experiment in this ES cell clone another loxP site and a VαJα element were introduced into the Jα region. Such ES cells were used to generate a mouse strain in which the TCRδ locus was deleted.

As a model system a H-2D^b restricted male (HY) specific TCR was chosen which is positively selected in female and negatively selected in male transgenic mice (Kisielow, 1988; Kisielow et al., 1988) of MHC haplotype H-2D^b.

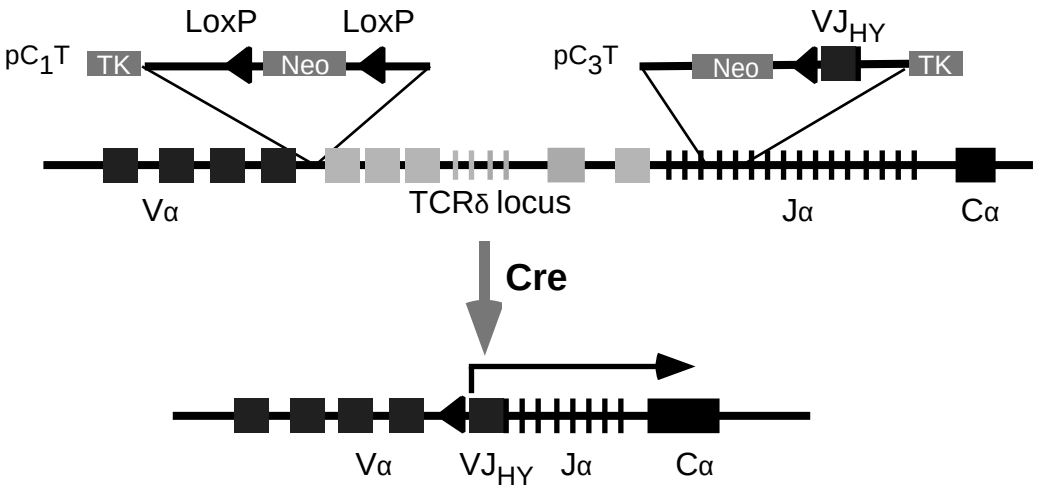


Fig. 8: Strategy to insert a recombined VαJα into its physiological position in the TCRα locus of the mouse. The targeting vector pC$_1$T is inserted by targeted mutagenesis in the Vα region. After removal of the neomycin resistance gene by Cre-mediated deletion, a second targeting vector pC$_3$T containing a recombined VαJα element is inserted in the Jα locus. The DNAfragment flanked by loxP sites is then removed by Cre-mediated recombination. TK: Thymidinkinase; Neo: Neomycin resistance gene; black triangles: loxP sites; arrow: transcriptional orientation

3.1.1 Targeted Insertion of the 5´loxP Site

First a neomycin resistance gene which was flanked by loxP sites was introduced in front of the Vα6/Vδ3 element which is located at the 3´end of the Vα cluster (Seto et al., 1994). Placing the upstream loxP site at this Vα elements would leave behind after deletion of the TCRδ locus the majority of other Vα elements that could be used in secondary rearrangements.

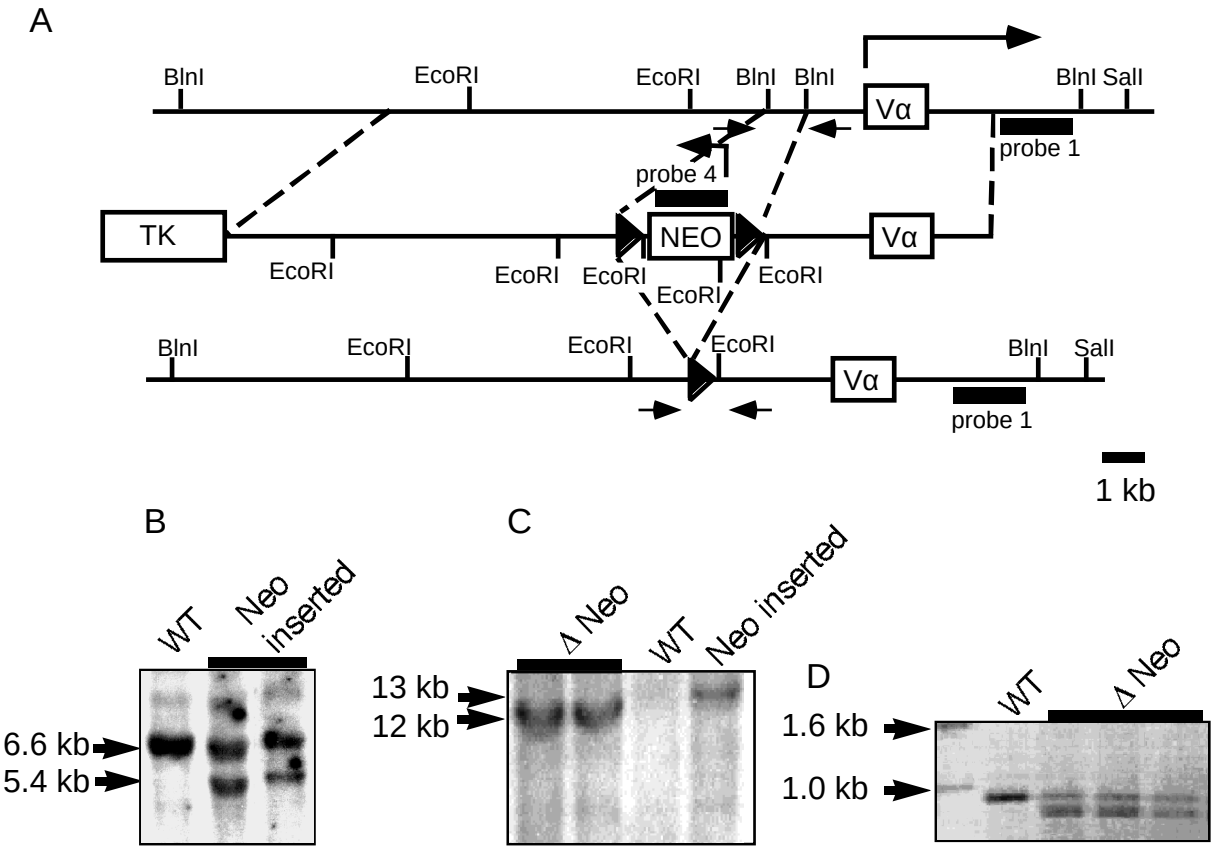


Fig. 9: Introduction of a loxP site into the Vα region;(A) scheme of the targeting event and the cre-mediated deletion ;(B) Southern blot analysis of pC_1T targeted ES cells after EcoRI/SalI digest, hybridisation with probe1; (C) Southern-blot analysis of of ES cells after cre-mediated deletion of the neomycin resistance gene after BlnI digest, hybridisation with probe 1; (D) confirmation of the cre-mediated deletion of the neomycin resistance gene by a PCR with primers flanking the Neomycin resistance gene; The predicted and confirmed sizes of the hybridised DNA fragments and the PCR products are shown in the table and indicated in (B) and (C) with arrows at the left side. TK thymidinkinase; Neo neomycin resistance gene; Va: Vα6/δ3 element; black triangels: loxP sites; bold bars: Southern blot probes; small arrows: PCR primers 1649 & 2126; large arrows: transcriptional orientation

Vα6/δ3 genotypes determined by Southern blot or PCR

	WT	**Targeted**	**ΔNEO**
EcoRI/SalI digest	6.6 kb	5.4 kb	5.4 kb
BlnI digest	5.2 kb	13 kb	12 kb
PCR Product	926 bp	2100 bp	837 bp

10^7 ES cells (E14.1) were transfected with 30 µg of the XbaI linearised targeting vector pC_1T and selected with G418 and gancyclovir (Capecchi, 1989). 96 double resistant colonies were picked and screened for homologous recombination by Southern blot (Fig. 9A). Genomic DNA was digested with EcoRI/SalI or BlnI and hybridized to the 3´ external probe 1 (fig. 9B, data not shown). Five homologous recombinant clones were identified (5% of G418/gancyclovir selected clones). Additional, random integration of the targeting vector was excluded by hybridisation with an internal probe specific for the neomycin-resitance gene (probe 4; data not shown).

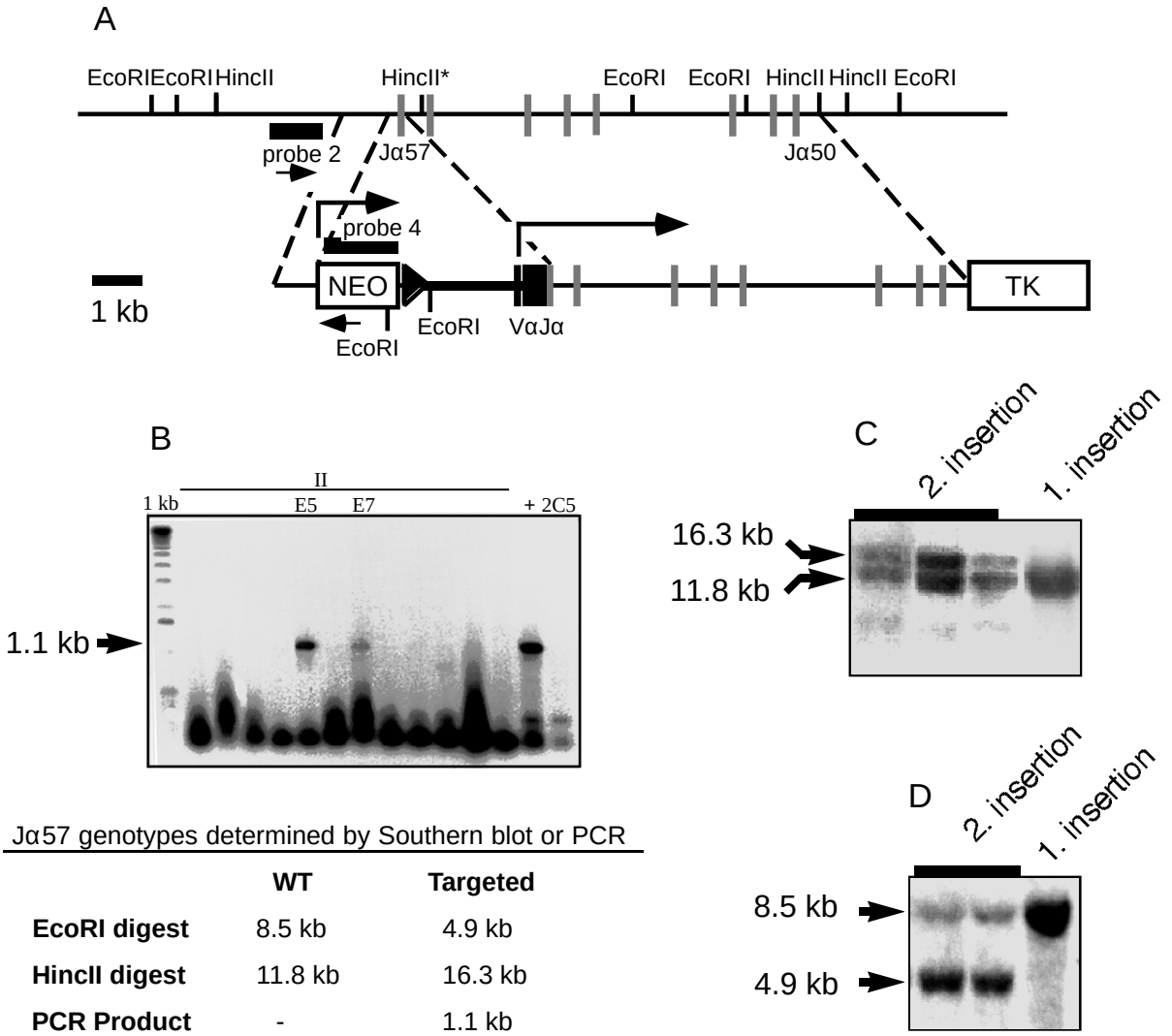
The neomycin resistance gene was removed by transient expression of the Cre recombinase (Torres and Kühn, 1997) in ES cells carrying the insertion of the targeting vector pC_1T. 10^7 ES cells were transfected with 50 µg of the Cre expression vector pGK-creBpA (K. Fellenberg, unpublished) and screened for G418 sensitive clones. In these clones the Cre-mediated deletion was confirmed by Southern blot analysis of BlnI digested DNA hybridised with the external 5´ probe 1 (fig. 9A & C) and PCR with primers 1649 & 2126 (table 3, fig. 9D). The recombination took place in 4% of the transfected cells.

This first targeting experiment was performed by F. Rieux-Laucat and me. Because his ES cell clones carrying the insertion of pC_1T were available earlier, the removal of the neomycin resistance gene was performed by F. Rieux-Laucat on his ES cells. which were then used by me for the following experiments.

3.1.2 Targeted Insertion of the Recombined VαJα Element

The VαJα element of the HY-TCR as well as a second loxP site were introduced into two ES cell clones which harbored the loxP site 5´ of Vα6/δ3. The Jα57 element which had joined with Vα9.2 to form the variable region of the α-chain of the HY-TCR (Uematsu, 1992; Arden et al., 1995) was replaced by this recombination event. It is located at the 5´end of the Jα cluster (Koop et al., 1994) and the 56 Jα elements remaining after deletion of the TCRδ locus could be used in secondary rearrangements.

The previously targeted ES cell clones 1F12 and 2C5 were transfected with 30 µg of the NotI linearized targeting vector pC_3T (fig. 6) and selected with gancyclovir and G418 for 8 days. The counter-selection by the gancyclovir/thymidinkinase system of of clones carrying random integrantions of pC_3T lead to a 8-fold enrichment of putative homologous recombinants. A PCR screening strategy was used for the identification of clones having integrated the targeting vector pC_3T into the genome by homologous recombination. This PCR screen was performed by using the primer 1992 (table 3) binding outside the short arm of homology of the targeting vector pC_3T and the primer 2688 (table 3) binding to the neomycin resistance gene (fig. 10 A). The



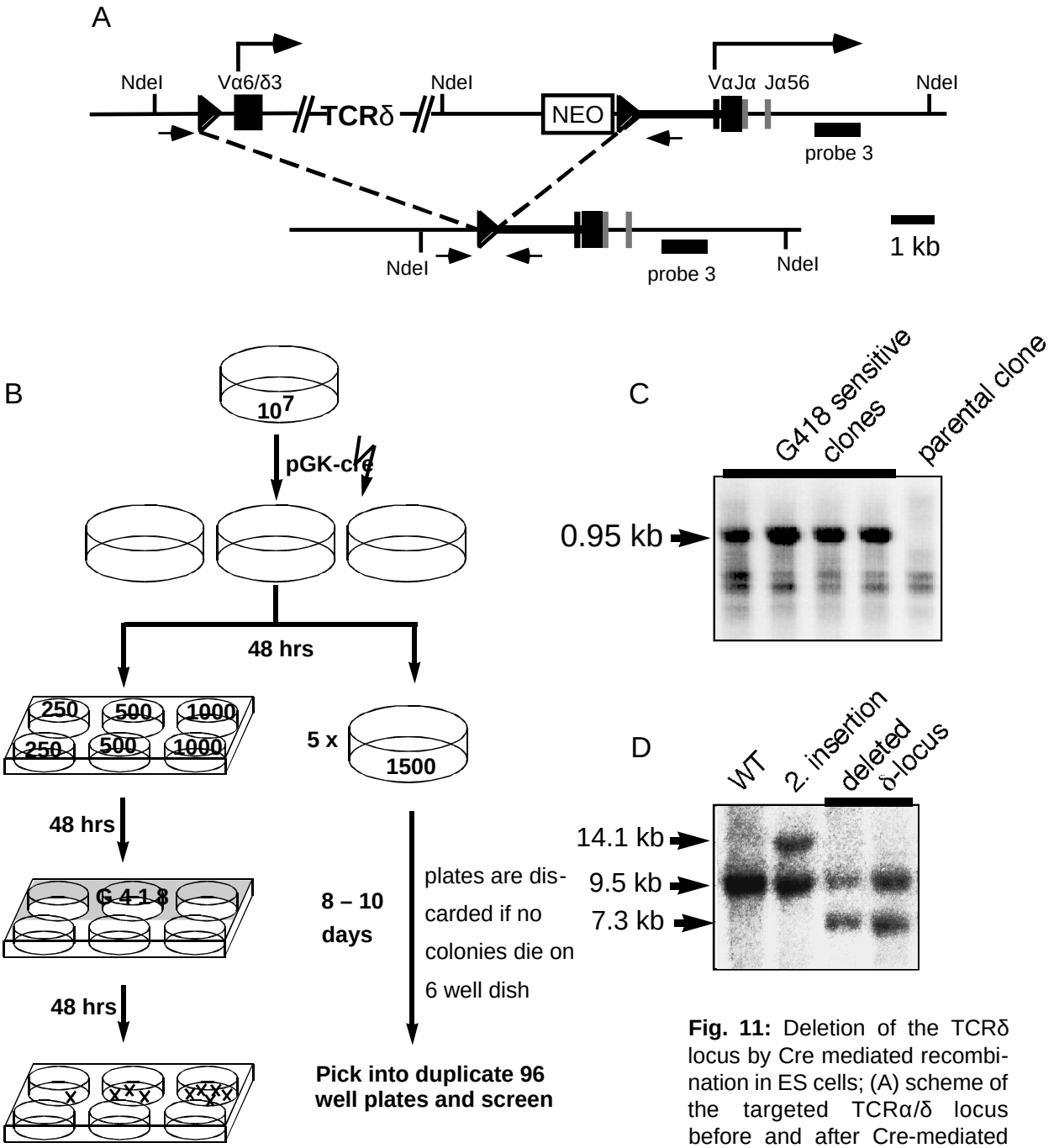
Jα57 genotypes determined by Southern blot or PCR

	WT	Targeted
EcoRI digest	8.5 kb	4.9 kb
HincII digest	11.8 kb	16.3 kb
PCR Product	-	1.1 kb

Fig. 10: Insertion of a recombined VαJα into the Jα locus by homologous recombination in ES cells containing a Vα loxP site; (A) scheme of the targeting vector and the locus; (B) PCR screen for homologous recombinants using the primers 1992 & 2688 (small arrows in A), the positive control is labeled with +, 2C5 was the parental ES cell clone, clones IIE5 and IIE7 were found to be homologous recombinants, (C) HincII and (D) EcoRI digested DNA of PCR positive clones was used for Southern blot analysis and hybridised to probe 2 to confirm the homologous recombinatin event, the * marked HincII site did not cut, although its presence had been confirmed by digestion of a PCR product. The predicted and observed sizes of the DNA fragments or PCR products are shown in the table. For a detailed legend see fig. 9.

specific conditions (94°C 30 s, 65°C 60 s, 72°C 120s, 35 cycles in a Biometra Triblock PCR thermocycler) for this PCR based screen had been established beforehand on ES cells transfected for a PCR target (pSANeoloxPtest) consisting of the neomycin resistance gene and an extended short arm of homology of pC_3T allowing the 5´ primer to anneal (data not shown).

The PCR screening for homologous recombinants was performed on half the cells of each colony (fig. 10A & B). In clones positive in the PCR screen the homologous recombination event was confirmed by Southern blot analysis of genomic DNA digest-



TCRα/δ lcous genotypes determined by Southern blot or PCR

	WT	Targeted	ΔTCRδ
NdeI digest	9.5 kb	14.1 kb	7.3 kb
PCR product	-	-	0.95 kb

Fig. 11: Deletion of the TCRδ locus by Cre mediated recombination in ES cells; (A) scheme of the targeted TCRα/δ locus before and after Cre-mediated recombination, small arrow: PCR primers 2636 and 2637, large arrows: transcriptional orientation, bold lines: Southern blot probes, black triangels: loxP sites, Neo: neomycin resistance gene, VαJα: Vα6Iδ3 element; (B) screening strategy for clones carrying both insertions in cis, numbers of plated cells are given in the wells or plates, the grey half of the 6 well plate is treated with G418, crosses in the lower 6 well plate represent dying colonies; (C) PCR using the primers depicted in A by small arrows on the parental clone or G418 sensitive clones after transfection with the Cre expression vector; (D) Southern blot analysis of DNA from G418 sensitive clones, NdeI digest and hybridisation with probe 4. The predicted and observed sizes of the detected fragments are shown in the table.

