Identification and Functional Characterization of a Mouse Lymphotoxin β-Receptor Like Protein

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by

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Abbreviations

aa	amino acid
ADAM	a disintegrin and metalloprotease
AIM	activation inducer molecule
AIRE	autoimmune regulator gene
AP	alkaline phosphatase
AP1	activator protein 1
APC	allophycocyanin
ASK1	apoptosis signal-regulating kinase 1
BLC	B lymphocyte chemoattractant
BMMC	bone marrow derived mast cells
bp	base pairs
CD	cluster of differentiation or Crohn's disease
cDNA	complementary DNA
CRD	cysteine rich domains
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
DC	dendritic cell
DcR	decoy receptor
DD	death domain
DED	death effector domains
DISC	death inducing signaling complex
DN	double negative thymocyte
dNTP	deoxyribonucleotide
DP	double positive thymocyte
DR	death receptor
ECD	extracellular domain
ECL	enhanced chemiluminiscence
EDTA	ethylenediaminetetraacetic acid
ELC	Epstein Barr-virus induced molecule 1 ligand chemokine
ELISA	enzyme linked immunosorbant assay
FACS	fluorescence activated cell sorter
FADD	Fas associated death domain
FCA	Flow cytometric analysis

FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
FSC	forward scatter
GC	germinal center
GPI	glycosylphosphatidylinositol
His	histidin
HRP	horseradish peroxidase
HSA	heat stable antigen
HVEM	herpes virus entry mediator
IBD	inflammatory bowel disease
ICD	intracellular domain
IFNγ	Interferon γ
Ig	immunoglobulin
IKK	I-κB kinase
IL-1R	Interleukin 1 receptor
IκB	inhibitor of NF-κB
IVC	individually ventilated cage
JNK	c-*jun* N-terminal kinase
kD	kilodalton
ko	knock out
LB medium	Luria Bertani medium
LIGHT	homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes).
LMP-1	latent membrane protein-1 (EBV)
lpr	lymphoproliferative A recessive mutation
LT	lymphotoxin
LTIC	lymphoid tissue inducing cell
LTβR	lymphotoxin β-receptor
mAB	monoclonal antibody
MAPK p38	mitogen activated protein kinase
MIP2	macrophage inflammatory protein 2
MKK	mitogen activated protein kinase (MAPK) kinase
mLTβR	mouse lymphotoxin β-receptor
mTEC	medullary thymic epithelial cell

MW-Std.	molecular weight standard
MZ	marginal zone
NALT	nasopharyngeal associated lymphoid tissue
NF-κB	nuclear factor-κ B
NIK	NF-κB inducing kinase 1
NK cells	natural killer cells
NP-40	Nonidet P-40
OD	optical density
PAA	polyacrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP-CY5.5	peridin chlorophyll protein-Cy5.5™
pfu	plaque forming unit
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
PMT voltage	photomultiplier voltage
PP	Peyer's patches
PVDF	polyvinylidene fluoride
RIP	receptor interacting protein
RIPA buffer	radioimmunoprecipitation buffer
ROS	reactive oxygen species
RTE	recent thymic emigrants
SAP	shrimp alkaline phosphatase
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SLC	secondary lymphoid tissue chemokine
sp	signal peptide
SP	single-positive thymocyte
SSC	side scatter
TACE	TNFα converting enzyme
TAE buffer	Tris acetate EDTA electrophoresis buffer
TBE buffer	Tris borate EDTA electrophoresis buffer
TBS	Tris buffered saline

TE buffer	Tris EDTA buffer
TGFβ3	transforming growth factor beta 3
TH1, TH2	T helper 1, 2 cells
THD	TNF homology domain
TLO	tertiary lymphoid organs
TLR	Toll like receptor
TMD	transmembrane domain
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR associated death domain
TRAF	TNFR associated factor
T_{Reg}	regulatory T cell
U	unit
UC	Colitis Ulcerosa
wt mice	wildtype mice