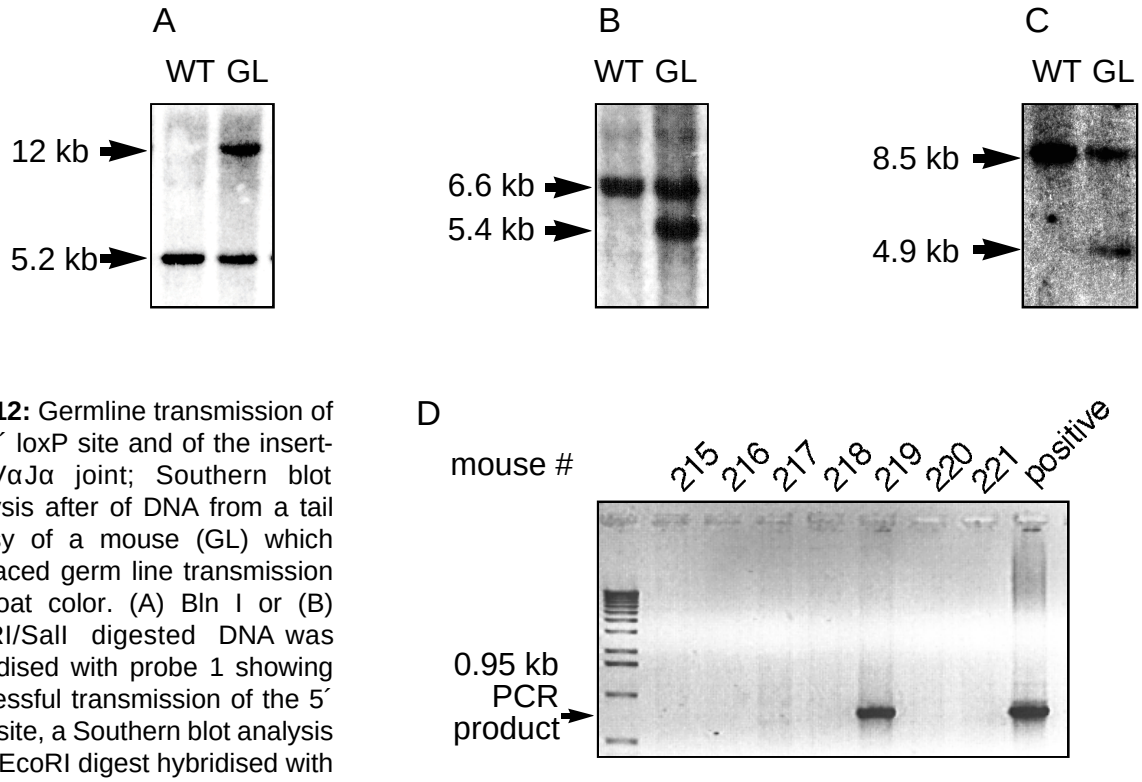


Fig. 12: Germline transmission of the 5´ loxP site and of the inserted VαJα joint; Southern blot analysis after of DNA from a tail biopsy of a mouse (GL) which displaced germ line transmission by coat color. (A) Bln I or (B) EcoRI/SalI digested DNA was hybridised with probe 1 showing successful transmission of the 5´ loxP site, a Southern blot analysis after EcoRI digest hybridised with probe 2 (C) confirmed transmission of the inserted VαJα in the same animal; (D) PCR which was used to identify offspring of $HY\alpha^{IF}$*deleter*-cre^{+} animals which had deleted the TCRδ locus in the germline; the strategies used for Southern blot analysis and PCR screen are the same as in fig. 9, 10 & 11.

ed with EcoRI or HindII digest and hybridized to the external probe 2 (fig. 10A, C & D). The frequency of homologous recombination in G418/gancyclovir selected cells was 8-10%. By hybridisation of the same blots with a probe for the neomycin resistance gene (probe 4) it was shown that the homologous recombinants contained no random integration of the targeting vector in addition (data not shown).

To exclude the possibility that the inserted VαJα joint had been mutated during homologous recombination the coding region was sequenced (data not shown) and no mutations in the exons or at splice sites were observed.

3.1.3 In-Vivo Deletion of the TCRδ locus

Since integration of the second targeting vector could have taken place on either one of the two homologous chromosomes, ES cell clones carrying both insertions on the same allele (in cis) had to be identified. The test for cis-position of the loxP sites was Cre mediated deletion of the TCRδ locus and the neomycin resistance gene. Five ES cell clones carrying both insertions were transfected with 50 µg of the Cre expression vector pGK-creBpA (Torres and Kühn, 1997). In a pre-experiment it was tested whether or not G418 sensitive colonies were generated by Cre mediated recombination in a respective clone (fig. 11B). Transfected cells of several clones were distributed in duplicates on 6 well plates and after the establishment of visible colonies

G418 selection was started on half the wells. The wells were then screened daily for dying colonies and only from those transfected ES cells which had shown to contain G418 sensitive cells, colonies were picked and screened for G418 sensitivity. The deletion of the TCRδ locus in G418 sensitive ES cell clones was verified by Southern blot analysis (fig. 11A). Genomic DNA was digested with NdeI and hybridized with the internal probe 3 (fig.11D). Sequencing of a PCR product generated with primers 2636 and 2637 which anneal 5´ and 3´ of the two loxP sites confirmed the juxtapunction of Vα and Jα region (fig. 11C, data not shown).

Since it is likely that the probability of an ES cell clone to keep its omnipotence decreases after several rounds of transfection/selection I used for the generation of chimeric mice ES cells which had gone through the fewest number of transfection experiments. This was achieved by a) generating a mouse strain which carried the 5´ loxP site as well as the 3´ VαJα insertion and b) deleting the TCRδ locus in vivo by crossing this strain to a mouse strain (*deleter*-cre) expressing the Cre recombinase early in embryogensis (Schwenk et al., 1995).

I generated chimeric mice by morula aggregation and blastocyst injection (Hogan et al., 1994) of 3 ES cell clones (ID8, IVA12, and IVF12) which carried the two insertions in cis location. Morula aggregation using 28 CD1 embryos yielded 4 mice with chimerism >90%. However, as judged by coat color these chimeras did not generate any offspring derived from the ES cells. Subsequently, I generated by blastocyst injection 16 chimeric mice of which 4 transmitted the mutated allele through the germline as shown by Southern blot analysis (fig. 12A, B & C).

The new mouse strains were called HYα^{IF}(A) (clone IVA12, germline transmission after breeding to CB20) and HYα^{IF}(F) (clone IVF12, germline transmission after breeding to C57BL/6). These mice were bred to a transgenic mouse strain expressing the β-chain of the HY-TCR to investigate whether theHYα^{IF} allele would be expressed already without deletion of the δ-locus. It was found that the HYα-chain was indeed expressed from this allele (fig. 13A).

In order to delete the TCRδ locus, HYα^{IF} mice were bred to the *deleter*-cre mouse strain (Schwenk et al., 1995). Mice carrying the *deleter*-cre allele as well as the mutated TCRα locus were found to be genetically mosaic in respect to the deletion of the TCRδ locus (data not shown). To establish the deleted mouse strain, I screened by PCR (fig. 12D) offspring of such mosaically deleted mice for absence of the deleter-cre allele and presence of the deletion of the TCRδ locus. The deleted allele, termed HYα$^{I\Delta}$, was transmitted through the germline of HY^{IF}(F) *deleter*-cre^{+} mice with a frequency of only 5%. Unfortunately, the deletion could not be confirmed by Southern blot analysis because in contrast to ES cell derived DNA, DNA from different organs of adult mice did not get digested completely by NdeI.

Finally, mice for the analysis of TCRα secondary rearrangements were obtained by

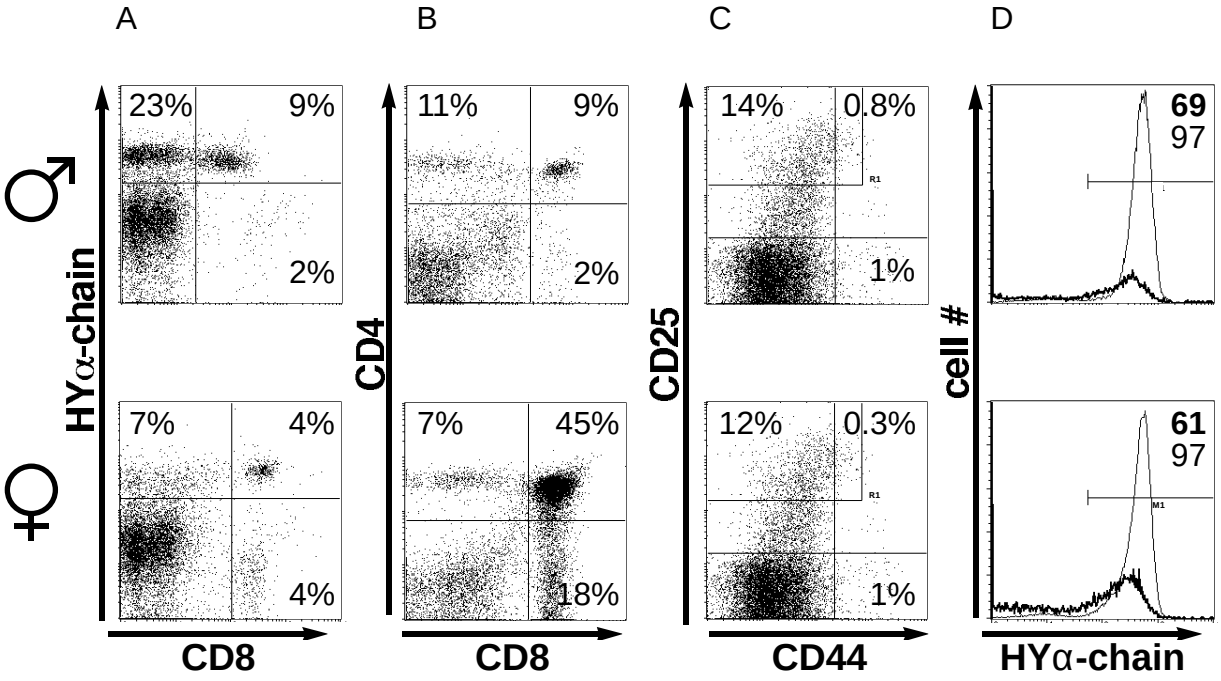


Fig. 13: Expression of the inserted transgene from a locus in which the large deletion to remove the TCRδ locus had not yet been performed, (A) LN cells of male and female HYα$^{IF/0}$ HYb^{+} mice; (B,C,D) thymocytes of HYα$^{IF/0}$ HYβ^{+} mice: (B) CD4 and CD8 staining of total thymocytes; (C) CD25 and CD44 staining of DN thymocytes; (D) HYα chain expression on CD25^{+} (bold line) and CD25^{-} (thin line) DN

breeding together the HYα^{IΔ} allele, the HYβ transgene (Uematsu et al., 1988) and TCRα deficient allele (Mombaerts, 1992). In these mice with the genotype HYα$^{IΔ/0}$HYβ^{+}, referred to as HY-I mice, expression of the TCRα gene was restricted to the insertion allele.

3.2 Expression of the Inserted HYα on Peripheral T Cells

3.2.1 HYα^{+}CD8^{+} Cells in Female HY-I Mice

Expression of the inserted HYα chain was monitored by use of the monoclonal antibody T3.70 (Teh et al., 1989) which is specific for the α-chain of the HY-TCR (Teh et al., 1990). In the analysis of expression of the inserted TCRα chain the conventional HY-TCR transgenic mouse strain was used as a positive control. Lymph node (LN) cells of (positively selecting) female HY-I and HYtg mice (H-2D^{b}) were analysed by FACS. The HYα-chain was detected on 18% of the CD8^{+} LN cells from the HY-I mouse compared to 10% of HYα^{+}CD8^{+} cell in LN cells from the HYtg mouse (fig. 14). These data suggest that HY-TCR^{+} cells can be efficiently positively selected in the thymus of HY-I mice and exported to the periphery.

A difference between HY-I and HYtg mice was observed in the population of CD8^{+}

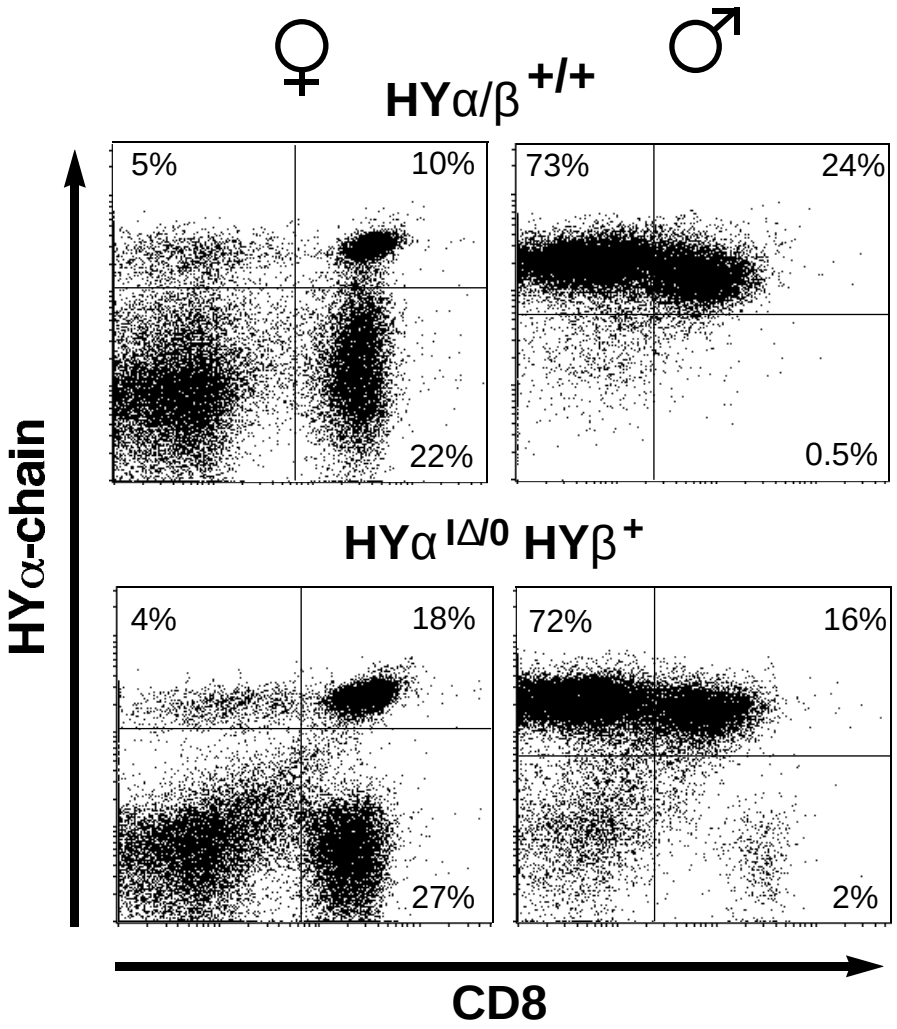


Fig. 14: Lymph node αβ T cells of HYtg and HY-I mice: total LN cells were stained for HYα-chain, TCRβ, CD4, and CD8 and analysed by FACS analysis. Shown are all $TCR\beta^+$ cells. The percentages of T cells in the subsets defined by the quadrants were indicated

cells which contained in female conventional transgenic mice a population of T cells with varying levels of the HY-TCR (fig. 14). In the insertion mice this population of $HY\alpha^{dull}$ cell could not be found and instead appeared as a clear distinct $HY\alpha^-$ population (fig. 14). The $HY\alpha^{dull}$ cells in the transgenic model have been described to coexpress a second α-chain, encoded by a VαJα joint in the endogenous TCRα loci. This process has been termed allelic inclusion (Borgulya et al., 1992; Malissen, 1992) . The two α-chains compete for surface expression with the transgenic β-chain, which in turn leads to variable intensity for HYα-chain staining on these cells. In the HY-I model this allelic inclusion cannot take place, because the transgenic TCRα was expressed by one TCRα locus and the other TCRα locus was genetically inactivated (Mombaerts, 1992). The $HY\alpha^-CD8^+$ cells in this model expressed TCRβ and can only have been generated by the replacement of the introduced VαJα joint with new VαJα rearrangements by RAG mediated recombination.

3.2.2 HYα^{+} Cells in Male HY−I mice

The majority of αβ T cells in LNs of male (negatively selecting) HY-I mice were CD8^{lo} or CD4^{-}CD8^{-} and expressed the HYα-chain (fig. 14). These cells can also be found in the HYtg and other transgenic mouse models and have been described to belong, although expressing the transgenic αβ TCR, to the γ/δ T cell lineage (von Boehmer et al., 1991; Bruno et al., 1996; Terrence et al., 2000).

A marked difference between the HY-I and HYtg model was an appearance of a distinct population of HYα^{-}CD8^{+} cells in the HY-I model (fig. 14). Again, these cells must had lost expression of the transgenically encoded a-chain by genomic replacement of the VαJα joint and expressed then endogenous α-chains. Similarly, a significant increase in the proportion of CD4^{+} cells which in their majority do not express the HYα-chain and can only be generated by expression of new receptor specificities is observed (fig. 14, data not shown).

3.3 Thymic Development In HYα Insertion Mice

3.3.1 Positive Selection in Female HY-I Mice

Based on developmental staging through the surface markers CD3, CD4, and CD8 thymic development was investigated in HY-I mice. The total number of thymocytes of thymi from female HY-I mice expressing the restricting MHC element H-2D^{b} was doubled compared to the one of thymi from female HYtg mice (H-2D^{b})(fig. 15). Thymic development in both models showed a bias towards the generation of CD8^{+} cells. However, the size of the DP and SP compartments was increased in the insertion model (fig. 15). This was accompanied by a reduction in proportion (7% vs. 32%) but not number (7x10^{6} vs. 7x10^{6}) of cells at the DN stage (fig. 15).

The thymi of female (but also male) HY-I mice were characterised by an almost complete loss of the DNII and DNIII stages and expression of the HYα-chain on 90% of DN cells, similar to the HYtg model (fig. 18, Buer et al., 1997). However, in contrast to the conventional model less CD25^{+} DN cells expressed the transgenic α-chain in the HY-I model (fig. 18, 37% vs. 86%) which could mean that the onset of TCRα expression from the HYα$^{I\Delta}$ allele is taking place later than from the conventional HY transgene.

In the DP compartment only a fraction of cells retained (mostly low) surface expression of HYα. Most DP cells lost expression of the inserted α-chain (fig. 19B). Some of these cells were shown to express TCRs encoded by endogenous Vα2, Vα3, Vα8 or Vα11 gene segments (fig. 19C, data not shown). The cells which express TCRs with new specificities may also account for the increase in cell number of the thymus in the insertion model over the conventional system, since these cells do possibly not compete with cells expressing the HY-TCR for the same positively selecting ligand

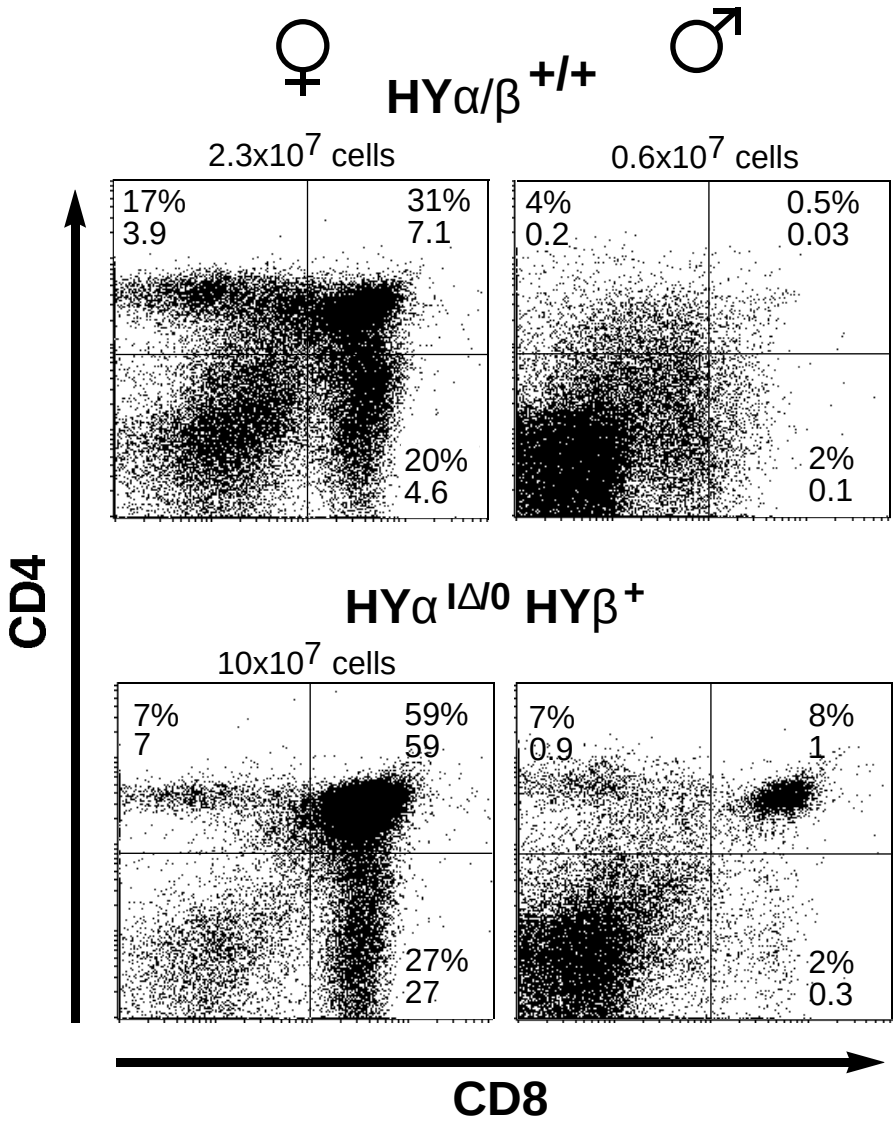


Fig. 15: Deletion of DP thymocytes in male HYtg and HY-I mice but efficient selection into the $Cd8^+$ compartment in female HY transgenics; All animals were expressing the restricting $H\text{-}2D^b$ molecule. Thymocytes were stained for CD4 and CD8 and analysed by FACS. Total thymic cell number is indicated above each dot plot. The percentage and absolute numbers ($x10^6$) of cells in the different subsets is given in the corner of the quad-

(Borgulya et al., 1992; Merkenschlager et al., 1994). Some of these $HY\alpha^-$ cells were selected into the CD4 SP compartment whose cells were shown to express only endogenous α-chains as evidenced by CD3 expression together with lack of HYα-chain expression (fig.19B). Some $CD4^+$ cells were also shown to express Vα2, Vα3, Vα8, and Vα11 (fig. 19B, data not shown). In contrast to the DP and $CD4^+$ cells nearly all of the $CD8^+$ cells (90%) expressed the HY-TCR. This may mean that the cells which retain the transgenic α-chain are efficiently positive-selected. It was observed that more $HY\alpha^+$DP cells than $HY\alpha^-$DP cells exhibit high surface expression of CD69 (fig. 16B) which correlates with receptor ligation and thus positive or negative selection (Swat et al., 1993; Kishimoto and Sprent, 1997).