1. Introduction

1.1. The TNF/TNFR superfamily

About 30 years ago tumor necrosis factor (TNF) and lymphotoxin (LT) were identified as proteins expressed by activated macrophages and T cells, respectively, and capable of inducing tumor cell lysis (Carswell *et al*., 1975; Kolb and Granger, 1968). The first studies on TNF revealed a broad spectrum of roles ranging from T lymphocyte differentiation to fever and wasting. Ten years later, with the cloning of TNF and LT it became obvious that both proteins belong to a superfamily of homologous ligands and receptors, the so called TNF/TNFR superfamily (Gray *et al*., 1984; Pennica *et al*., 1984). Currently 19 different ligands and 29 different receptors are known in humans, summarized in figure 1. Over the years many different functions of the TNF/TNFR superfamily members were elucidated demonstrating critical roles in organogenesis, apoptosis, autoimmunity, inflammation and host defense. Members of the TNF/TNFR superfamily have also been found to be involved in diseases like arteriosclerosis, autoimmune diseases (e.g. rheumatoid arthritis, diabetes and inflammmatory bowel disease), osteoporosis and cancer (Locksley *et al*., 2001). Knowledge of the way the ligands and receptors function would provide an opportunity to find pharmaceuticals for treating these diseases.

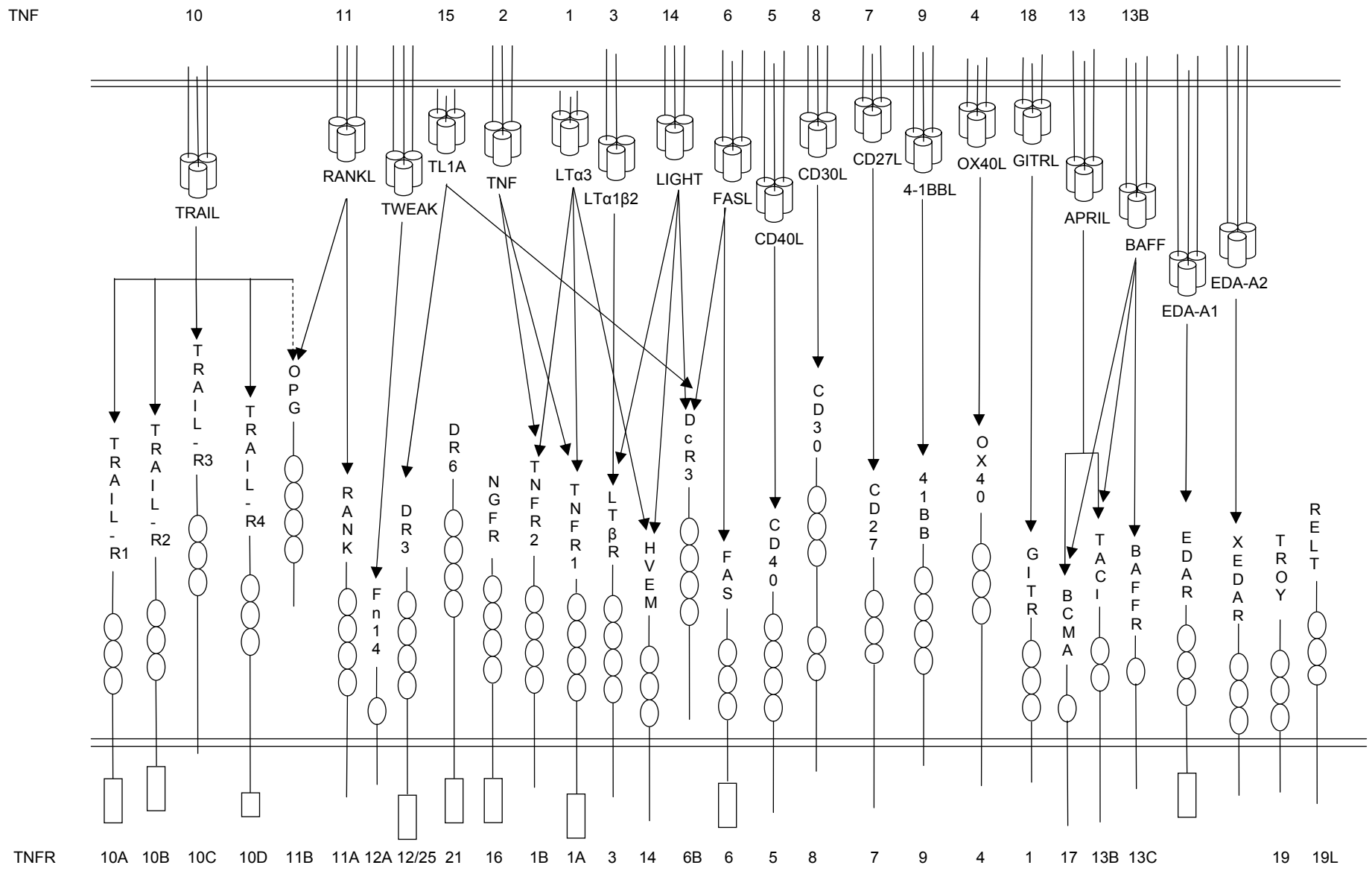
In order to gain this knowledge, many different kinds of studies on different biological levels were performed with the purpose of clarifying the structure and function of each member.