3.3.2 Replacement of The Inserted VαJα on Non-Selecting Background

The HY-TCR was shown to be restricted to the MHC haplotype H-2D^b; it is not positively selected in thymi of mice with H-2^d background (Kisielow et al., 1988). To investigate whether secondary recombination events can efficiently rescue thymocytes from death by neglect I bred the HY-I model onto H-2^d background (data not shown). The thymic cellularity of 7.2x10^7 cells in H-2D^d HY-I mice was comparable to the one of female H-2D^b HY-I mice (fig 17A). As in HY-I mice carrying the MHC haplotype H-2D^b, expression of the transgenic receptor was detected on most DN cells (Fig. 19B). Thus, the majority of these HY-TCR expressing CD4$^-$CD8$^-$cells are generated independent of MHC restriction as described in the HYtg model already (von Boehmer, 1990; von Boehmer et al., 1991) and may actually belong to the γδT cell lineage (see discussion). Some cells in the DP compartment which had a similar size as the one in positively selecting HY-I animals (fig. 17B & 19A) expressed low levels of the HY-TCR (fig. 17B & 19B). The lack of HYα^{high} DP or CD69$^+$HYα$^+$DP cells (fig. 16A & 19B) reflects the absence of positively selecting ligand (Swat et al., 1993; Kishimoto and

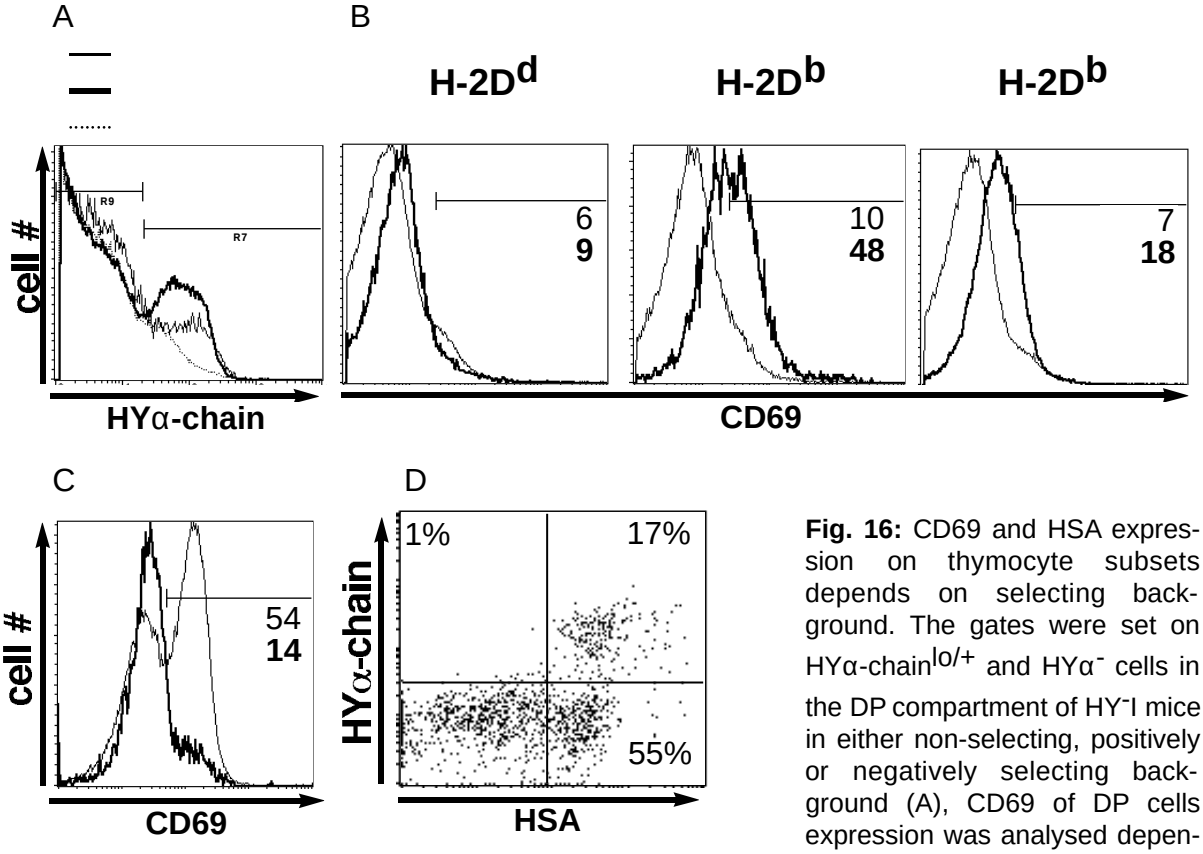


Fig. 16: CD69 and HSA expression on thymocyte subsets depends on selecting background. The gates were set on HYα-chain$^{lo/+}$ and HYα$^-$ cells in the DP compartment of HY$^-$I mice in either non-selecting, positively or negatively selecting background (A), CD69 of DP cells expression was analysed dependent on HYα expression in HYα$^-$ (thin line) or HYα$^{lo/+}$ DP cells (thick line)(B). CD69 expression was assessed on HYα$^-$ (thin line) or HYα$^{lo/+}$ CD4$^+$ cells of male H-2D^b HY-I thymocytes (C). HYα-chain

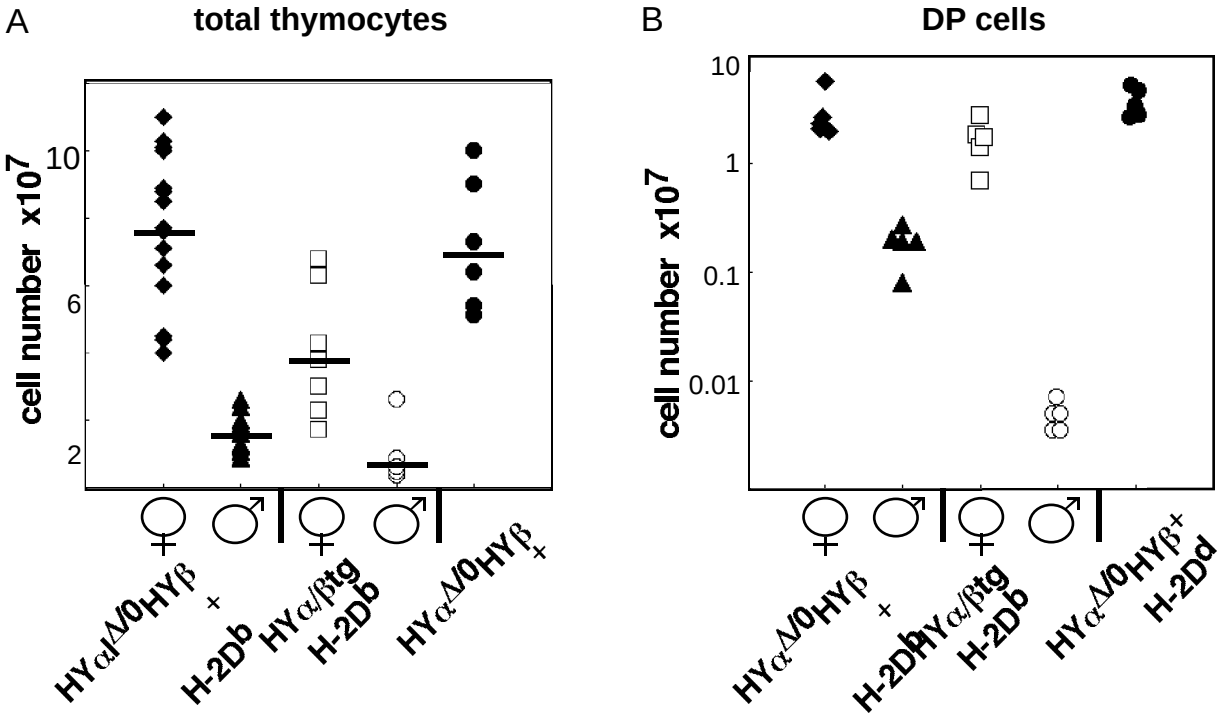


Fig. 17: Negative selection reduces total thymic cellularity in HY transgenic mouse strains (A), the effect is due to a drastic decrease in the number of DP cells (B), shown are the cell numbers of mice of an age of 6-8 weeks. A single cell suspension of thymocytes was counted and stained for CD4 and CD8. The number of DP cells was calculated using the percentage of lymphocytes which were found by FACS analysis to be $CD4^+CD8^+$.

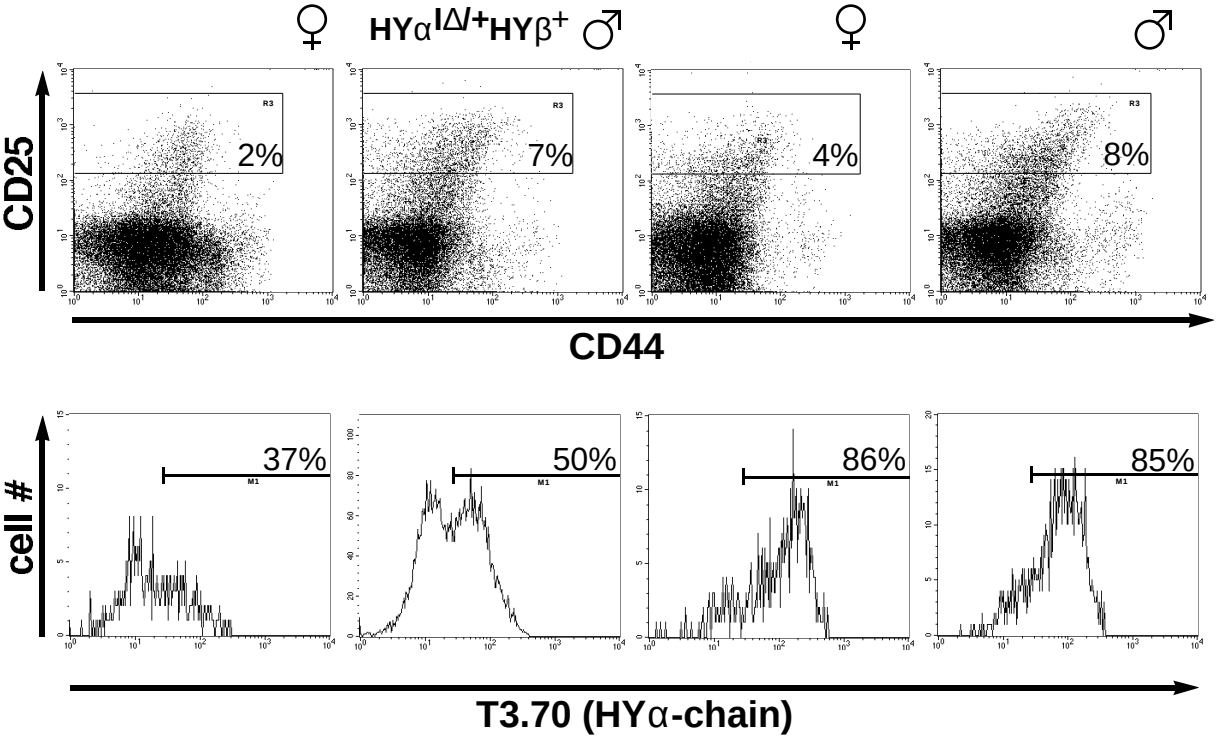


Fig. 18: Fewer HYα-chain expressing cells were found in the $CD25^+$ DN compartment of HY-I mice than in conventional HY transgenics. Thymic cell suspensions were stained with CD4/8, CD25, CD44, and HYα-chain specific antibodies and analysed by FACS, a life gate was set on CD4/8 negative cells. The percentages in the CD25/CD44 dot plots (upper row) are given for cells that were lying within the shown $CD25^+$ gate with respect to total DN numbers. The percentage of HYα chain expression is

Sprent, 1997). Thus, these cells either just had entered the DP compartment and had no time for secondary recombinations or were unable to replace the inserted α-chain.

The transgenic α-chain was also virtually absent on SP cells of these mice (fig. 19B). However, albeit transgenic α-chain could not be detected on the surface of SP

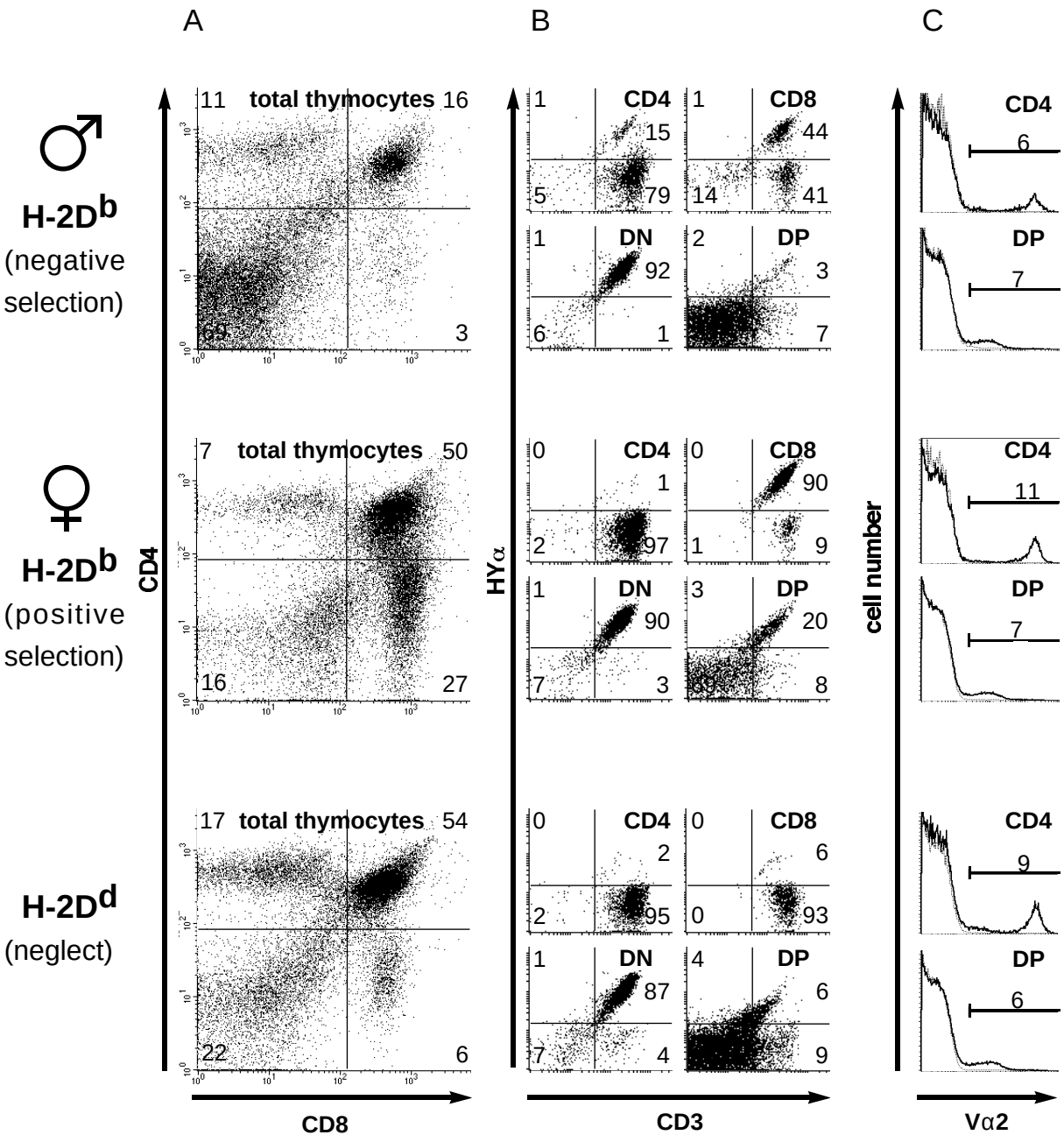


Fig. 19: T cell development in HY-I mice which express different MHC haplotypes allowing positive, negative and no selection; The cells were stained with antibodies for CD4, CD8, CD3, and HYα-chain, or CD4, CD8, Vα2, and HYα-chain and analysed by FACS. To allow investigation of CD3 and HYα-chain expression (B) gates were set on thymic subsets as defined by CD4 and CD8 expression (A).The percent of cells which lied in the quadrants of the dot plots are indicated in the corners. Vα2 expression was analysed in histogram overlays (C) by gating on $CD4^+CD8^-HY\alpha^-$ (CD4) or $CD4^+CD8^+HY\alpha^-$ (DP) cells. The thin line in the histograms shows the staining by an isotype control antibody.

cells, they expressed surface TCR as shown by staining of CD3ε and TCRβ (fig. 19B, data not shown). Staining with antibodies against V elements different from the one used in the HYα chain (Vα2, Vα3, Vα8, and Vα11) confirmed the expression of endogenous V elements (Fig. 19C, data not shown). The CD8 to CD4 ratio (1/3) was similar to WT mice, indicating a large repertoire of MHC class I and class II restricted specificities.

Thus, thymocytes which expressed a TCR specificity that does not react with MHC were able to undergo secondary recombinations at the mutated TCRα allele until they obtained a combination of TCRβ and TCRα chains that can initiate positive selection.

3.3.3 Negative Selection in Male HY-I Mice

In male HY-I mice expressing the MHC molecule H-2D^b the cognate ligand for the HY-TCR is recognised by the thymocytes and induces negative selection of them (Kisielow et al., 1988) . This leads to complete absence of DP and SP compartments in the thymus of male conventional HYtg mice and dramatically reduced cellularity (fig. 15 & 17A, Kisielow et al., 1988). In contrast to this, small DP and SP compartments were found in the negatively selecting thymi of male HY-I mice (fig. 15, 17B & 19A). The thymic cellularity was, however, also strongly reduced when compared to female mice (8-10 times, fig. 17A). This reduction was most pronounced in the DP compartment with a cell number reduced to 4% of the number found in female mice (fig. 17B). The DN compartment was large in size (83% 11x10^6 cells), mainly due to cells expressing the HY-TCR as it has been observed before in HYtg males (fig. 15, 19A, & 19B; Teh et al., 1990; von Boehmer et al., 1991; Nikolic-Zugic et al., 1993).

Nearly all of the DP and SP cells had lost expression of the HYα$^{I\Delta}$ allele and some of these HYα$^-$ cells expressed instead TCRα chains using Vα2, Vα3, Vα8, and Vα11 elements (fig. 19B &19C, data not shown). Thus, these cells had undergone recombination events that lead to excision of the transgenic and the generation of endogenous VαJα joints. Unexpectedly, some SP and DP cells retained expression of the inserted α-chain (fig 19B). The receptor of the HYα^{low}DP cells had obviously been engaged as judged by the increased CD69 staining (fig. 16B). It is not clear whether these cells die or are in the process of undergoing secondary recombinations, but positively selected HYα^{high} cells were not observed (fig. 19B). All SP HYα$^+$ cells exhibited an immature phenotype as judged by strong staining for HSA (fig. 16D, data not shown). They are in their majority cells before selection because most of them had a CD69$^-$ phenotype (fig. 16C, data not shown). All mature HSA^{low}CD4$^+$ cells had lost expression of the HYα-chain (fig. 16D).

Coreceptor function is essential for the induction of thymic selection (Rahemtulla, 1991; Killeen, 1992; Fung-Leung et al., 1993; for review: Kisielow and von Boehmer, 1995). Thus, expession of an autoreactive TCR before onset of coreceptor surface

expression may not result in apoptosis and it is conceivable that cells which rearrange the insertion allele shortly after β-selection at the DN IV stage could give rise to the population of HYα$^-$DP cells observed in negatively selecting HY-I thymi. Assuming that such „early recombiners“ could undergo 9 rounds of cell division (factor of 2^9; Falk et al., 1996) and have to generate a DP compartment of $1.5x10^6$ cells (15% of total thymocytes in male HY-I mice), it can be calculated that a mere $3x10^3$ DN cells need to acquire a new VαJα joint and develop into DP cells within 3.5–4 days (life time of a DP cell; Egerton et al., 1990; Huesmann et al., 1991; Grusby, 1993). It is possible that HY-TCR expressing DN thymocytes aquired a new VαJα joint only after β-selection has rendered the TCRα locus accessible for recombination and transcription (Sleckman et al., 1997; Villey et al., 1997; Hozumi et al., 1998; Monroe et al., 1999) and triggered differentiation into DP cells (Mombaerts et al., 1992a). The time an „early recombiner“ spends in the DN compartment may thus be lower than 3.5–4 days and the number of DN cells which carry a new VαJα joint detectable at a given time may be below $3x10^3$ cells. It seems to be difficult to detect such cells by FACS analysis.

Thus, it appears that only few autoreactive thymocytes escape from apoptosis by secondary recombination. Editing seems not to be a general rescue pathway in thymic development in this particular model.