Figure 1:

Summary of all known human TNFs, linked to their receptors, according to Locksley *et al*., 2001; Bodmer *et al*., 2002; Ashkenazi, 2002 and Ware, 2003.

 Ligand;  Cysteine rich domain, contributing to the extracellular domain (ECD) of the receptor;  Death domain, part of the intracellular domain (ICD) of some receptors

Arrows indicate the binding between ligand and receptor.


TNF
10
11
15
2
1
3
14
6
5
8
7
9
4
18
13
13B
TRAIL
RANKL
TWEAK
TL1A
TNF
LTα3
LTα1β2
LIGHT
FASL
CD40L
CD30L
CD27L
4-1BBL
OX40L
GITRL
APRIL
BAFF
EDA-A1
EDA-A2
TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
OPG
RANK
Fn14
DR3
DR6
NGFR
TNFR2
TNFR1
LTβR
HVEM
DcR3
FAS
CD40
CD30
CD27
4 1BB
OX40
GITR
BCMA
TACI
BAFFR
EDAR
XEDAR
TROY
RELT
TNFR
10A
10B
10C
10D
11B
11A
12A
12/25
21
16
1B
1A
3
14
6B
6
5
8
7
9
4
1
17
13B
13C
19
19L

1.1.1. The ligands of the TNF/TNFR superfamily

Although the members of the TNF/TNFR superfamily act in variable and pleiotropic manners they share remarkably similar structures and conserved interactions.

The TNF ligands are type II transmembrane proteins with their extracellular domain (ECD) at the carboxy terminus of the protein. The ECD contains a so-called “TNF homology domain” (THD) with a sequence homology between the family members of ~20-30%. These THDs form trimers which are responsible for the receptor binding (Bodmer *et al*., 2002). Nearly all ligands are expressed as membrane bound proteins and some of them are cleaved by proteases such as metalloproteases of the ADAM family (adamalysins) in the case of TNF (TACE: TNFα converting enzyme) (Black *et al*., 1997) and RANKL (Lum et al., 1999), by members of the furin family in the case of BAFF, APRIL, TWEAK and EDA (Schneider et al., 1999; Chen *et al*., 2001) or by matrisylin, a matrix-degrading metalloproteinase, in the case of FasL (Powell *et al*., 1999). Some ligands need to be soluble to fulfill their physiological role such as EDA (Chen *et al*., 2001) whereas others are inhibited after shedding, for example FasL (Tanaka *et al*., 1998). LTα is directly secreted as a soluble homotrimer ($LT\alpha_3$) (Ashkenazi, 2002) or expressed on the surface of cells as a heterotrimer ($LT\alpha_1\beta_2$) together with lymphotoxin β (LTβ) to which it is associated during biosynthesis (Ware *et al*., 1995).

1.1.2. The receptors of the TNF/TNFR superfamily

Nearly all TNFRs are type I transmembrane proteins with their ECDs at the amino termini of the proteins. One to four (CD30: five) cysteine rich domains (CRD), so-called “cysteine rich pseudorepeats”, build the ECDs of the TNFRs. With few exceptions in the fourth CRD each domain has six cysteine residues which build three intrachain disulfide bonds (Naismith and Sprang, 1998). For TNFRI, Fas and TRAILR2 (DR5) ligand binding in a 3:3 stoichiometry occurs in the second and the third CRD (Banner *et al*., 1993; Locksley *et al*., 2001; Ashkenazi, 2002).

According to the intracellular domains (ICDs) the TNFRs can be divided into three different groups: first, the TNFRs with a TNFR-associated factor (TRAF) binding motif leading to NF-κB- (nuclear factor-κB), AP1- (activator protein 1), or NF-κB/AP1-activation. Cell death, however, is also infrequently induced. Second, the “death receptors” (DR) which share a so-called “death domain” (DD), a sequence

of 68 and 80 amino acids in Fas (Itoh and Nagata, 1993) and TNFRI (Tartaglia *et al*., 1993), respectively, to which adaptor molecules bind mainly for the induction of caspases-dependent apoptosis and rarely for the activation of NF-κB and/or AP1. Third, the "decoy receptors" (DcR), which lack a (functional) ICD and therefore a signaling pathway. None of the cytoplasmic tails of the TNFRs contain any enzymatic activity (Ware *et al*., 1995).

Apart from these common structures there are some exceptions in the TNFR superfamily. BCMA, TACI, BAFFR and Xedar lack a signal peptide and are therefore called "type III transmembrane proteins". TRAIL R3 (DcR1) is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor while OPG and DcR3 are secreted as soluble receptors because they have no membrane-interacting domain (Bodmer *et al*., 2002). Proteolytic cleavage is found for CD27, CD30, CD40, TNFRI and TNFRII leading to an additional soluble active form (Gruss and Dower, 1995). TNFRII, CD40, Fas, and 4-1BB also exist in a membrane bound and in a soluble form due to alternative splicing (Lainez *et al*., 2004; Papoff *et al*., 1996; Smith *et al*., 1994). These proteolytically cleaved or alternatively spliced receptors funtion as decoys sequestering the ligands from the membrane bound receptors. The low affinity p75-NGFR is the most divergent TNFR with no known TNF-like ligand and a propensity to dimerize rather than trimerize.

1.1.2.1. TRAF binding receptors

Many members of the TNFR superfamily initiate intracellular signaling by recruiting TNFR-associated factors (TRAFs) through their cytoplasmic tails.

TRAFs constitute a family of genetically conserved adapter proteins that have been identified in mammals, *Drosophila*, *Caenorhabditis elegans* and *Dictyostelium discoideum*. Mammalian TRAFs (TRAF1-6) have emerged as the major mediators for the cell activation, cell survival, and antiapoptotic functions of the TNF receptor superfamily and the interleukin-1 receptor/Toll-like receptor superfamily (IL-1R/TLR). Binding of TRAFs to members of these two superfamilies, directly or indirectly via TNFR associated DD (TRADD), leads to the activation of NF-κB and/or AP1 (Chung *et al*., 2002), with one exception: the complex of TRAF3 and LTβR starts the apoptotic pathway (Van Arsdale *et al*., 1997). Binding of TRAFs to TNFRs or IL-1Rs/TLRs takes place between conserved amino acid residues on the surfaces of TRAF1, 2, 3, and 5 and a

structural supermotif ((P/S/T/A)x(Q/E)E) in the ICDs of the receptors. This supermotif and a minor motif, (PxQxxD), were found in TNFR2, CD27, CD30, CD40, OX40, 4-1BB, HVEM and in the human Epstein-Barr virus protein LMP-1 (Ye *et al*., 1999). Binding to this supermotif was shown for TRAF2 (Ye *et al*., 1999) and TRAF3 (Ni *et al*., 2000).