3.3.3.1 Thymic Selection in Homozygous Insertion Mice

Analysis of male mice (H-2D^b) carrying the HYα$^{I\Delta}$ allele homozygously and the HYβ allele revealed a phenotype (fig. 20) which resembled strongly the one observed in the HYtg model (fig. 15). No distinct DP and SP compartments were found. The thymus consisted mainly of DN cells (91.2%, $1.7x10^7$ cells). Homozygous and heterozygous females on the other hand were similar with respect to their thymic phenotypes (fig. 20A). The observed difference in the stringency of negative selection could be due to higher surface expression of the receptor resulting in higher sensitivity of the cells to peptide/MHC. I excluded this possibility by staining for the TCR on the surface of DP thymocytes, CD8$^+$ thymocytes and peripheral T cells of female heterozygous and homozygous HY-I mice. Lower surface expression in heterozygous mice compared to homozygous mice was not observed (fig. 20B, data not shown) by the use of different anti-TCR reagents (anti-HYα-chain, anti-Vβ, anti-Vβ8, and anti-CD3ε).

Thus, escape from negative selection was almost completely abolished when the HYα$^{I\Delta}$ allele was bred to homozygosity.

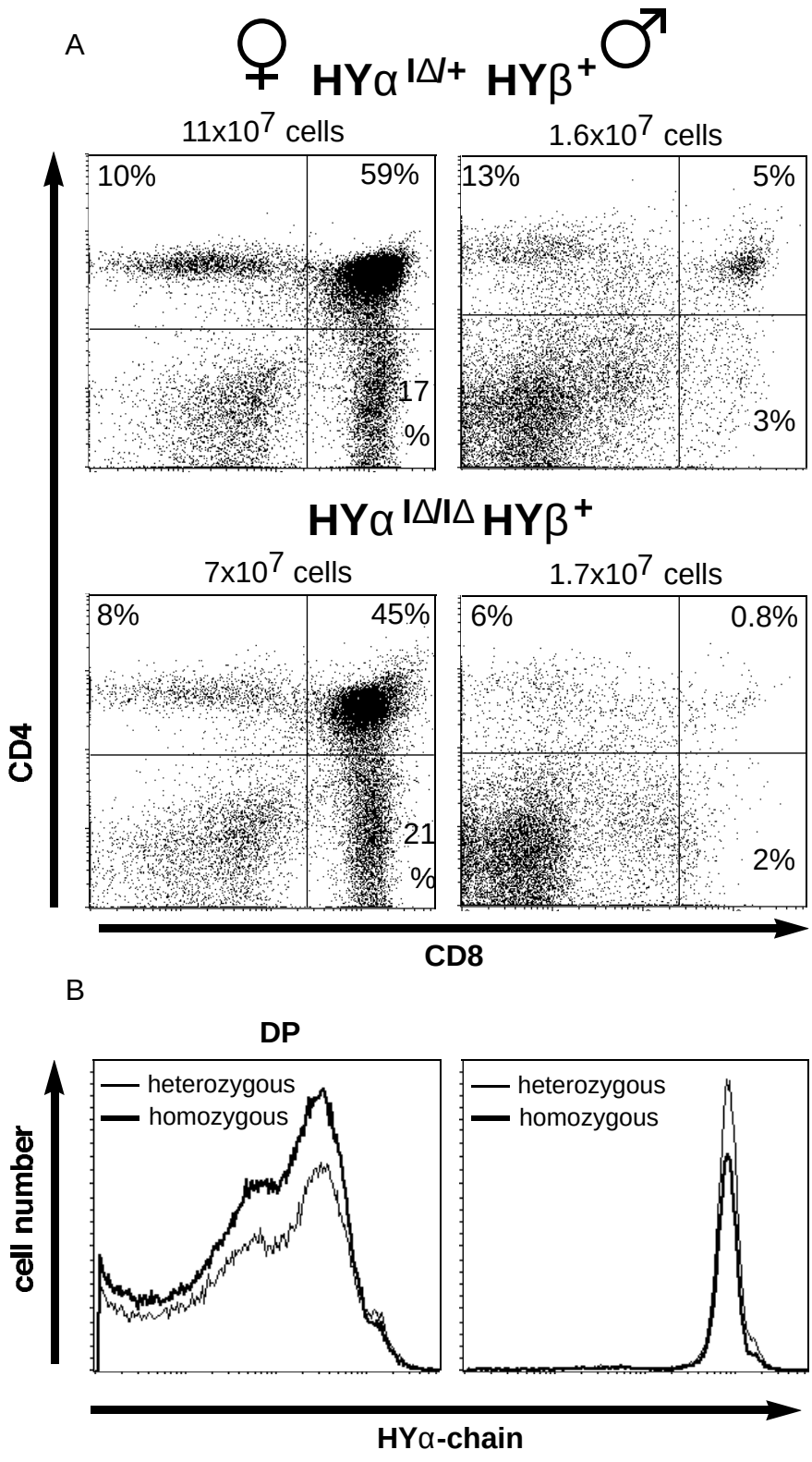


Fig. 20: Complete deletion of DP and SP cells in homozygous as compared to heterozygous HY-I males (A); total thymocytes were stained for CD4, CD8, and HYα-chain and analysed by FACS. The percentage of cells in the different subsets is indicated in the corners of the dot plots. The levels of α-chain expression on DP and $CD8^+$ thymocytes were identical between homozygous and heterozygous HY-I females as assessed gating on the respective populations and analysing HYα chain in histogram overlays (B).

3.4 Negative Selection in Fetal Thymic Organ Cultures

In a WT thymus rearrangement and initiation of expression of the TCRα locus happens in the DP compartmen, but in HY-I mice the inserted α-chain was shown to be expressed already in DN cells (Fig. 19B). It seems possible that cells carrying the HY-TCR received the negatively selecting signal during transition from the DN to the DP stage. Because they would not have entered the proper stage for V(D)J recombination at the TCRα locus, replacement of the autoreactive TCR was not possible and the cells died by apoptosis. To circumvent this problem I investigated the reaction of HYα$^+$DP cells to activating peptide/MHC complexes in fetal thymic organ cultures (FTOC; Hogquist et al., 1993).

In FTOC it is possible to perform studies on the first αβ lineage thymocytes which appear in the fetal thymus. It was assumed that most of the cells which entered the DP compartment in fetal development would express the transgenic TCR, because they had just entered the stage for TCRα recombination. In a pre-test I determined at which day in embryogenesis the DP compartment is formed in HY-TCR transgenic mice. For this pre-test thymi from embryos of conventional HYtg animals of day 15 after mating (E15.5), E16.5, and E17.5 were analysed by FACS for CD4, CD8, and HYα chain expression (fig. 21, data not shown). In the thymi from female E16.5 HYtg embryos only 5% but from female E17.5 embryos already about 30% of cells were CD4$^+$CD8$^+$ (fig. 21). For the actual experiments the HY-I model was used and also in thymi from female E17.5 HY-I embryos large DP compartment (52%) were found (fig. 25). As predicted, most (68%) of the cells at this stage were found to express the HY-TCR (fig. 26).

The optimal concentration of HY peptide (Markiewitz et al., 1998) was determined by titration on E17.5 thymi from female conventional HYtg embryos. It was observed that 50 µM and 5 µM of HY-peptide deleted DP cells efficiently (fig. 22). However, cell loss in the CD8$^+$ was more pronounced with 50 µM than 5 µM HY-peptide. This difference in negative selection dependent on peptide concentration may be caused by different sensitivities of DP and SP cells for the induction of apoptosis by an activating ligand (Kishimoto

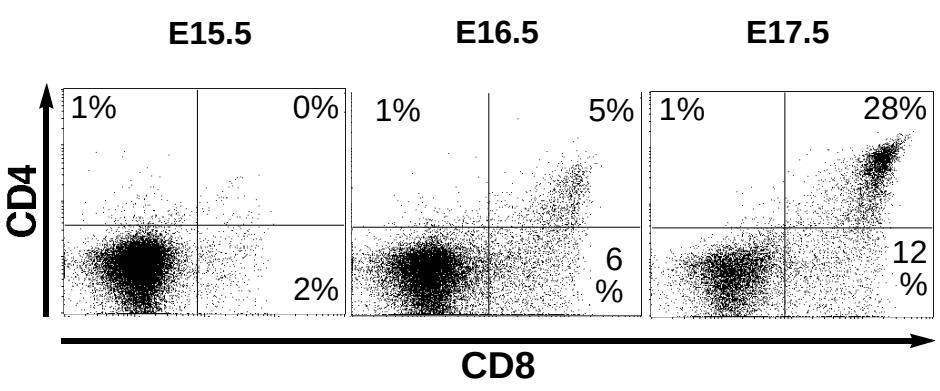


Fig. 21: Onset of αβ T cells lineage development in the thymus of HYtg embryos; thymoctes from HYtg embryos of the indicated gestational days were analysed by FACS for expression of CD4 and CD8. Only thymi from female embryos are shown.

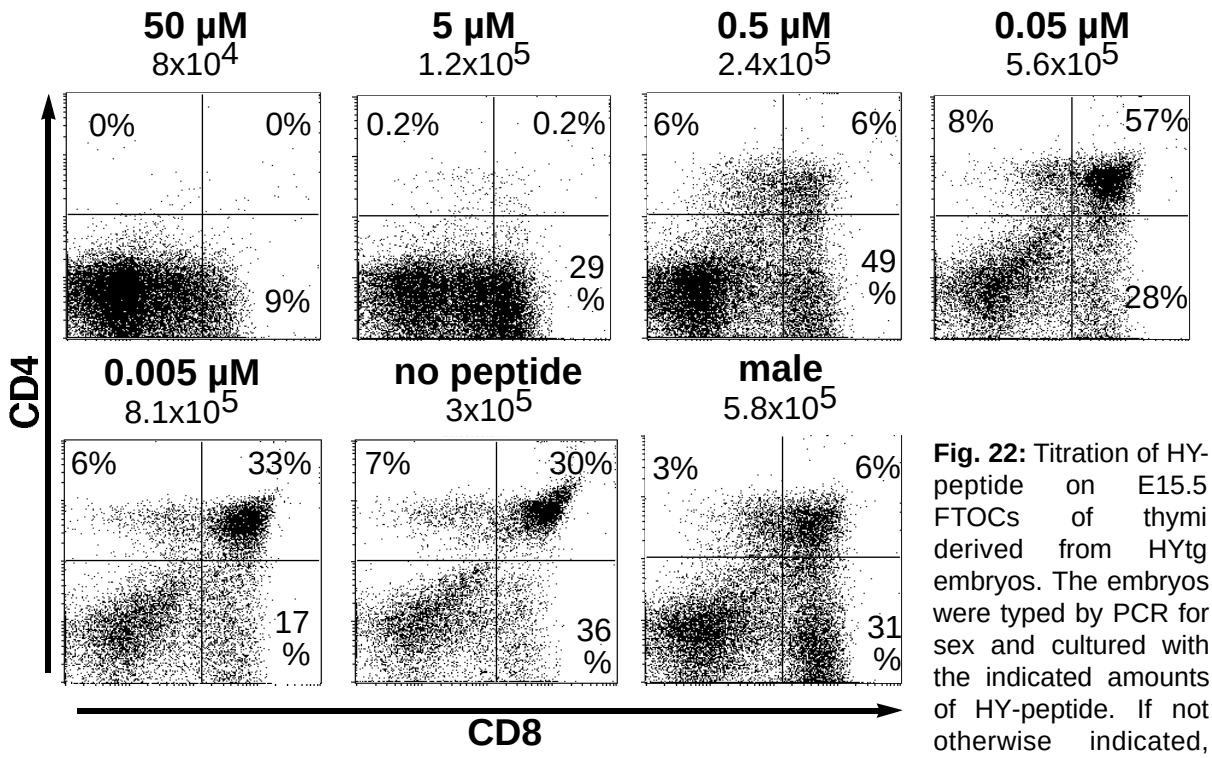


Fig. 22: Titration of HY-peptide on E15.5 FTOCs of thymi derived from HYtg embryos. The embryos were typed by PCR for sex and cultured with the indicated amounts of HY-peptide. If not otherwise indicated, only cells of FTOC of thymi from female embryos are shown. Analysis was performed after 5 days of culture by FACS after antibody staining for CD4 and CD8. The optimal peptide concentration was assessed by deletion of cells in the DP and CD8 compartments. The number of cells recovered from FTOC of the two thymic lobes from one embryo are indicated above the dot plots.

and Sprent, 1997; Davey et al., 1998). To be certain that negative selection would be induced it was decided to use in most experiments 50 µM of peptide, since it had been shown that especially in FTOC low concentrations of some activating peptides could induce positive selection (Hogquist and Bevan, 1996; Fukui et al., 1997) or alternatively a low number of activating ligand and thus selection „niches“ could allow some cells to escape selection at all (Merkenschlager et al., 1994). However, some experiments were also performed with 5 µM of peptide and generated similar results (data not shown). Nonspecific toxicity of the peptide at 50 µM was excluded by culture and analysis of E17.5 thymic lobes which lacked the transgenic β-chain (and thus specific reactivity to the HY-peptide). No difference was observed in FTOCs from $HY^{I\Delta/0}$ embryos with or without peptide after 5 days (fig. 24). Thus, any effect which would be observed upon peptide administration in FTOC of thymocytes expressing the HY-TCR would be due to the transgenic specificity.

To generate embryos with the HY-I genotype homozygous $HY\alpha^{I\Delta/I\Delta}HY\beta^{+}$ males were mated with TCRα deficient females. At E17.5 genotype and sex of the embryos were determined by PCR for the HYβ transgene (see: table 4), a y-chromosomal and a x-chromosomal gene (fig. 23; table 4; Kunieda et al., 1992). Thymi were prepared from these embryos and cultured with or without HY peptide for up to 5 days. In thymic lobes of

E17.5 female HY-I embryos up to 68% of the DP thymocytes expressed the transgenic α-chain (fig. 26). After culture for one day this was reduced to 52%, probably due to replacement of the inserted α-chain in cells which did not immeditately receive a positively selecting signal. When 50 µM of HY peptide had been added to such 1-day cultures the number of HYα$^+$ cells among DP cells was reduced to 15% (fig. 26 & 27). The HYα$^-$ cells had replaced the receptor, as shown by the use of Vα2 elements in the TCRs on these cells (fig.26). The reduction of DP thymocytes expressing the HY-TCR under negatively selecting conditions was accompanied by a reduction in total cell number, which was caused specifically by the deletion of the HY$^+$ cells (fig. 26 & 27).

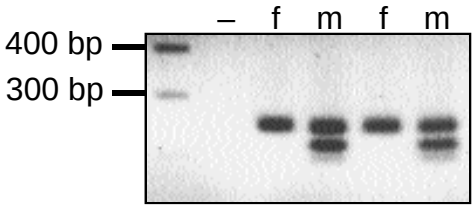


Fig. 23: Sexing PCR: Tail DNAwas prepared from embryos and PCR reactions performed with the primers Nds3/4 and Zfy11/12 amplifying an X-chromosomal (upper band) and a Y-chromosomal (lower band) DNA fragment, respectivley. The products of the PCRs were separated on 2% agarose gels.. m: male, f: female, -: no DNA

To test whether the surviving HYα$^+$DP cells had been induced to undergo apoptosis as well, these cells were stained with Annexin V, a reagent which detects changes in

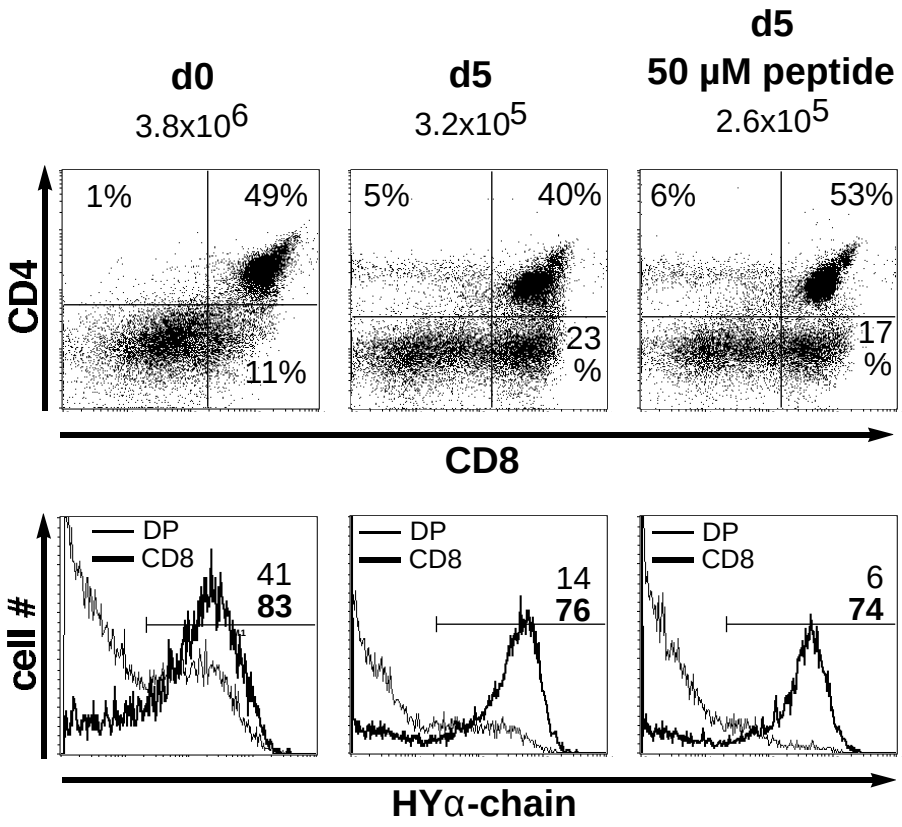


Fig. 24: Application of 50 µM HY peptide does not affect FTOC unspecifically, thymi of E17.5 embryos which had the genotype HYα$^{I\Delta/0}$ were cultured in the presence or absence of peptide for up to 5 days and then analysed for cell number, CD4, CD8, and HYα-chain expression. The total number of cells recovered is indicated above the dot plots.The percentages shown in the corners refer to the number of total thymocytes lying in the quadrant regions. The numbers given in the histograms are percentages of cells lying in the indicated region, bold number with respect to CD8$^+$ cells, plain numbers to DP cells.

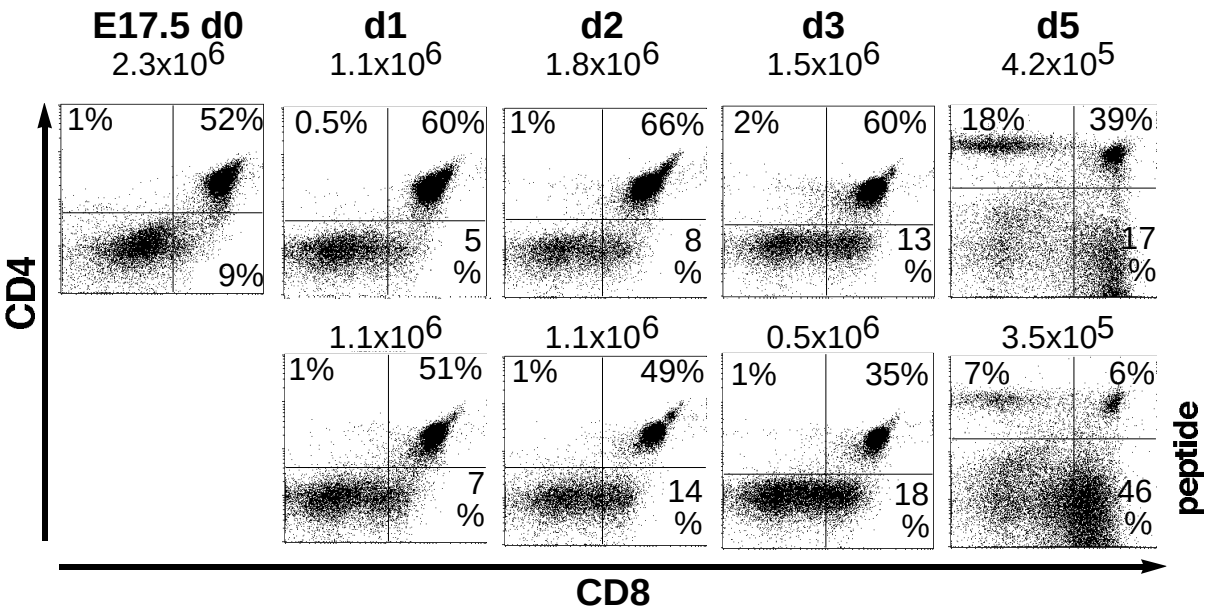


Fig. 25: Reduction in the size of the DP compartment over time in FTOC of female HY-I E17.5 thymi cultured in the absence (upper row) and presence (lower row) of 50 µM (5 µM for d5) of HY peptide, single cell suspensions were prepared on the indicated days and stained with the following antibodies: CD4 FITC and CD8 PE for d1–d3; CD4 Cyc and CD8 PE for d5.

membrane phospholipid symmetry characteristic of induced cell death (Koopman et al., 1994; Martin et al., 1995). A 4 times increase in the proportion of Annexin V^+ cells was observed among $HY\alpha^+DP$ cells after peptide administration (fig. 26).

Over a culture time of 5 days it was found that the percentage of cells in the DP compartment as well as thymic cellularity decreased with each day of culture with peptide as compared to cultures without peptide (fig. 25). This effect may be caused by the failure of progenitor cells to develop into DP cells as they are immediately deleted upon coreceptor upregulation (fig. 15).

Unexpectedly, after addition of peptide in these long-term cultures accumulation of $CD8^+HY\alpha^+$ cells was observed (fig. 25). It was found that the majority of these cells expressed low HSA levels, indicating that they were mature T cells. These cells could have been αβ T lineage cells which were positively instead of negatively selected (Hogquist and Bonnevier, 1998) or activated γδT lineage cells expressing the transgenic αβ TCR (Terrence et al., 2000). If they were belonging to the γδT lineage, they would upregulate CD8 upon activation without the need to develop through the DP compartement as αβ T lineage cells. A test for the lineage relationship was therefore to use young fetal thymi (E14.5) which do not yet contain DP cells and add peptide. As expected, peptide treatment in these cultures almost complete abrogated the development of a DP compartment (fig. 28). It was observed that the total number of $CD8^+HY\alpha^+$ cells after culture with peptide for 5 days cultures was comparably low ($0.4x10^5$ cells) as compared to the $CD8^+HY\alpha^+$ cells in E14.5 cultures of HY-I thymi from male embryos ($1.5x10^5$

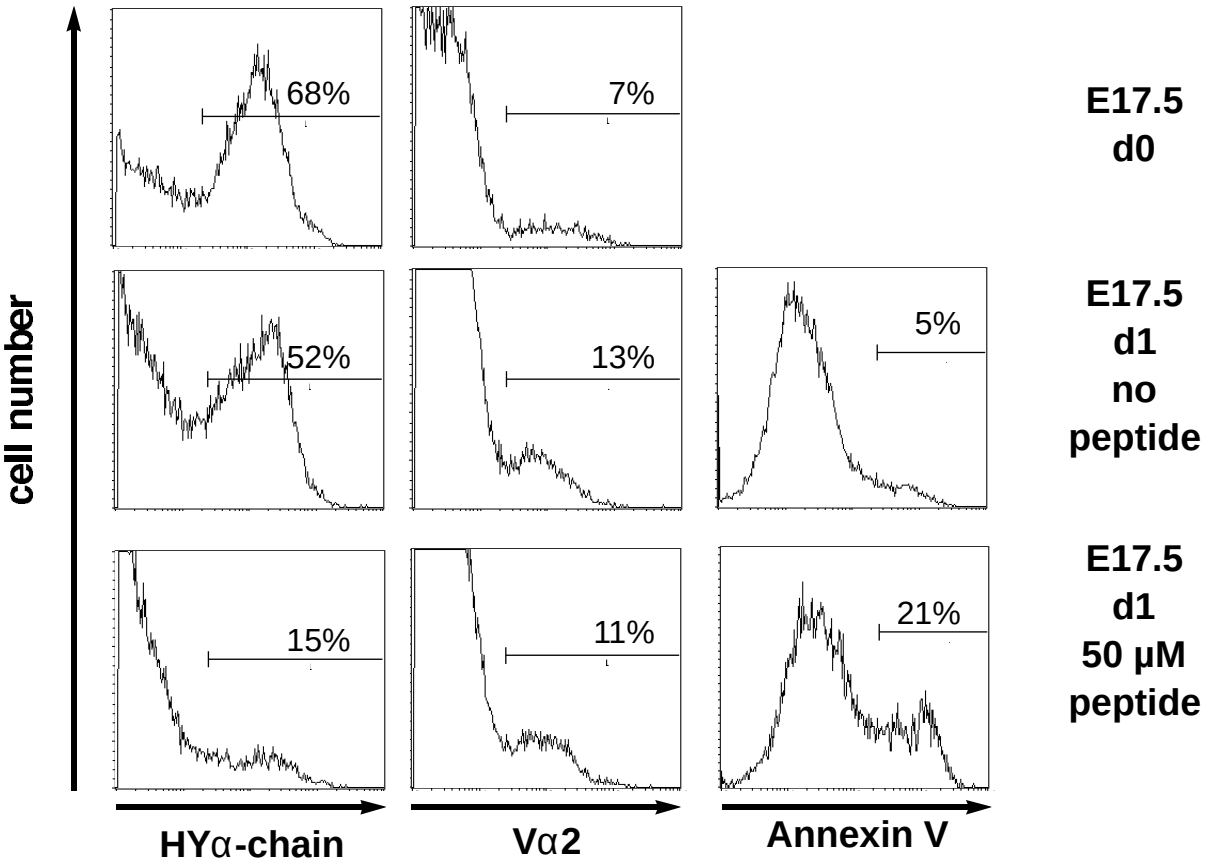


Fig. 26: Deletion of HYα$^+$ cells from the DP compartment of E17.5 HY-I FTOCs during the course of a one day culture with 50 µM HY-peptide is associated with increased expression of the apoptosis marker AnnexinV on remaining HYα$^+$ DP cells, but not increased numbers of Vα2 expressing cells. Gestation day 17.5 HYα$^{I\Delta}$ embryos were typed for sex and presence of HYβ-chain. The thymi of female HYβ$^+$ embryos were cultured for one day and analysed by fluorocytometry for CD4, CD8, HYα,

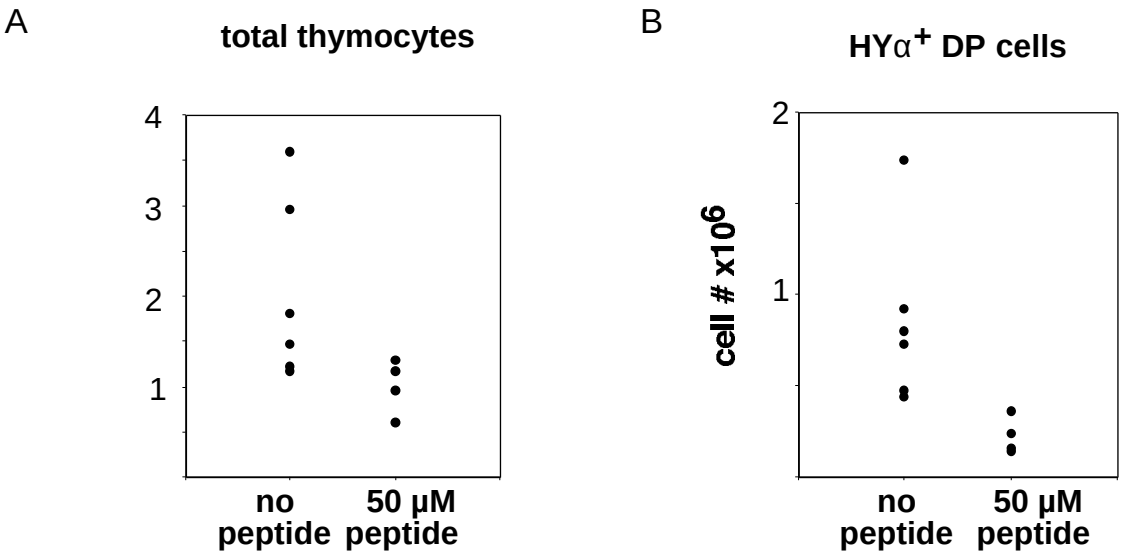


Fig. 27: The total cell numbers of FTOCs of female E17.5 HY-I embryos cultured in the presence or absence of HY-peptide (A). The numbers of DP cells expressing the HYα was calculated from total cellularity and the percentage of cells found by FACS analysis to expressing CD4, CD8 and the HYα-chain (B).

cells) (fig. 28). As described before (Teh et. al., 1990) despite the presence of activating ligand in FTOC from male HYtg embryos a DP compartment was generated. Thus, the majority of $CD8^+HY\alpha^+$ cells seems to be dependent on development through the DP compartment and belong to the αβ T cell lineage. However, it cannot be excluded that some of the $CD8^+HY\alpha^+$ cells which remained in the cultures after addition of peptide

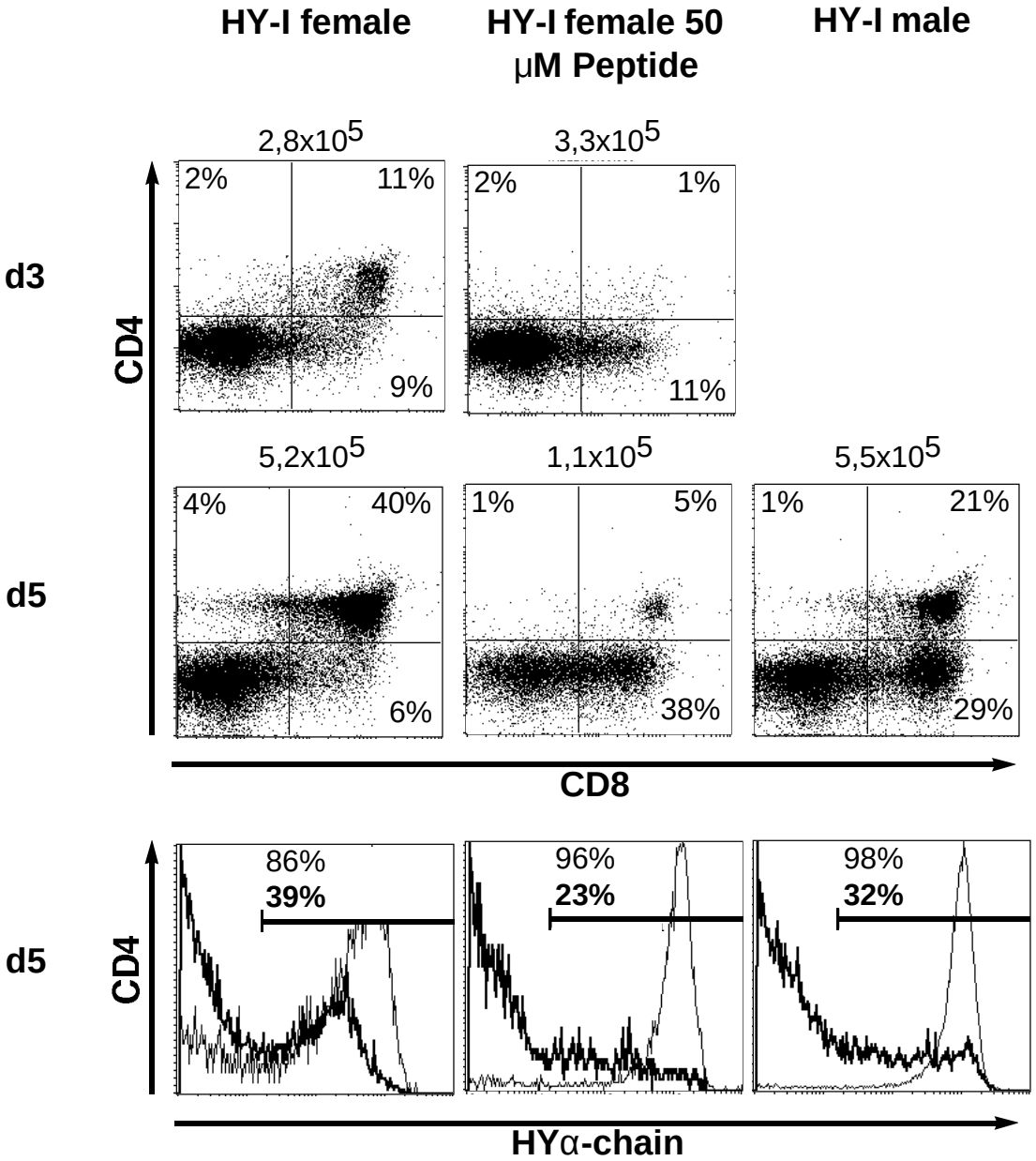


Fig. 28: Few $CD8^+HY\alpha$-chain$^+$ cells accumulate in E14.5 FTOC of HY-I embryos; Gestational day14.5 $HY\alpha^{I\Delta/0}$ embryos were prepared and typed for sex and HYβtg. The thymi were placed on membranes, and cultured with or without 50 µM of HY-peptide for up to 5 days. Single cell suspensions were prepared on d3 and d5 of culture and expression of CD4, CD8, and HYα-chain assessed by FACS analysis under exclusion of dead cells by Topro-3. Total cell numbers recovered from the cultures are indicated above the dot plots. The percentages shown were calculated in respect to the quadrants in the dot plots.The histograms show HYα-chain expression on DP cells (bold line) or $CD8^{+/lo}$ cells (thin line) of d5 FTOCs. The percentages show the number of cells in the indicated region, bold numbers for DP

were belonging to the γδT lineage.

These results confirmed that DP thymocytes expressing the HY-TCR undergo apoptosis when encountering cognate peptide/MHC. Editing can also in FTOC not prevent cell loss upon induction of negative selection. Interestingly, some of the autoreactive cells can differentiate into mature SP cells retaining expression of the HY-TCR.

3.5 Thymic Reaggregation Culture

The use of FTOC to investigate TCR editing in the DP compartment had only shown that a certain fraction of HY-TCR expressing DP cells were deleted when they were stimulated with cognate MHC/peptide complex. Some cells, however, seemed to have changed receptor specificity. Since this receptor change was also observed in cultures without peptide it cannot be said, whether peptide/MHC had induced the process. A thymic reaggregation culture strategy (Anderson et al., 1993) was used to study the effect of negative selecting thymic environment on a pure population of HYα$^+$DP thymocytes. The thymic reaggregation culture system was modified to contain all stromal and antigen presenting cell types of a mature thymus. To obtain the stromal cells, thymi of mature C57BL/6 mice were digested with collagenaseD/DNaseI and subsequently trypsin/EDTA and T cell precursors were removed by magnetic cell sorting (MACS) with CD4 and CD8 magnetic beads followed by a second depletion using CD90 (Thy-1.2) beads. Remaining cells were considered thymic stromal and antigen presenting cells. This cell suspension contained a high percentage of large granular cells and expression of the antigen presenting molecules MHC class I and class II on these cells differed in subsets defined by CD11b and CD11c expression (fig. 29), corresponding to macrophages and DC cells, respec-

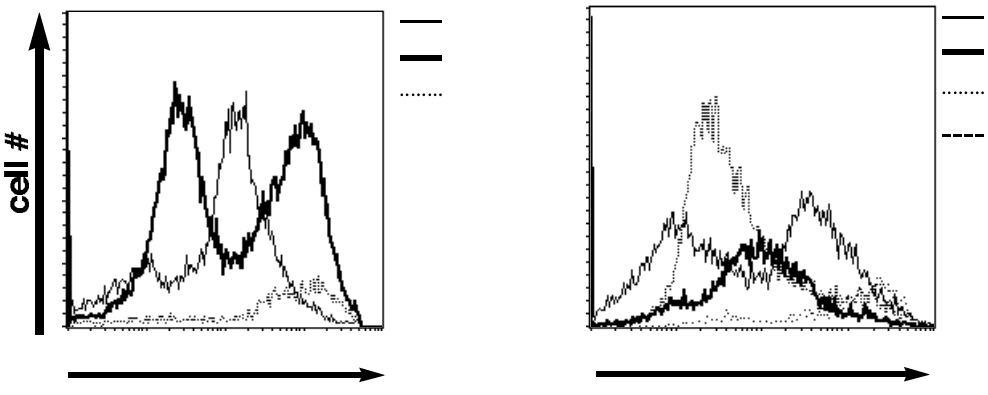


Fig. 29: Expression of MHC class I and class II on subpopulations of thymic stromal and APC cells as defined by expression of CD11c and CD11b. Cells were prepared by digestion of C57Bl/6 thymi with collagenaseD/DNaseI and trypsin/EDTA. The susension was depleted of CD4$^+$ and CD8$^+$ cells, then of CD90$^+$(Thy-1.2) cells by MACS. The remaining cells were stained with ab´s against H-2 K^b, H-2 I-

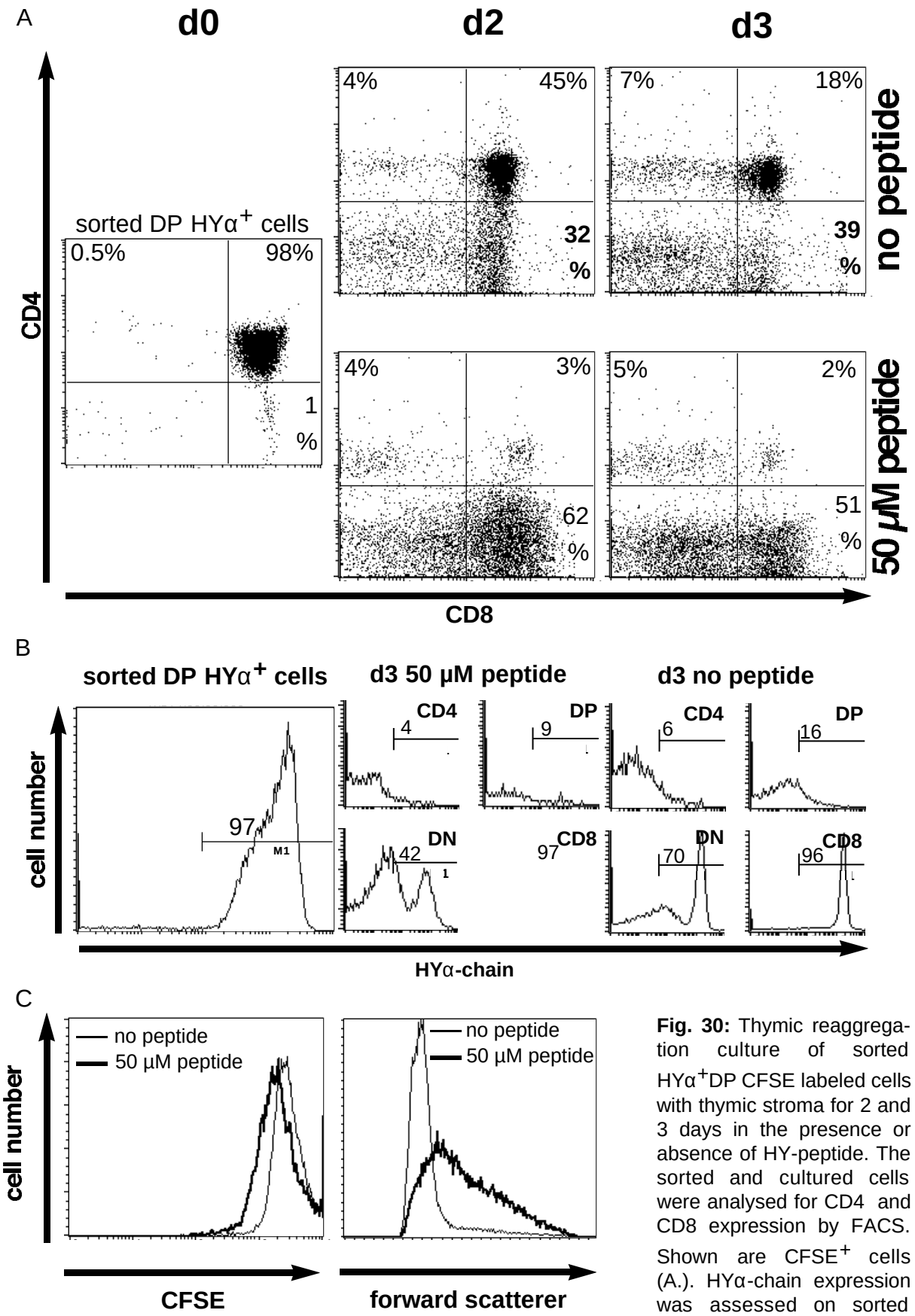


Fig. 30: Thymic reaggregation culture of sorted HYα$^+$DP CFSE labeled cells with thymic stroma for 2 and 3 days in the presence or absence of HY-peptide. The sorted and cultured cells were analysed for CD4 and CD8 expression by FACS. Shown are CFSE$^+$ cells (A.). HYα-chain expression was assessed on sorted cells and on the subsets indicated by the the quadrants in the CD4/CD8 dot plots (B). Loss of CFSE label in CD8$^+$ cells (d3) as well as increase in general cell size was observed in cultures after addition of peptide (C).

tively.

In parallel to the preparation of thymic stromal cells HYa^+DP^+ cells were sorted by FACS from thymocytes of female HY-I ($H\text{-}2D^b$) mice. The sorted cells were labeled with CFSE (Lyons and Parish, 1994) and mixed in the ratio 3:1 with thymic stromal cells. After centrifugation the pellet was transferred to membranes which were placed on media with or without 50 µM peptide. The drops containing thymocytes and thymic stromal cells formed disc-like reaggregates over night.The transfer of the cells to the membranes was performed by pipetting with a drawn-out glas capillary and distributing half of the content to two membranes. Since a substancial variation in the number of distributed cells must be assumed in this method, absolute cell numbers were not indicated in fig. 30 & 31. Only large changes in cellularity were considered significant and are reported.

After 2 and 3 days cells were harvested and analysed for expression of CD4, CD8, and HYα-chain to investigate whether some of the cells had lost expression of the transgenic α-chain which would indicate that these cells had undergone editing to escape negative selection (fig. 30A & B).

It was found that the cultures without peptide showed efficient development of $CD8^+HY\alpha^+$ cells (fig. 30A & B), but a large DP compartment remained in these cultures over the 3 day period (fig. 30A). The majority of DP cells had lost expression of the HYα-chain (fig. 30B) but may have been unable to be positively selected with their new receptor into either the CD8 or CD4 compartments. $CD4^+$ cells which had lost the expression of the transgenic α-chain were also observed in these cultures (fig. 30A & B). Their new TCR allowed them to be positively selected and to develop into $CD4^+$ cells. Thus, sorted $HY\alpha^+$DP cells in the reaggregation culture system can principally perform secondary recombinations to change receptor specificity.