The signal transductions of TNFRs via TRAFs have been studied intensively but have not yet been clarified in every detail. Signaling via TNFR2 and TRAF2 is shown here as an example and in a simplified model according to Song *et al*., 1997 and Wajant *et al*., 2001.

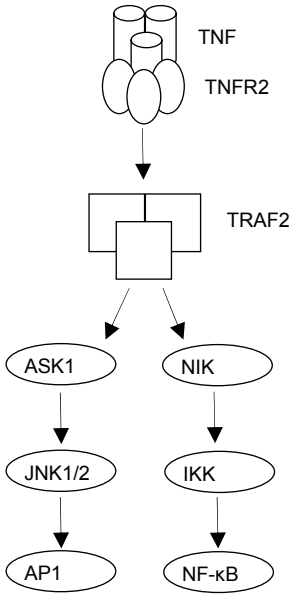


Figure 2:

Signal transduction pathway via TNFR2 / TRAF2

TRAF2 binds the activated TNFR2 and recruits either ASK1 for AP1 activation or NIK for NF-κB activation.

AP1 (activator protein 1), ASK1 (apoptosis signal-regulating kinase 1), IKK (inhibitor of NF-κB (IκB) kinase), JNK (c-*jun* N-terminal kinase), NF-κB (nuclear factor-κB), NIK (NF-κB inducing kinase 1)

1.1.2.2. Death receptors

Apoptosis/programmed cell death is a central event during several physiological processes like organogenesis, inflammation and cellular immune response. Cell shrinkage, cytoplasmic membrane blebbing, chromatin condensation, DNA fragmentation and finally destruction of the cell by its disassembly into fragments are the characteristics of this event. The eight death receptors (DRs) of the TNFR superfamily (TNFR1, Fas, TRAMP, TRAILR1, TRAILR2, DR6, p75-NGFR, and

EDAR) can trigger the apoptotic pathway through their common conserved death domain (DD) on their ICD.

Upon activation of the DRs their DDs recruit various adaptor proteins (TRADD, FADD, and RIP) that mediate preferentially programmed cell death but in the case of TNFRI also proliferation. These proteins in turn recruit other proteins via their DDs or death effector domains (DEDs). The destruction of the cell is accomplished by activation of a family of proteases referred to as caspases (Ashkenazi, 2002).

The two best studied DRs, Fas and TNFRI, are depicted in figure 3 as an example of the DD-depending signal cascades according to Ashkenazi, 2002, in a simplified model with additions described subsequently.

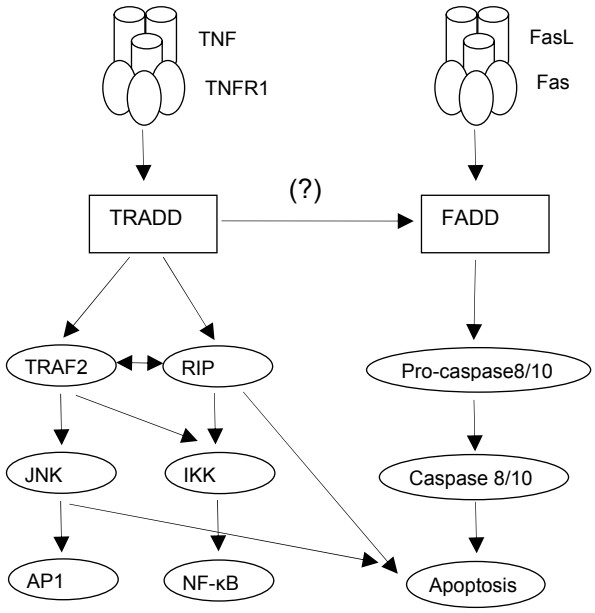


Figure 3:
Signaling cascade via death domains
FADD (Fas-associated DD), TRADD (TNFR1-associated DD), RIP (receptor interacting protein)

After binding of FasL, Fas assembles a death-inducing signaling complex (DISC): the recruitment of FADD activates the apoptosis-initiating proteases 8 and/or 10 via proteolysis of their pro-caspases. TNFR1 can trigger a similar pathway after recruiting TRADD as an intermediate adaptor protein to its DD. TRADD finally binds FADD and induces apoptosis.