A different picture was observed in cultures in the presence of 50 µM of HY peptide. After 3 days the cellularity of these cultures was reduced by 30-50%. Only a small DP compartment remained (fig. 30A). $HY\alpha^-CD4^+$SP cells were generated in similar number as in cultures without peptide (fig. 30A & B). The cultures with peptide were dominated by $CD8^+$ cells (fig. 30A & B) similar to the accumulation of such cells in the FTOC experiments. Most of these $CD8^+$ cells expressed the transgenic TCR, had a large, blast-like phenotype and had been dividing as seen by loss of CFSE label (fig. 30C). It cannot be excluded that some of these $CD8^+HY\alpha^+$ cells were generated by proliferation of cells with this phenotype that were contaminants from the sorting (fig. 30A). However, already after 12 hrs >70% of the cells in thymic reaggregates had developed into $CD8^+HY\alpha^+$ cells in the presence or absence of HY-peptide (data not shown) which did not leave contaminating mature T cells enough time to become activated and start proliferation. In addition, 46% of $HY\alpha^+$DP cells were able to differentiate into $CD8^+HY\alpha^+$ cells within 36 hrs in suspension cultures (Swat et al., 1991) in

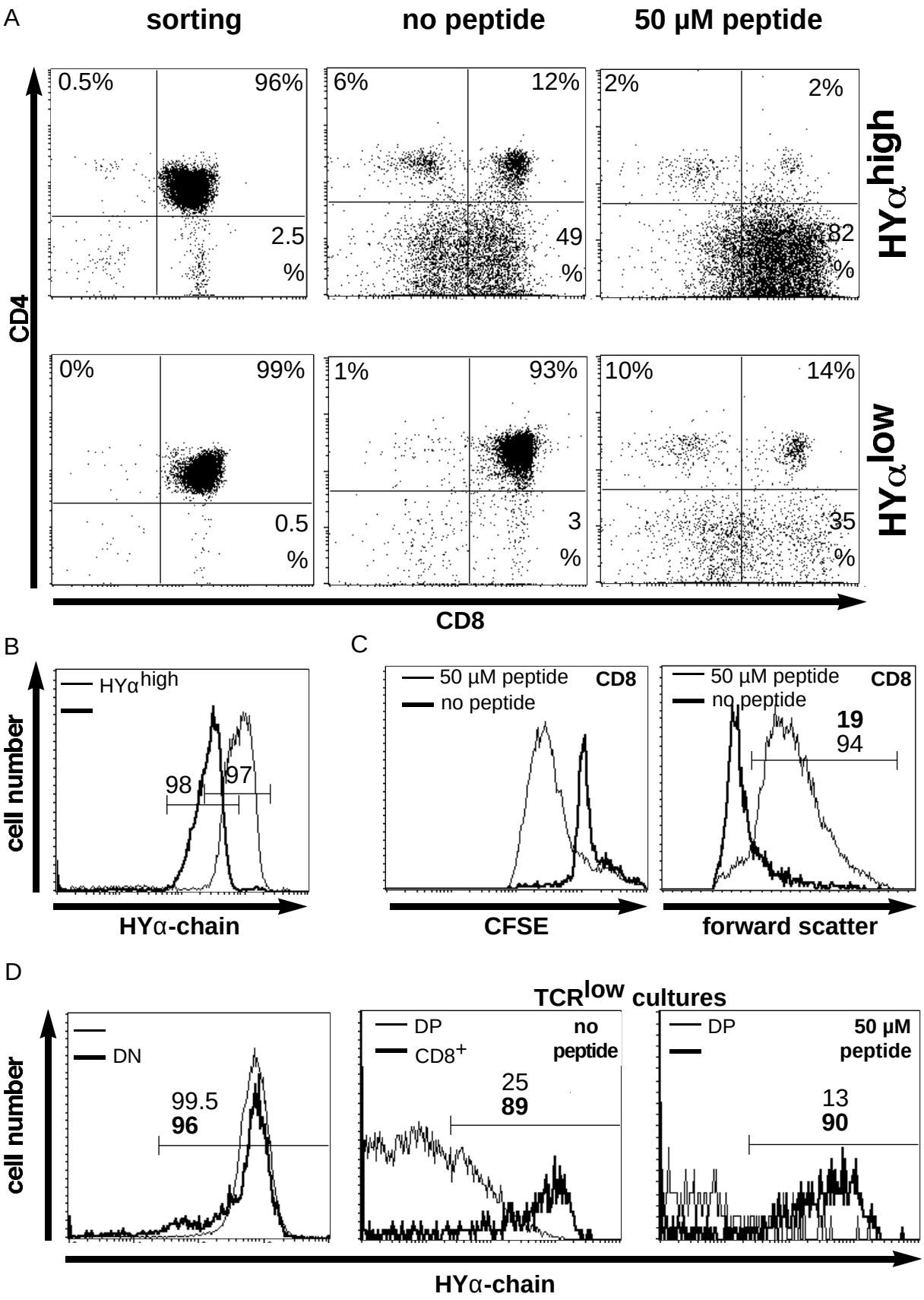


Fig. 31: Thymic reaggregates of sorted CFSE labeled HYα$^+$DP cells and thymic stroma after culture for 3 days in the presence or absence of HY-peptide. The sorted and cultured cells were analysed for CD4 and CD8 expression by FACS. Shown are CFSE$^+$ cells (A). HYα-chain expression was assessed on sorted cells and on CD8$^+$ and DP cells (B)(D). Loss of CFSE label in CD8$^+$ cells as well as increase in cell size was observed in cultures after addition of peptide (C).

the presence of APCs not expressing the restricting MHC haplotype (data not shown). These cells must have been positively selected in vivo already, since their development was independent of further signaling induced by the restricting MHC element. Thus, it seems that some HYα^{+}DP cells cells can develop into HYα^{+} CD8^{+} cells after addition of peptide. Escape from deletion by change of specificity (editing) was seen in thymic reaggregates of HYα^{+}DP cells only in a minority of cells (fig. 30A & B).

It has been reported that negative selection may preferentially apply to non-positively selected TCR^{low}DP thymocytes (Ghendler et al., 1997; Kishimoto and Sprent, 1997; Ghendler et al., 1998; Tarazona et al., 1998; for review: Page et al., 1996). Sensitivity for induction of apoptosis by triggering with cognate ligand seems to decrease with maturation (Davey et al., 1998).

Therefore, DP cells from thymi of female HY-I mice were sorted into HYα^{high} and HYα^{low} subsets (Fig. 31B) and these populations were taken into reaggregation culture to follow their developmental fate. The majority of cells in TCR^{high} cultures developed either into CD4^{+}, CD8^{+}, or CD8^{low} cells (fig. 31A). After addition of 50 µM peptide only slight reduction in the cell number was observed. Most cells in this culture had differentiated into CD8^{+}HYα^{+} cells (fig. 31A & B) which had aquired a blast-like phenotype and started to proliferate (fig. 31C). However, as discussed already, it cannot be excluded that some of these cells are derived from contaminating SP cell from the cell sorting (fig. 31A).

A different picture was seen upon analysis of HYα^{low} cultures. Only few cells in these cultures differentiated in the absence of peptide into SP cells (fig. 31A), similar to previous reports (Swat et al., 1992). A large fraction of the DP cells had lost expression of the transgenic α-chain (fig. 31B). They may have undergone secondary recombinations at the insertion allele. When peptide was added, only around 1/20 – 1/30 of cells could be recovered from the reaggregates compared to cultures without peptide. Most dramatic was the loss of thymocytes in the DP compartment (fig. 31A). There only some cells survived which obviously had changed receptor specificity and lost expression of the HY-TCR (fig. 31D). No accumulation of a CD8^{+}HYα^{+} cells could be observed in this culture (fig. 31A & B).

Thus, HY-TCR^{low}DP cells are highly susceptible to deletion induced by negative selection. HYα^{high} cells on the other can at least partially develop into CD8^{+}HYα^{+} cells. Secondary recombinations and surface expression of new receptors (and thus loss of HYα expression) were not observed to allow rescue of thymocytes from apoptosis in cultures to which HY peptide was added.

3.6 Editing in Peripheral T Cells

It is believed that most T cells develop in the thymus and migrate into the periphery after having undergone all necessecary recombination, selection, and commitment steps (for review: Kisielow and von Boehmer, 1995). Mature T cells were not thought to express the RAG proteins and change TCR specificity (Monroe et al., 1999; Yu et al., 1999; Nagaoka et al., 2000). However, reports from two groups questioned this dogma (McMahan and Fink, 1998; Lantelme et al., 2000a; Lantelme et al., 2000b). In one case (McMahan and Fink, 1998) a β-chain transgenic mouse model was used, in which T cells were under selective pressure by a peripherally expressed weak superantigen. In peripheral T cells which were characterised by lower surface expression of the transgenic β-chain rag gene expression, germline transcripts of TCR loci, reexpression of the pTα gene, and DNA breaks at the TCR loci were observed (McMahan and Fink, 1998). In a population of $TCR^{low}CD4^{+}$ human peripheral T cells another group identified similar signs of ongoing recombination (Lantelme et al., 2000a; Lantelme et al., 2000b).

Peripheral editing may either allow T cells to generate a new α-chain during the course of an immune response or it enables T cells specific for a peripheral autoantigen to change to an innocent TCR specificity. The HY-TCR insertion model allowed me to investigate peripheral editing by observing not indirect signs of recombination (e.g. rag expression, pTα expression, DNA breaks) but rather change of receptor specificity.

3.6.1 In Vitro Activation of Peripheral $HY\alpha^{+}$ T Cells

Peripheral T cells can be activated in vitro by stimulation with APCs expressing the restricting MHC element loaded with antigenic peptide. This activation system was used to observe replacement of the HYα-chain by another endogenously encoded α-chain among cells of a proliferating transgenic T cell population. $CD8^{+}HY\alpha^{+}$ peripheral T cells from female HY-I mice were labeled with CFSE and taken into culture with Mitomycin C treated splenocytes of C57/BL6 or BALB/c mice that were loaded with different concentration of HY peptide. After 3 days of culture the cells were harvested and analysed for HYα-chain and CD8 expression as well as for the intensitiy of the CFSE label (fig. 32). T cells that had either been cultured with APCs from C57BL/6 or BALB/c females in the absence of peptide did not become activated and did not proliferate. Peptide in the concentration range from 0.5 µM to 50 µM activated the transgenic cells. Similarly, splenocytes of C57BL/6 males induced proliferation of HY-TCR expressing cells (fig. 32). However in no case an increase of $HY\alpha^{-}$ negative cells as compared to non-activated cultures ,especially among the cells having undergone the most cell divisions, was observed (fig. 32). Thus, although transgenic thymocytes were activated to proliferate, they did not perform secondary recombinations. All cells

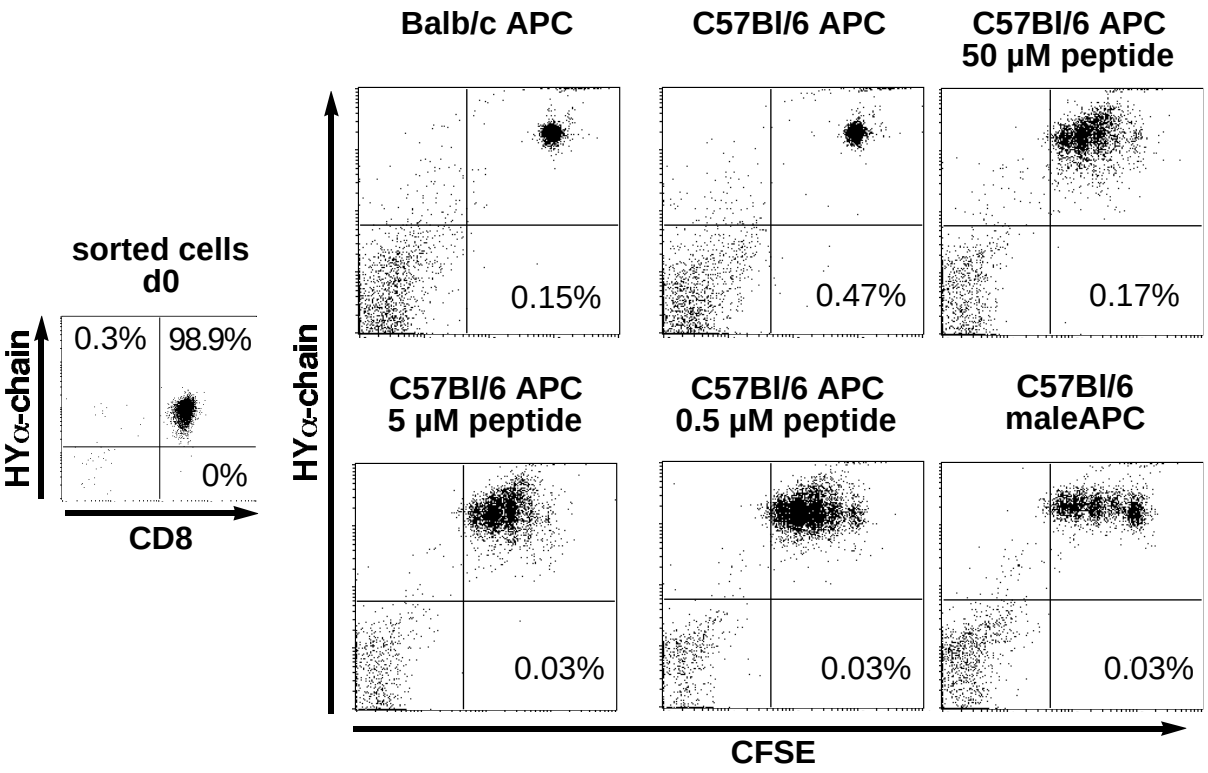


Fig. 32: Analysis of secondory recombination after in-vitro T cell activation. A single cell suspension of LN and spleen cells was marked with T3.70 biotin ab and streptavidin-beads, HYα-chain$^+$ cells were enriched through MACS, subsequently the cells were incubated with Streptavidin-Cychrome, CD4 FITC, and CD8 PE. In a FACSstar cell sorter CD8$^+$HYα$^+$ cells were sorted. The sorted cells were labeled with CFSE and 10^5 of these cells added to $5x10^5$ Mitomycin C treated peptide labeled APC of the indicated mouse strains. Three days later the cells were harvested, and analysed for the CFSE labe and expression of CD8 and HYα-chain by FACS. Dead cells were excluded by Topro-3.

retained surface expression of the trangenic α-chain. However, it is conceivable that in this artificial culture system cell types or anatomical structures were missing which usually would support peripheral editing in WT mice. Therefore an in vivo model was used to further study peripheral editing processes.

3.6.2 In Vivo Activation of HYα$^+$CD8$^+$ T Cells

Adoptive transfer of HYtg T cells has been used in a number of experiments to investigate life span, activation, and memory formation of T lymphocytes (Rocha and von Boehmer, 1991; Kirberg et al., 1994; Bruno et al., 1995). However, it has to be kept in mind that in adoptive transfer systems a bias may be generated by selection for cells which are better suited to migrate into lymphoid organs of the host organism. The advantage of using T cell deficient mouse strains in adoptive transfer studies is that the transferred T cells do not have to compete with host T cells to populate the lymphoid system and that later analysis is not disturbed by peripheral T cells of host

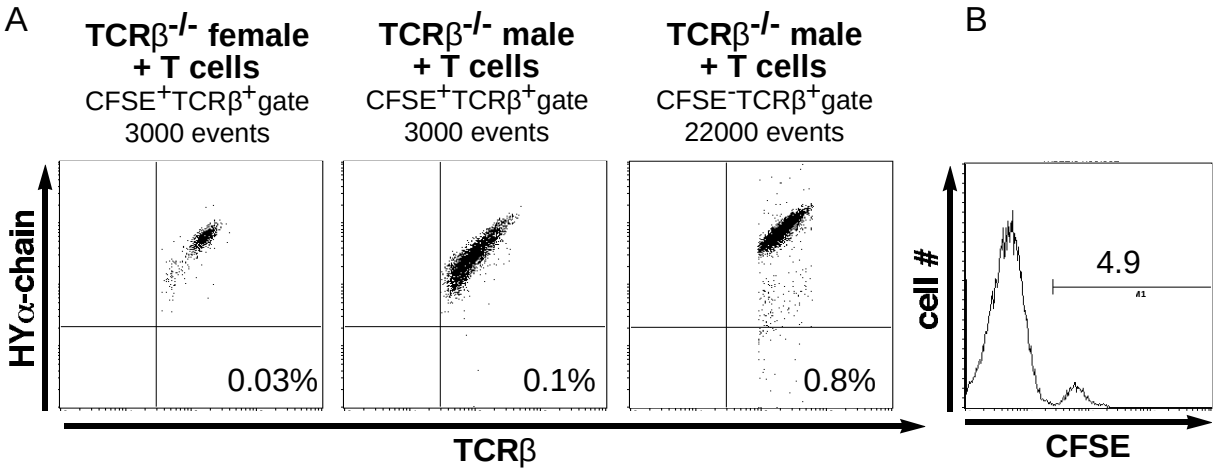


Fig. 33: Analysis of peripheral editing in an adoptive transfer experiment. LN and spleen cells were marked with T3.70 Biotin antibody and Streptavidin-MACS beads. HYα-chain$^+$ cells were enriched by MACS. Subsequently, the cells were stained with Streptavidin-Cychrome, CD4-FITC, and CD8-PE . CD8$^+$HYα$^+$ cells were sorted and labeled with CFSE. 10^7 of these cells were injected i.v. into TCRβ$^{-/-}$H-2D^b mice. Three days later spleen and LN cells were prepared from the hosts and depleted of B cells by MACS with CD19 beads. The remaining cells were stained with T3.70 Biotin (streptavidin-cychrome) and TCRβ PE and analysed by FACS. Dead cells were excluded by Topro-3 staining. For analysis of HYα chain expression it was gated on TCRβ expressing cells (A). Cells recovered from male hosts are shown in two plots gated on CFSE$^+$ or CFSE$^-$ cells as shown in the histogram. The histogram displays the CFSE label of all T cells recovered from male hosts (B). In the dot plot showing CFSE- cells a tighter TCRβ gate had to be set because TCRβ was the only marker distinguishing transfered and host lymphocytes. In female hosts no loss of CFSE label was observed.

origin. Therefore, TCRβ deficient mice (Mombaerts et al., 1992) which are characterised by the complete absence of peripheral αβ T cells were used as host animals for CD8$^+$HYα$^+$ peripheral T cells. The transferred cells were obtained by FACS sorting from female HY-I animals (H-2D^b) and labeled with CFSE to follow proliferation. They were injected i.v. into TCRβ deficient males and females. Three days later LN cells and splenocytes depleted of B cells by MACS were analysed for HYα, TCR, and CD8 expression as well as for the intensity of the CFSE label (fig. 33). Only 0.03% of the transfered cells were recovered. In these cells it was observed that despite TCR and co-receptor crosslinking during the sorting process the cells which had been injected into female hosts did not become activated to proliferation as seen by the retention of the CFSE label (data not shown). In addition, basically all cells retained surface expression of the transgenic α-chain. In male host on the other hand, seven times more T cells were recovered after the three days and complete loss of CFSE label was observed among the majority (95%) of these cells. These cells had become activated to proliferate. The number of HYα$^-$ cells remained below 1% in this population as well as in the CFSE$^+$ population (fig. 33). Thus, in vivo activation of peripheral T cells expressing the HY-TCR did not lead to any observable peripheral editing in this experimental system.

4 DISCUSSION

4.1 The TCRα insertion model

Editing processes which result in the replacement of an autoreactive specificity offer an opportunity for B- or T lymphocyte precursors to escape deletion by negative selection. They were shown to contribute significantly to the generation of the B cell repertoire (for review: Radic and Zouali, 1996; Nemazee, 2000). Most insights into the function of editing in B cell development were gained by model systems in which recombined V(D)J joints had been inserted into the Ig loci of the mouse (Chen et al., 1995; Prak and Weigert, 1995; Pelanda et al., 1997; Qin et al., 1999). A similar strategy was designed to investigate the involvement of secondary recombination events in T cell development. A VαJα joint was introduced into the TCRα locus and secondary rearrangements were analysed on this mutated allele.

4.1.1 Targeted Insertion of a VαJα in Murine ES Cells

During T cell development the initial Vα to Jα joining results in the excision of all intervening Vα and Jα elements as well as the entire TCRδ locus (Petrie, 1993; Chien, 1987). I used targeted mutagenesis (Hooper et al., 1987; Capecchi, 1989) and Cre-loxP meditated recombination (Sauer and Henderson, 1988; Gu et al., 1994) in murine ES cells to imitate Vα-Jα recombination which is usually carried out by the V(D)J recombinase system. A TCRα locus was generated which resembled the chromosomal organisation of a recombined TCRα gene (fig. 3 & 8) and expressed the introduced α-chain of the HY-TCR (fig. 8).

The TCRδ locus was flanked with loxP sites in two consecutive targeting experiments. To allow the use of G418 selection in the second targeting experiment, the neomycin resistance gene of the first targeting vector was removed by transient transfection with a Cre expression plasmid. However, a better strategy for future experiments involving two consecutive targeting experiments in the same cell line would be if removal of the first selection marker by Cre-mediated recombination was not necessary. Each transfection of ES cells can result in reduced pluripotency and the cells may no longer be able to take part in the generation of germ cells. Thus, the combination of different selection markers for the two targeting vectors (e.g. neomycin and puromycin resistance genes, Watanabe et al., 1995) seems advisable for future experiments.

In addition, the use of two different resistance genes may allow an easier strategy to find cis-location of the insertions. Hybridization of metaphase chromosomes with fluorescent DNA probes (Lichter et al., 1990) specific for unique sequences in the two

targeting vectors (i.e. resistance genes) may reveal whether both genes are located on the same allele, making an in-vitro Cre-mediated deletion unneccessary.