The complex of TNFR1 and TRADD can also activate AP1 and NF-κB. Herr *et al*., 1999 described the induction of apoptosis after JNK-activation and Wu *et al*., 1997 also found RIP as an inducer of apoptosis. TRAF2 recruits IKK thereby enabling RIP to activate the kinases of the IKK complex (Wajant *et al*., 2003). So TRADD

seems to be the central platform where committment of the cell into either apoptosis or survival, proliferation and differentiation takes place.

However, it needs to be mentioned here that recent studies have revealed that FADD and caspase 8 are not recruited to the TNFRI/TRADD complex for the induction of apoptosis and that there might be another mechanism for programmed cell death after TNFRI activation (Harper *et al*., 2003).

1.1.2.3. Decoy receptors

DcRs compete with membrane bound receptors for their common ligand. Three different forms of decoy exist. The soluble receptors DcR3 and osteoprotegerin (OPG) sequester their ligands either intracellularly or in the extracellular milieu respectively (Bhardwaj and Aggarwal, 2003). TRAIL R3 (DcR1) is anchored in the membrane but lacks an ICD whereas TRAIL R4 (DcR2) has only an incomplete DD and therefore signaling does not occur (Ashkenazi and Dixit, 1999). All these decoy receptors prevent binding of their ligands to membrane bound receptors which would start further signaling pathways.

1.2. The lymphotoxin β receptor (LTβR) and its ligands

The LTβR and its ligands are members of the TNF/TNFR superfamily and play an important role in the development of lymphoid cells and tissues, the maintenance of their structures and in cellular immunity.

1.2.1. Structure of the LTβR

The mouse LTβR gene encodes for an open reading frame of 1,248 base pairs (415 amino acids) spanning 10 exons with 6.9kb on chromosome 6, closely linked to the TNFRI locus. Translation is supported by a Kozak sequence adjacent to the first methionine codon. Lacking a canonical TATA or a CCAAT box and with a high G/C content the LTβR promoter resembles a non-inducible housekeeping gene promoter and suggests that it is constitutively regulated (Force 1995). However it has been reported that the transcription of the LTβR is upregulated in NIH 3T3 cells, a mouse fibroblast cell line, upon stimulation with dexamethasone (Muller *et al*., 2001) and that the expression is downregulated on L929, another mouse fibroblast cell line, and WEHI 164 cells, a mouse fibrosarcoma cell line, after ligand binding or treatment with an agonistic antibody (Rennert *et al*., 1998).

As a typical type I transmembrane protein, the LTβR protein consists of an amino terminal ECD with a signal peptide (sp) of 30 amino acids and four CRDs (193 amino acids), a transmembrane domain (TMD) of 21 amino acids and an ICD of 171 amino acids as depicted in chapter 5.3.2, figure 15. The ligand binding domain is similar to that of TNFRI in the positional arrangement of cysteine residues in a CxxCxxC motif and the predicted size of the loops created by the disulfide bonds in CRD1 and CRD2. The major contact domains for the functional ligand $LT\alpha_1\beta_2$ are CRD2 and part of CRD3. In contrast CRDs 3 and 4 of the LTβR resemble those of TNFRII in the positioning of the cysteine residues and by the presence of five additional amino acids adjacent to the ligand binding domain in CRD3. TNFRI, CD27, CD40, Ox40, Fas and 4-1BB contain a gap in this region. An additional similarity between LTβR and TNFRII is the membrane-proximal prolin-rich region in the ECD, which might form a stalk extending the CRDs from the cell surface (Force *et al*., 1995).