The in-vitro deletion of the 263 kb (Malissen, personal communication) genomic fragment containing the TCRδ locus (see 3.1.3) was performed without any enrichment for deleted clones. It was not clear at that time whether it would be possible to find deleted clones easily because other authors had carried out Cre-mediated recombinations of similar size only by using negative selection system to enrich for recombined clones. Using a HPRT based selection cassette one group (Ramirez-Solis et al., 1995) observed a 90 kb deletion with a frequency of $1.5x10^{-5}$. Another group (Li et al., 1996b) had included the thymidinkinase gene into the sequence which was flanked by loxP sites and reported a recombination frequency of $6.6x10^{-6}$ for deletion of a 200 kb fragment. In contrast to these observations, the deletion of the TCRδ locus upon transient Cre expression took place with a frequency of 1.5% of surviving transfected cells. In addition, another deletion of the TCRδ locus performed by F. Rieux-Laucat occured with a frequency of 10%. Thus, compared to previously published results these frequencies were unexpectedly high and are not different from deletion frequencies observed in experiments in our laboratory with smaller DNA segments flanked by loxP sites (unpublished observations). Our data indicate that it may not be necessary to introduce a negative selection system for the enrichment of cells which have undergone a Cre-mediated deletion of genomic fragments of up to several hundred kb.

4.1.2Establishment of the HY-I Mouse Strain

I used ES cell clones with the two targeted mutations in cis-position to generate chimeric mice by morula aggregation and blastocyst injection (Hogan et al., 1994). At first morula aggregation seemed preferencial because it had been reported to be an efficient strategy for the generation of chimeric animals (Wood et al., 1993a; Wood et al., 1993b). In one experiment which was performed at the same time in our laboratory morula aggregation resulted in the establishment of a transgenic mouse strain (Hein et al., 1998). In contrast, aggregation of my ES clones did not yield chimeric mice which transmitted the mutations through the germ line. Fortunetely, chimeras generated by blastocyst injection allowed the establishment of a mouse strain (HYa^{IF}) which carried the VαJα joint of the HY-TCR in the Jα region. It is possible that blastocyst injection is the superior method if ES cells have been transfected multiple times, but the lower number of aggregation chimeras (4) compared to injection chimeras (14) does not allow definitive conclusions.

By breeding of HYa^{IF} mice, which have a TCRδ locus flanked by loxP sites, to a mouse strain expressing the Cre recombinase early in embryogensis (Schwenk et al., 1995), I wanted to remove the TCRδ locus. However, double transgenic mice were

genomically mosaic mice in terms of deletion of the TCRδ locus (data not shown). They gave rise to offspring which carried deletion of the TCRδ locus in the germline with a frequency of only 5% (see 3.1.3). The strain with the deletion of the loxP flanked genomic segment (fig.5, bottom) was termed HYa$^{I\Delta}$ and used for subsequent analysis. In the HY-I model expression of the HY-TCR was facilitated by the HYa$^{I\Delta}$ allele and a conventional HYβ transgene (Uematsu et al., 1988). By use of a Cα deficient allele (Mombaerts et al, 1992b) on the second chromosome, analysis of secondary recombination processes was restricted to the inserted allele.

4.2 Expression of the Inserted TCRα Allele

4.2.1Peripheral Expression

In initial experiments it was observed that the proportion of HYα$^+$CD8$^+$ T cells in the periphery of positively selecting female HY-I mice was almost doubled as compared to the conventional transgenic HYtg strain (see 3.2.1). The larger population of peripheral HYα$^+$ T cells may be the result of higher thymic output or prolonged lifespan of these cells in the insertion system. Since the peripheral HYα$^+$CD8$^+$ population has been described in the HYtg system to be short lived and highly dependent on continuous thymic output (Rocha and von Boehmer, 1991), it is possible that the higher number of HYα$^+$CD8$^+$ cells in the thymus of HY-I females as compared to HYtg females (see 3.3.1) results in increased thymic output and thus a larger peripheral population of HYα$^+$CD8$^+$ T cells.

In the periphery of male (negatively selecting) HY-I mice, a population of HYα$^+$CD8$^{-/lo}$ cells developed which had already been described in a variety of αβ TCR transgenic models. Such cells were shown to be γδ T lineage cells forced to express the transgenic αβ TCR (von Boehmer et al., 1991; Nikolic-Zugic et al., 1993; Bruno et al., 1996; Brabb et al., 1997; Fritsch et al., 1998; Terrence et al., 2000). An almost complete absence of γδTCR$^+$ cells was observed in HY-I mice, but already the transgene encoding for the HYβ-chain alone was shown to suppress development of bona-fide γδT cells (von Boehmer, 1988, data not shown). Expression of the inserted α-chain in cells of the γδT cell lineage may be a transgenic artefact which cannot be avoided in the case of TCRα insertion transgenes, because in peripheral γδ T cells transcription of the TCRδ genes is facilitated by the TCRα enhancer (Sleckman et al., 1997; Monroe et al., 1999).

4.2.2Thymic Expression

The inserted α-chain together with the transgenic HYβ-chain was expressed as early as at the CD25$^+$ stage in the DN compartment of HY-I mice. However, the proportion of these HYα$^+$CD25$^+$DN cells was lower when compared to conventional HYtg mice (see 3.3.1) indicating either later onset of expression of the inserted trans-

gene or faster disappearance of HYα$^+$ cells from this compartment (by differentiation or cell death). Since the fraction of CD25$^+$ cells in the DN compartment was not decreased compared to conventional HYtg´s, no evidence supports the latter point of view (see 3.3.1).

One way to determine whether the HYα-chain is indeed expressed later from the inserted allele than from the conventional transgene may be the investigation of its capacity to drive thymic development through a block at DN III imposed by pTα deficiency (Fehling et al., 1995; Buer et al., 1997). Preliminary data indeed suggest that the inserted transgene is less efficient in reconstituting thymic cellularity in preTCR deficient animals than the conventional HY-TCR transgene (data not shown), indicating onset of expression in most cells after β-selection.

Another way to analyse the onset of expression of the inserted HYα-chain is offered by the observation that the DN II and DN III compartments in mice transgenic only for the inserted α-chain appear similar to the same stages in WT mice (data not shown). The depletion of these thymic subsets in TCR transgenic animals depends solely on the expression of a transgenic TCRβ chain (data not shown). Thus, by Northern-blot or quantitative RT-PCR analysis the onset of HYα-chain expression could be investigated in thymocytes from mice carrying only the HYα insertion allele.

In another TCRα insertion model (Wang et al., 1998), expression of the inserted α-chain was also observed in the majority of DN cells already. Unfortunetely, the published data do not include an analysis of transgene expression in the DN subsets. The authors suggest that either a recombined VαJα joint is sufficient to allow expression at the DN stage or that the introduction of additional regulatory elements (promotor and enhancer of the neomycin resistance gene, IgHε enhancer) into the locus facilitated the early transcription of the gene.

Which mechanisms could result in expression of an inserted recombined TCRα gene as early as at the CD25$^+$DN cells?

It is possible that premature TCRα enhancer function facilitates transcription earlier in development due to the deletion of the other enhancer element in the locus, the TCRδ enhancer. Another possibility is that upon expression of transgenic β-chain a preTCR is formed and the DN cells immediately receive the signal for further differentiation, including transcription of the TCRα locus.

If activity of the TCRδ enhancer suppresses TCRα enhancer function in DN III, mice which retain the TCRδ locus and carry an inserted α-chain in the Jα locus should not express the gene at the CD25$^+$ stage. However, I could observe similar expression of the HYα-transgene in the CD25$^+$ subset of thymocytes of the HYα^{IF}HYβ$^+$ mouse strain, which still contains the complete TCRδ locus on the targeted allele (fig. 13). Thus, it seems unlikely that deletion of the TCRδ enhancer has allowed prema-

ture expression of the inserted HYα gene. However, since an artificial element (Polyoma Virus enhancer) which could have influenced transcriptional activity of the HYα-element is also present in the locus (fig. 11A), this conclusion has to be viewed with caution.

As mentioned above, it is also possible that the product of the transgenic β-chain gene induces further differentiation by a preTCR dependent signal. Rapid loss of CD25 expression which leads to a depletion of DN III and activation of TCRα enhancer function may be the result. Function of the TCRα enhancer is necessary for germline transcription at the TCRα locus after β–selection and the TCRα enhancer may also support expression of an inserted TCRα transgene at this stage (Sleckman et al., 1997; Villey et al., 1997; Hozumi et al., 1998; Monroe et al., 1999). This hypothesis could be tested by analysis of expression of the HY-TCR in pTα deficient mice since pTα deficiency would be expected in that case to also induce a block at DN III in TCR transgenic animals (Fehling et al., 1995). The cells trapped at this stage would be mostly negative for expression of the transgenic α-chain, because they have not received a signal to start α-chain expression. This observation has already been made in pTα deficient conventional HYαβ transgenic mice (Buer et al., 1997) and should be extended to HY-I mice, because transcription of the $HY^{I\Delta}$ allele is solely controled by endogenous elements.

Thus, it seems likely that HYα chain expression still depends on signals generated during β-selection. Experiments are under way to study the influence of pTα deficiency on the expression of an inserted α-chain in the HY-I model, but have been delayed by a breeding problem.

4.3 Positive Selection of $HY\alpha^{+}CD8^{+}$ Thymocytes

Conventional TCR transgenic mouse strains do not allow the investigation of secondary recombination events at the TCRα locus, because transgenic TCRα chain expression is independent of the endogenous loci. However, in these models it was observed that transgenic TCRα chain expression per se did neither result in efficient shut-down of recombination nor did it lead to allelic exclusion of the endogenous loci. Instead, endogenous α-chains were observed to be rearranged and expressed together with the transgenic α-chain, a process termed allelic inclusion (von Boehmer, 1990a; Borgulya et al., 1992; Malissen, 1992; Boyd et al., 1998). Receptor change by allelic inclusion has occasionally also been termed editing (McGargill et al., 2000), but should be distinguished from receptor editing based on secondary recombination (see 4.6.2) because it involves rearrangement of loci in germ line configuration and does not result in genomic loss of the gene coding for the primary specificity. If autoreactivity resulted in reinduction of recombination at the TCRα locus, it should in most

cases lead to new VαJα joints on both chromosomes and thus loss of the joint coding for the autoreactive specificity.

By use of the HY-I model it was possible to investigate whether positive selection prohibits secondary recombination (see 3.3.1). Preferential development of $CD8^+HY\alpha^+$ thymocytes indicated efficient positive selection of cells expressing the transgenic receptor as reported before (fig. 15 &19, Kisielow et al., 1988; Teh et al., 1988; Pircher, 1989). However, in contrast to $CD8^+$ thymocytes the majority of DP cells did not express the HYα chain anymore (fig. 19B). Instead, as a result of secondary rearrangements low levels of different Vα chains were found on the surface of these cells (fig 19C). The $HY\alpha^-$DP cells efficiently developed into $HY\alpha^-CD4^+$ and even some $HY\alpha^-CD8^+$ cells (fig. 19B). As was observed in male, negatively selecting HY-I mice, most thymocytes expressed the HY-TCR and were deleted (fig. 19). However, in female HY-I mice not all these HY-TCR expressing thymocytes received a positively selecting signal which precluded recombinational excision of the introduced VαJα.

Incomplete positive selection was also observed in conventional TCR transgenic mice (von Boehmer, 1990b). It was suggested that only a limited number of T cell precursors with a particular specificity can receive a positively selecting signal, because it seems that transgenic T cells compete for an interaction with APCs expressing the selecting MHC/peptide complex. Thus, the availability of positively selecting “niches” serves in the thymus as a rate limiting step as also shown in a thymic reaggregation model. Efficiency of positive selection was found to be linearly dependent on the number of thymic stromal cells (Merkenschlager et al., 1994). In the HYtg mouse model it was observed that only in a situation when transgenic T cells had been diluted 1:20 by WT cells in bone marrow chimeras, all transgenic T cells were positively selected (Huesmann et al., 1991). However, whether the availability of selection „niches“ is also rate limiting in positive selection in a WT thymus and could thus be the cause of secondary rearrangements remains to be investigated.

Assuming that cells with non-transgenic specificities do not compete with cells expressing the transgenic specificity for selection “niches” and contribute to the higher cellularity seen in the positively selecting thymi of the insertion compared to the conventional HY-TCR model (fig 15).

Rag expression ceases in several model systems only after positive selection (Turka, 1991; Brändle et al., 1992; Groves et al., 1997). To investigate whether continued rag expression results in secondary recombination, HY-I animals in non-selecting H-$2D^d$ background were analysed. It was observed that thymic cellularity was not reduced (fig. 17) and SP thymocytes were generated in a ratio similar to WT mice. The inserted α-chain was replaced by endogenous α-chains (fig 19B & C). Thus, thymic development and lineage commitment relies in this situation and in contrast to posi-

tively selecting HY-I animals solely on randomly generated receptors. The observed efficient replacement of a VαJα joint fits to the observation that in fetal but not adult thymocytes the repertoire is biased towards the use of 5´Jα elements (Thompson et al., 1990b; Rytkönen et al., 1996; Rytkönen-Nissinen et al., 1999) which is predicted to be the case when first VαJα joints are generated with these 5´Jα elements but then replaced by secondary rearrangements.

4.4 Negative Selection of HY-TCR Expressing Thymocytes

Editing of receptors with autoreactive specificity seems to play a role in B cell development as shown in Ig insertion models (for review: Radic and Zouali, 1996; Nemazee, 2000). The HY-I system allowed investigation of editing as a rescue mechanism for thymocytes which have generated an autoreactive specificity. It was found that the possibility to replace the receptor did not allow the majority of HY-TCR expressing thymocytes to escape apoptosis induced by the activating ligand. Only few DP cells (4% of the number of DP cells compared to positively selecting female HY-I mice) escaped and the majority of them had lost expression of the transgenic α-chain and aquired a new TCRα-chain. It has been found that already in the earliest stage after β−selection, the DN IV stage, germline transcripts of the TCRα locus and even VαJα joints are generated (Villey et al., 1997; Hozumi et al., 1998; Hernandez-Munain et al., 1999; Rytkönen-Nissinen et al., 1999). It is possible that the few DP cells which are generated in the thymus of male HY-I mice are derived from cells which have performed Vα-Jα recombination before upregulation of the CD8 molecule, since CD8 is required for selection of MHC class I restricted thymocytes (Killeen, 1992; Fung-Leung et al., 1993). Due to the high proliferative capabilites of thymocytes when differentiating into DP cells (Falk et al., 1996), only 3–4x10^3 of these cells need to perform such early recombinations within 3-4 days (life span of DP cells) to generate a DP compartment of the size observed in negatively selecting animals (see also: 3.3.3).

Immature $CD4^+HY\alpha^+$ and $CD8^+HY\alpha^+$cells (see: 3.3.3) may also contribute to the generation of $HY\alpha^-$DP cells in male HY-I mice as it was shown by their low CD69 level (fig. 16) that these cells had not received a selecting signal (Bendelac et al., 1992; Swat et al., 1993).

Neither SP nor DP thymocytes developed in mice homozygous for the TCRα insertion allele (fig. 20). It was shown that TCR levels were similar on thymocytes in these mice to thymocytes in heterozygous HY-I mice (fig. 20), excluding increased signaling as a cause for this stringent deletion. Thymoctes which recombine the $HY\alpha^{I\Delta}$ allele at the DN stage already could result in loss of the autoreactive specificity before the onset of negative selection (see above). But some studies suggest that TCRα recom-

bination starts only on one of the two alleles (deVillartay, Nussenzweig, personal communications) which in mice homozygous for the HYα$^{I\Delta}$ allele would leave behind the second allele resulting in continued HY-TCR expression and thus negative selection. Complete deletion as seen in homozygous HY-I animals could be the result. This observation supports the hypothesis that most of the remaining DP cells have replaced the HYα chain early in development.

Taken together, editing of TCR to escape negative selection does not appear to play a major role in T cell in contrast to B cell development.

4.4.1In Vitro Studies of Negative Selection

The data obtained from male, autoreactive HY-I mice argued against editing in T cells as an efficient mechanism to evade deletion by negative selection. However, it seemed possible that the premature expression of the HY-TCR in HY-I mice resulted in the lack of secondary recombination. Negative selection may have led to apoptosis before the cells had entered the DP stage at which TCRα recombination usually occurs (Wilson et al., 1994; Kisielow and von Boehmer, 1995). Outcomes of negative selection dependent on the onset of TCR expression in TCR transgenic mouse models have been observed before. Berg and coworkers (Berg et al., 1989) compared the superantigen induced deletion in the αβ TCR transgenic mouse model 2B4 to the deletion induced in mice expressing only the β-chain of the same TCR. Interestingly, deletion of the DP compartment was only found in the αβ TCR transgenic strain which expressed the receptor prematurely. In the β-chain transgenic mouse the autoreactive cells were not deleted but developmentally arrested.

In vitro experiments were performed to investigate whether premature expression of HY-TCR had resulted in deletion of the cells instead of receptor change. It was found that peptide treatment of FTOC could indeed limit the number of precursors developing to the DP stage (fig. 25 & 28). In addition, FTOCs and thymic reaggregation cultures were used to assess whether cells expressing CD4, CD8 and HYα could escape deletion by editing of the TCR when they encountered the cognate ligand.

In FTOC of thymi from female E17.5 HY-I embryos deletion of the HYα^{+}DP population was observed after peptide administration (fig. 26). Induction of apoptosis was shown by staining of the remaining HYα^{+}DP cells with Annexin V (fig. 26; Martin et al., 1995). Pronounced deletion was also observed when sorted HYα^{low}DP thymocytes encountered peptide loaded thymic stroma in reaggregation cultures (fig. 31A; see 3.5). In both systems editing of the TCR did not allow the majority of thymocytes to escape apoptosis induced by the negatively selecting signal, supporting the conclusion that the observation made in vivo was a transgenic artefact.

Unexpectedly, after addition of peptide some cells in both culture systems developed into HYα^{+}CD8^{+} cells (fig. 25 & 30, data not shown) of a mature phenotype. The

majority of these HYα$^+$CD8$^+$ cells were not γδ lineage cells having upregulated CD8 after activation (Terrence et al., 2000) because their development depended on the DP stage (fig. 28). Increased positive selection in fetal thymi despite the presence of the activating ligand has been observed before (Teh et al., 1990; Hogquist and Bonnevier, 1998). Perhaps the APCs which induce negative selection are themselves still immature and therefore unfuntional in the fetal thymus (Wu et al., 1995). Alternatively, the HYα$^+$CD8$^+$cells have experienced the selecting ligand after previous positive selection in vivo and therefore became activated, not deleted. This hypothesis is supported by the observation that HYα^{high}DP cells appeared to be at least partially resistant to negative selecting signals and contained already positively selected cells (see 3.5, fig. 31). Observations from other groups also suggest that negative selection takes place mostly on DP cells with intermediate TCR levels (Murphy et al., 1990; Shortman et al., 1991; Lucas et al., 1994; Ghendler et al., 1997; Peterson et al., 1999; for review: Page et al., 1996) and that thymocytes with high TCR expression have a lowered sensitivity for deletion (Kishimoto and Sprent, 1997; Baldwin et al., 1999; Davey et al., 1998).

In conclusion, the data generated by use of the HY-I model showed that in this model thymocytes do not change receptor specificity to escape deletion, neither when they receive the negatively selecting signal upon upregulation of coreceptors in vivo nor when the peptide is administered in vitro to DP cells expressing the HY-TCR.

4.6 Editing in Other TCR Transgenic Mouse Models

4.6.1The 2B4 TCR Insertion Model

Secondary recombination processes during thymic development have also been investigated by others in a TCRα insertion model (Wang et al., 1998). The VαJα joint of the 2B4 TCR specific for a peptide from pigeon cytochrome c presented on the MHC class II molecule H-2 I-A^k was inserted into the Jα locus of the mouse. As in the HY-I model, the β-chain was expressed from a conventional transgene. Similar to my observations, in a non-selecting background their transgene was efficiently replaced by secondary recombinations and allowed normal thymic development using endogenously encoded VαJα joints. However, in contrast to my data, the inserted transgene was also efficiently replaced after introduction of the cognate antigen. No reduction in total thymocyte number or number of DP cells was seen in this model under the condition of negative selection.

Unfortunately, the conclusions that can be drawn from this model are limited. Since the authors did not observe expression of a VαJα introduced into the Jα locus, another strain was generated which carried the IgHε enhancer in the TCRα lcous as well. The TCRδ locus and the neomycin resistance gene were also retained. It cannot be

excluded that these elements allowed inappropriate accession of the V(D)J recombinase to the locus. Early replacement of the introduced VαJα element during β-chain recombination at DN III could have generated a significant population of idiotype negative cells before acquisition of the coreceptors. These cells could have entered the DP compartment, not being affected by the negatively selecting ligand. And indeed, compared to the HY-I model (90%) a low number of DN cells (43%) expressed the transgenic receptor in the 2B4 insertion model. Further analysis of 2B4 idiotype expression, especially in the DN III subset, are needed to resolve this issue.

4.6.2Editing, Allelic Inclusion and TCR Internalization

Interestingly, editing of TCRα genes as a mechanism to escape negative selection has recently been suggested to play a role in a conventional TCR transgenic mouse model (McGargill et al., 2000). Expression of a peptide derived from ovalbumin was targeted to thymic cortical epithelial cells by means of the K14 promotor. In a mouse strain expressing the ovalbumin peptide and an ovalbumin specific MHC class I restricted TCR (OT-1) thymocytes were not deleted. Instead, they lost surface expression of the transgenic α-chain by allelic inclusion of endogenous α-chains, a process which the authors termed editing.

Sha and coworkers also observed allelic inclusion and only partial deletion in a TCR transgenic model reactive to the MHC class I molecule H-2 L^d. SP cells were efficiently generated and peripheral T cells shown to express TCR, but not the idiotype (Sha et al., 1988). Sarukhan and colleages (Lanoue et al., 1997; Sarukhan et al., 1998) observed allelic inclusion in a mouse model of a H-2 I-E^d restricted TCR specific for influenca hemagglutinin coexpressing the cognate antigen. In this model DP cells are not deleted and SP cells generated efficiently by the use of endogenous α-chains as evidenced by the lack of SP cells in TCRα deficient background. Interestingly, in this model allelic inclusion allows autoreactive DP thymocytes to proceed in development, without allelic inclusion the cells become developmentally arrested (Sarukhan et al., 1998). The DP compartment was also not depleted upon autoreactivity in two TCR models reactive to the mixed-lymphocyte stimulatory (Mls) a antigen (Berg et al., 1989; Pircher et al., 1989). A similar observation was made by another group in RAG-deficient αβ TCR transgenic mice (F5) specific for the nucleoprotein NP68 of the influenca virus. Chronic administration of the cognate peptide leads initially to complete deletion of the DP cells, however, after 7 days a population of antigen resistant DP cells starts to develop (Wack et al., 1996; Tarazona et al. 1998). These studies showed that negative selection does not necessarily result in the absence of DP cells. At which stage autoreactive thymocytes undergo apoptosis appears to depend on a range of factors including the onset of TCR expression, the affinity of the interaction between TCR and peptide/MHC, and the cell type presenting

the antigen (see 1.4). Under some circumstances cells may receive a signal which results in termination of TCRα recombination but eventually also in apoptosis late enough to have generated endogenous TCRα joints. The autoreactive specificity may in this case be removed from the cell surface and allelic inclusion allow some cells to proceed in development. Models in which autoreactive cells are not deleted early in development may be predisposed to this escape mechanism (Sha et al., 1988; Berg et al., 1989; Pircher et al., 1989; Lanoue et al., 1997; Sarukhan et al., 1998, McGargill et al., 2000).

The importance of allelic inclusion in T cell development is supported by the observation that up to 30% T cells in WT mice express two TCRα chains (Casanova et al., 1991; Padovan, 1993; Alam and Gascoigne, 1998). One study even suggests that such cells are more frequent than expected from random rearrangements on two chromosomes. Notably, only pre-selection TCR^{low}TCRα double producing DP thymocytes surface stain for both α-chains while the majority of post-selection TCR^{high} thymocytes or peripheral T cells stain only intracellularly for a second α-chain (Alam et al., 1995; Alam and Gascoigne, 1998; Boyd et al., 1998). Thus, T cells seem to have mechanisms to restrict the expression of one α-chain to the cytoplasm. It was suggested that this restriction to the cytoplasm is either the result of competition for β-chain (Alam et al., 1995; Alam and Gascoigne, 1998) or of active internalization (Boyd et al., 1998). The fact that upregulation of a second, autoreactive receptor can be observed upon loss of tolerance in some models (Schonrich et al., 1991; Sarukhan et al., 1998) argues in favor of the second model. Several different mechanism which could regulate TCR surface levels dependent on TCR triggering have been described to date (Cenciarelli et al., 1992; Molina et al., 1992; Wiest et al., 1993; Dietrich et al., 1994; Ericsson and Teh, 1995; Stone et al., 1997; Boyd et al., 1998; Lauritsen et al., 1998).

Thus, it appears that allelic inclusion is an additional mechanism which increases the probability of thymocytes to express a TCR which allows positive selection. TCRs which do not signal at all (non-selected) as well as TCRs which signal too strongly (negative selected) could become actively internalized. It seems possible that only TCR stays on the cell surface which is constantly triggered by a positively selecting ligand. This mechanism would allow “editing” of surface TCR in cells which after termination of TCRα locus recombination express two specificities. It ensures that only the specificity which triggered positive selection remains on the cell surface. Internalization of allelically included receptors operates later in development than secondary recombination processes because V(D)J recombination must have ceased

already.

4.7 RAG Expression and Editing

RAG1 and RAG2 proteins are necessary for V(D)J recombination (Schatz, 1989; Oettinger et al., 1990) and expression of the rag genes is associated with V(D)J recombination in thymic development (Turka et al., 1991; Brändle et al, 1992). McGargill and coworkers (McGargill et al., 2000) observed higher levels of rag transcripts in DP cells from „editing“ K14Ova/OT-1 mice as compared to DP cells from positively selecting OT-I animals. They reasoned that this result demonstrated upregulation of rag transcription in order to change receptor specificity. However, the observed level of rag transcripts in the „editing“ population was lower than in a non-selected population and could also be interpreted as the result of incomplete positive selection of all these cells or completed positive selection in only some of the cells in this population. Similarly, engagement of TCR on DP cells in vitro and in vivo has been shown to result in lower levels of rag gene transcripts without the observation of complete absence (Turka et al., 1991; Brändle et al., 1992; Groves et al., 1997). Only artificial stimuli were able to abrogate rag expression completely (Turka et al., 1991). In addition, measured levels of rag transcripts do not neccessarily correlate to the protein expression, because RAG proteins are kown to be regulated posttranslationally (Li et al., 1996a). But also the presence of RAG protein does not guarantee its activity, the target locus has to display a chromatin structure which is accessible to the V(D)J recombinase (Wayne et al., 1994; Stanhope-Baker et al., 1996; Cherry and Baltimore, 1999; for review: Sleckman et al., 1996; Hempel et al., 1998). Thus, it is not clear whether the investigation of rag transcript levels allows the conclusion that V(D)J recombination is taking place at a particular locus or has even been reinitiated (McGargill et al., 2000).

In the HY-I model rag expression was not assessed because the advantage of this model is that it allows the direct observation of products from secondary recombination events.

4.8 Peripheral Editing

Signs of V(D)J recombinase activity in mature peripheral T lymphocytes have recently been brought forward by two groups (McMahan and Fink, 1998; Lantelme et al., 2000a; Lantelme et al., 2000b). The HY-I model was used to investigate whether peripheral editing changes the receptor specificities of mature T cells.

In a first experiment HYα^{+}CD8^{+} T cells were activated in vitro with peptide loaded APC to see whether loss of HYα chain expression as a sign of editing could be observed within three days. Since triggering of the TCR results in its internalization

and degradation (Valitutti et al., 1997), it can be ruled out that cells had genomically edited the TCRα gene but retained surface expression of the HY-TCR. Editing was not observed in these experiments (fig. 30).

Peripheral editing was reported in transgenic T cells being chronically stimulated by a weak peripheral superantigen (McMahan and Fink, 1998) leading also to downregulation of TCR. This situation was mimicked in the second experimental system used. $HY\alpha^{+}CD8^{+}$ T cells were injected into male and female TCRβ deficient mice. Neither in male nor female hosts a significant $HY\alpha^{-}$ T cell population developed, although the cells were activated and proliferated strongly in male recipients (fig. 31). However, it is possible that peripheral editing depends on additional signals by other T lymphocytes (like helper T cells) which were absent in the TCRβ deficient recipients. The experiment was therefore repeated using wildtype C57BL/6 as hosts. In female recipients again only $HY\alpha^{+}$ transferred T lymphocytes were observed (data not shown). Unfortunately, transferred T cells were not recovered from male C57BL/6 recipients (data not shown). In these mice the cells had either been deleted or lost CFSE label and expression of the α-chain simultaneously. Thus, it cannot be excluded that cells had undergone peripheral editing. Further experiments have to be performed which allow identification of transferred cells by a surface marker (i.e. Ly-5.1).

Taken together no signs of peripheral editing were observed in experimental systems using the HY-I model, but a) it cannot be excluded to take place in WT host and b) it may require chronical stimulation over a longer period of time.

4.9 Conclusions

A mouse model was generated which allowed the investigation of secondary recombination events at the TCRα locus. In this model the inserted VαJα joint was readily replaced by another one in thymi not supporting selection. However, secondary recombinations did not allow thymocytes to efficiently escape from apoptosis induced by a negatively selecting ligand.

Observations by other researchers have shown that allelic inclusion followed by receptor downregulation allows cells which originally express a non-selectable or autoreactive specificity to differentiate into mature T cells.

Thus, the current data indicate that thymocytes undergo secondary recombination processes at TCRα loci when they carry out-of frame VαJα joints on both chromosomes or encode a specificity which does not allow positive selection. Selection, however, results in termination of recombination. Cells which express two distinct TCR may retain only surface expression of the TCR mediating positive selection. Thymocytes that express only TCRs with high affinity for self-peptide/self-MHC or TCRs whithout reactivity towards self-MHC die by apoptosis and appear not to be efficiently rescued by receptor editing.

5 SUMMARY

Thymocytes are selected for recognition of self-MHC and against autoreactivity. I investigated whether thymocytes failing in thymic selection are able to revise their TCR by secondary rearrangements at the TCRα locus. To study secondary rearrangements, the VαJα gene segment of an H-2D^{b} restricted male (HY) specific TCR was introduced into the germline TCRα/δ locus of the mouse. Using the Cre-loxP system a 263 kb deletion within the TCRα/δ locus was performed to mimic the natural recombination process. Expression of the HY-TCR was achieved by use of this HYα insertion allele and a conventional HYβ transgene.

In positively selecting animals T cells expressing the insertion transgene were observed. In non-selecting background exchange of the transgenic receptor for secondary rearrangements efficiently rescued thymocytes from death by neglect.

In the (negatively selecting) thymus of male HY-TCR expressing animals a 25 fold reduction in the number of CD8^{+}CD4^{+} (DP) cells as compared to positively selecting HY-TCR expressing mice was observed. Addition of antigenic peptide to fetal thymic organ cultures induced apoptosis in HY-TCR expressing DP cells. The deletion was restricted to HY-TCR^{low}DP cells as was shown in thymic reaggregation cultures.

Thus, thymocytes can efficiently escape „death by neglect“ but not negative selection by use of secondary rearrangements at the TCRα locus.

6 ZUSAMMENFASSUNG

T Zellen erstellen während ihrer Entwicklung im Thymus durch Umlagerung von Gensegmenten die Gene für den T Zellrezeptor (TCR). Jeder Thymozyt wird anschließend Selektionsprozessen unterzogen, die sicherstellen, daß ihr TCR körpereigene MHC Moleküle erkennt (positive Selektion), aber keine Autoreaktivität gegen körpereigene Antigene besitzt (negative Selektion). Ich habe untersucht, ob Thymozyten ihren TCR durch sekundäre Umlagerungen am TCRα Locus verändern können, wenn sie nicht positiv selektiert werden oder aber ein negativ selektierendes Signal erhalten. Um diese sekundären Umlagerungsprozesse zu untersuchen wurde das VαJα Exon eines männchenspezifischen, H-2D^b restringierten TCR (HY-TCR) in den TCRα/δ Locus der Maus eingebracht. Mit Hilfe der Cre-loxP Technologie wurde anschließend *in-vivo* eine 263 kb große genomische Deletion, die den TCRδ locus sowie Teile der Vα und Jα Regionen einschloß, durchgeführt, um die natürliche Vα-Jα Umlagerungsreaktion nachzuahmen. Dieser TCRα-Insertionsmausstamm wurde mit einem Mausstamm verpaart, der ein für die β-Kette des HY-TCR kodierendes konventionelles Transgen besaß. Auf diese Weise wurde die Expression des HY-TCR ermöglicht. Die Analyse sekundärer Umlagerungsprozesse wurde auf das Insertionsallel beschränkt, indem Tiere verwendet wurden, die auf dem Schwesterchromosom ein TCRα defizientes Allel besaßen.

Im Thymus von weiblichen (positiv selektierenden) Tieren des HY-Insertionsstamms (H-2D^b) wurde beobachtet, daß HY-TCR ausprägende Thymozyten, sich preferentiell zu CD8$^+$ Zellen entwickelten. Einige Zellen prägten aber den HY-TCR nicht mehr aus. Hier konnte nachgewiesen werden, daß diese Zellen eine neue TCR-Spezifität besaßen und Vα Elemente endogenen Ursprungs verwendeten.

Nach Kreuzung in den nicht-selektierenden H-2D^d Hintergrund wurde beobachtet, daß die Thymozyten effizient die nutzlose TCR Spezifität duch sekundäre Umlagerungsprodukte veränderten. Die Zellzahl des Thymus war nicht reduziert, obwohl alle Zellen zunächst eine nicht-selektierbare Spezifität besassen.

Im Thymus negativ selektierender Mäuse des HY-Insertionsstammes (H-2D^b), war die Thymozytenzahl im Vergleich zu positiv selektierenden Mäusen 8 mal niedriger. Auch die Zahl an CD4$^+$CD8$^+$ Zellen war stark reduziert (25 fach). Überlebende Zellen hatten das Insertionstransgen durch ein sekundäres Rekombinationsprodukt ersetzt. Dieser Prozess war aber ineffizient und erlaubte nicht die Bildung eines Thumus normaler Größe. In fetalen Thymuskulturen starben HY-TCR ausprägende CD8$^+$CD4$^+$ Zellen nach Zugabe des HY-Peptides durch Apoptose. Dies wurde anhand reduzierter Zellzahlen und der Detektion apoptotischer Zellen mittels Annexin V festgestellt. In

Thymusreaggregationskulturen wurde außerdem beobachtet, daß durch Zugabe von HY-Peptid nur in $CD8^+CD4^+$ Zellen mit schwacher Ausprägung des HY-TCR Apoptose ausgelöst wurde. Eine Änderung der TCR-Spezifität erfolgte nicht.

Offensichtlich können in diesem Modell nur wenige Thymozyten, die einen TCR mit autoreaktiver Spezifität ausprägen, der Apoptose aufgrund negativer Selektion durch Änderung der Rezeptorspezifität mittels sekundärer Umlagerungen entrinnen. Sie sind also nur in der Lage effizient ihre Rezeptorspezifität durch sekundäre Umlagerungen zu wechseln, wenn sie keinen selektierbaren TCR ausprägen.

7 KURZZUSAMMENFASSUNG

Thymozyten werden Selektionsprozessen unterzogen, die sicherstellen, daß ihr T Zellrezeptor (TCR) körpereigene MHC Moleküle erkennt (positive Selektion), aber keine Autoreaktivität besitzt (negative Selektion). Um untersuchen zu können, ob Thymozyten ihren TCR durch sekundäre Umlagerungen am TCRα Locus verändern können wurde das VαJα Exon des HY-TCR in den TCRα/δ Locus der Maus eingebracht und die natürliche Vα-Jα Umlagerungsreaktion mittels einer 263 kb große genomische Deletion nachgeahmt. Die Ausprägung des HY-TCR wurde durch Coexpression des HYα-Insertionsalleles mit einem konventionellen HYβ Transgen ermöglicht.

In positiv selektierenden Tieren des HY-Insertionsstamms wurde die Entwicklung von T Zellen mit HY-TCR beobachtet. In nicht-selektierenden H-2D^{d} Mäusen aber wurde die hier nutzlose TCR Spezifität effizient duch sekundäre Umlagerungsprodukte ersetzt.

Im Thymus negativ selektierender Mäuse war die Zahl der CD4^{+}CD8^{+} (DP) Zellen 25 fach reduziert. In fetalen Thymuskulturen und Thymusreaggregationskulturen wurde gezeigt, daß in HY-TCR^{low}DP Zellen nach Zugabe des HY-Peptides Apoptose ausgelöst wurde. Eine Änderung der TCR-Spezifität erfolgte nicht. Also können Thymozyten mit autoreaktiver Spezifität in diesem Modell negativer Selektion nicht effizient durch Änderung der Rezeptorspezifität mittels sekundärer Umlagerungen entrinnen. Hierzu sind sie nur in der Lage, wenn sie keinen selektierbaren TCR ausprägen.

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9 ABBREVIATIONS

ab	antibody
APC	antigen presenting cell
ATP	adenosine-tris-phosphate
BSA	bovine serum albumin
bp	base pairs
cre	„causes recombination“
CD	cluster of differenciation
CFSE	5 (and 6) -carboxyfluorescein diacetate succinimidyl ester
Cyc	cychrome
d	day
D element	diversity element
DC	dendritic cell
DMEM	Dulbecco´s modified eagles medium
DN	double negative ($CD4^-CD8^-$)
DNA	desoxyribonucleic acid
DNaseI	desoxyribonuclease I
dNTP	2-deoxyribonucleosidtriphosphate
DP	double positive ($CD4^+CD8^+$)
Δ	deleted
E	gestational day
EDTA	ethylendiamin-tetra-acedic acid
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorting
FITC	fluorescein-isocyanate
FTOC	fetal thymic organ culture
fig.	figure
G418	geniticin
GL	germ line
H-2	histocompatibility locus 2
HEPES	N-(2-Hydroethyl)piperazin-N´-2-ethylsulfonic acid
HSA	heat stable antigen
HY-I	genotype $HY\alpha^{I\Delta/0}HY\beta^+$
HYtg	HY-TCR α/β transgene
Ig	immunoglobulin
i.v.	intravenously
J element	joining element

kb	kilo base pairs
LN	lymph node
MACS	magnetic cell sorting
MHC	major histocompatibility locus
MΦ	macrophage
Neo	neomycine resistance gene
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PerCP	peridinin chlorophyll protein
pTα	pre Tα
rag	recombination activating gene
rpm	rounds per minute
SDS	sodium dodecyl sulfate
SP	single positive ($CD4^+$ or $CD8^+$)
TCR	T cell receptor
TEA	T early alpha
tg	conventional transgene
TK	thymidinkinase
V element	variable element
V region	variable region
WT	wild type

Acknowledgements

The research described in this thesis was carried out from April 1997 through October 2000 at the Institute for Genetics of the University of Cologne under the supervision of Prof. Dr. Klaus Rajewsky. The project was funded by the Sonderforschungsbereich 243 of the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie through a stipend to me. I would also llike to thank Boehringer Ingelheim Fonds, the Deutsche Gesellschaft für Immunologie and the Universität zu Köln for travel fellowships.

I am grateful for the help and supervision of Klaus Rajewsky, Irmgard Förster, and Frédéric Rieux-Laucat.

I want to acknowledge the technical help of Anke Leinhaas and Angela Egert for ES cell culture, aggregation, blastocyst injection, and reaggregation culture, Gabriele Zoebelein and Simone Wilms for sequencing, Claudia Uthoff-Hachenberg and Brigitte Hampel for antibody preparation, Christine Königs for genotyping, Christoph Göttlinger for cell sorting, Werner Müller for the computer network, and Åsa Böhm, Gisela Schmall, and Ursula Lichtenberg for running the laboratory. The mouse work would not have been possible without the care of Gina Piper, Tanja Tropartz, Agathe Stark, Kerstin Marohl, and Gisela Küster. Alot of people shared with me the laboratory A-206: I had a lot of fun including scientific and non-scientific discussions during the time with Robert Rickert, Harry Schroeder, Greg Ippolito, Bojan Polic, Marat Alimzhanov, and Tanja Novobrantseva.

The generation of the HY-I mouse strain was supported by the critical discussions with all the people in the ES cell club of whom I want to thank especially Ralph Kühn. The analysis of my mouse was guided by the assistance of the members of the Tcell club: Irmgard Förster, Christian Schmedt, Sasha Tarakovsky, Ivo Lieberam, Bjoern Clausen, Holger Babbe, Ari Waisman, Dietrich Grimme, Constanze Burkhardt and many more.

I am grateful to L. Hood, J. Fehling, K. Fellenberg, U. Betz, and H. von Boehmer for supplying material and to M. Malissen for supplying me with a map of the TCRα/δ locus prior to publication. The critical discussions with H. von Boehmer and D. Mathis were extremely helpful during the course of the thesis work.

I would like to thank Ari Waisman, Marc Schmidt-Supprian, Dominik Schenten, Manfred Kraus, Irmgard Foerster, Greg Ippolito, my friend Bruno Martinez, and especially Tanja Novobrantseva and Marat Alimzanov for proofreading of bits and pieces of my thesis. Phillip Oberdörffer, Thomas Wunderlich, Mario Zierden, Manfred Kraus and many other people made lunch time enjoyable by joining me on the daily lunch in the Mensa.

Ferner möchte ich all meinen Freunden danken, die mich während meiner Arbeit in Köln unterstüzt haben und immer wieder reges Interesse am „Mäusemachen“ gezeigt haben. Hervorheben möchte ich vor allen Dingen Friederike Krech, die mir erklärte, daß auch meine Maus 4 Beine, Schwanz und Schnäuzer brauche, Eva Gwosdz, die mich häufig auch nach verunglückten Experimenten aufmuntern konnte und Bartholomäus Schade, der mich aus so manchem Computerproblem rettete. Ferner möchte ich meine Eltern danken, die mein Interesse an der Biologie immer gefördert und mir mein Studium ermöglicht haben. Ohne die Unterstützung und Liebe von Ulrike Faber wäre sicher vieles in den letzten Jahren, einschließich der Doktorarbeit, anders verlaufen.

Finally, I would like to thank Drs. K Rajewsky, P. Dröge, G. Plickert, and U. Lichtenberg for agreeing to be in my thesis committee.

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. K. Rajewsky betreut worden.

Köln, den 15.11.2000

Thorsten Buch

Teilpublikationen: keine