1.2.2. Expression of the LTβR

Constitutively expressed mouse LTβR mRNA was found in the major visceral organs and lymphoid tissues including liver, lung, kidney, heart, spleen and lymph nodes with low expression in testis, and in cell lines of monocytic, thymic medullary and epithelial origin but was found neither in a cytotoxic T cell line (PMMI) nor in several other T cell lines, the main source of the ligand $LT\alpha_1\beta_2$ (Force *et al*., 1995). In addition Degli-Esposti *et al*., 1997 also found the human 2.1 kb mRNA in melanoma cell lines but not in B cells or in the brain. Recently the LTβR was detected on bone marrow derived mast cells (BMMCs) (Stopfer *et al*., 2004) and on activated macrophages (Ehlers *et al*., 2003). Taken together the LTβR seems to be expressed on all cell types except B and T lymphocytes; these cells express the ligands $LT\alpha_1\beta_2$ and LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes).

1.2.3. Ligands of the LTβR

Up to now two functional ligands are known for the LTβR: the heterotrimeric lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) and the homotrimeric LIGHT. $LT\alpha_1\beta_2$ exclusively binds to the LTβR whereas LIGHT also binds to HVEM and DcR3 (Ware *et al*., 1995; Mauri *et al*., 1998). The other possible heterotrimer $LT\alpha_2\beta_1$ does not seem to bind to the LTβR but to TNFRI (Browning *et al*., 1995). This complex was only found *in vitro* and its physiological role needs to be determined. There is no evidence for a naturally occuring $LT\beta_3$ homotrimer either (Kuprash *et al*., 2002). A third ligand has been postulated since recent studies of Boehm *et al*., 2003 and Muller and Lipp, 2003 where the phenotype of mice double deficient in LTβ and LIGHT is less severe than the phenotype of $LT\beta R^{-/-}$ mice.

1.2.4. Expression of the ligands

Activated T, B and natural killer (NK) cells express soluble $LT\alpha_3$ and membrane bound $LT\alpha_1\beta_2$ (Ware, 1992).

Transcripts of LTβ were found in adult spleen and thymus and on lower levels in bone marrow, lung, liver, and skeletal muscle. Little or no expression was found in brain, heart, and testis. With *in situ* hybridization LTβ was also detectable in embryonic thymi, spleen, liver, bone marrow, and skin, and in the brain and cerebellum of three-week-old mice.

The pattern for LTα was similar in spleen and thymus (Pokholok *et al*., 1995).
Interestingly, LTβ expression was detected on a subset of $CD45^+CD4^+CD3^-$ $IL2R\gamma c^+IL7R\alpha^+CXCR5^+\alpha_4\beta_7integrin^+\alpha_4\beta_1integrin^+$ precursor cells from fetal liver, called lymphoid tissue-inducing cells (LTIC), which can become natural killer cells, dendritic antigen-presenting cells, and eventually follicular dendritic cells, but do not become T or B lymphocytes. These cells are involved in the organogenesis of lymph nodes, Peyer's patches (PPs) and tertiary lymphoid organs (TLO) (Mebius *et al*., 1997, 2004; Ruddle, 1999, 2004).
LIGHT mRNA was detected in heart, placenta, liver, lung, appendix, kidney, brain, peripheral lymphoid tissues and predominantely in spleen but not in embryonic tissue, endocrine glands or tumor lines of non hematopoietic and myeloid origin (Mauri *et al*., 1998). Tamada *et al*., 2000 showed LIGHT expression on the surface of immature dendritic cells (DCs) and Morel *et al*., 2000 determined the intracellular expression of LIGHT in resting T cells and, together with the surface expression, in activated T cells, mainly $CD8^+$ T cells.

1.2.5. Signal transduction

The LTβR has no DD but a TRAF binding motif in its ICD. Binding of TRAF2 or TRAF5 leads to the activation of NF-κB and AP1 (Nakano *et al*., 1996; Chang *et al.*, 2002).
Interestingly, in complex with TRAF3 LTβR activation - via agonistic antibodies or its ligands $LT\alpha_1\beta_2$ and LIGHT - triggers IFNγ-dependent cell death in a subclone of an adenocarcinoma cell line (HT29.14S) (VanArsdale *et al*., 1997; Rooney *et al*., 2000).

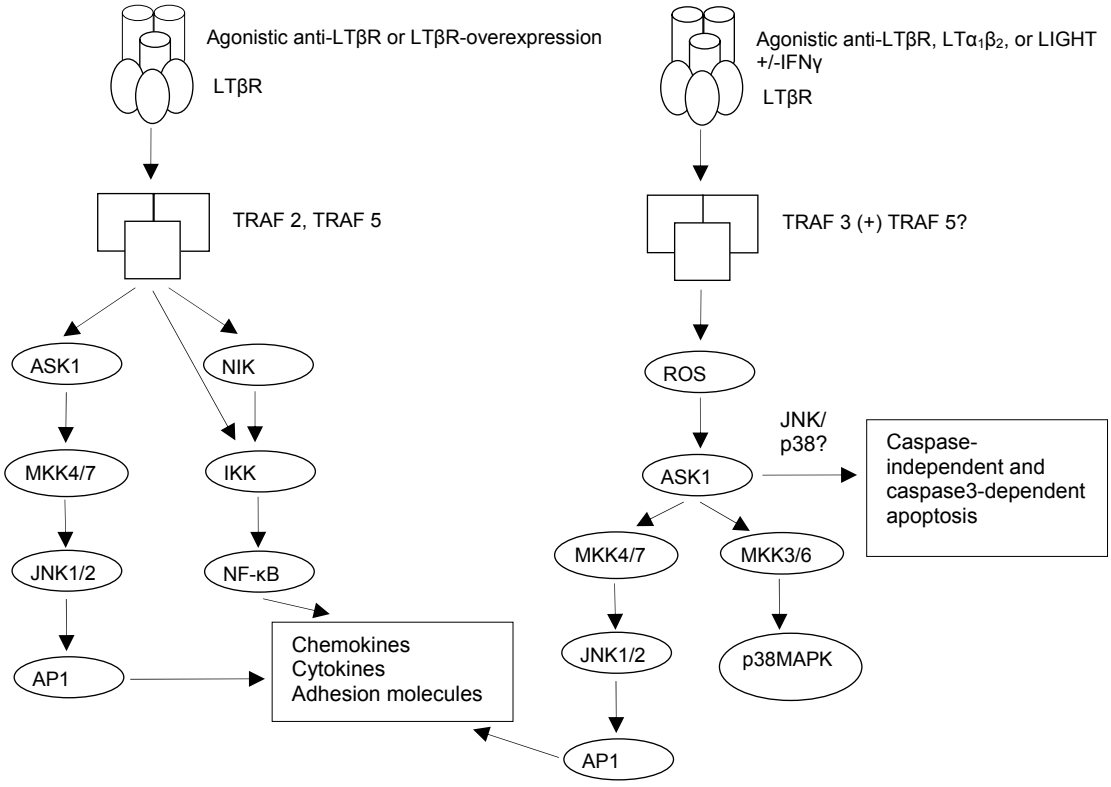


Figure 4:

Signaling cascade after the activation of the LTβR, according to Chang *et al*., 2002; Dejardin *et al*., 2002 ; Chen *et al*., 2003

These signaling cascades were shown for certain cell lines and might depend on the different cell types. The induction of IL8 via AP1 and NFκB activation was found in HEK293 (a human embryonic kidney cell line) (Chang). Cell death in HT29 and Hep3BT2 (a human hepatoma cell line) was inducible via ROS (reactive oxygen species) with IFNγ and without IFNγ in HeLa (human cervical carcinoma cell line) (Chen). TRAF3 also binds NIK but does not activate NFκB (Song *et al*., 1997).

MAPK (mitogen activated protein kinase); MKK (MAPK kinase)

1.2.6. Biological functions of the LTβR

The function of the LTβR was determined not only in cell culture systems but also by the generation of mice deficient in the receptor itself or in its ligands. Tumanov *et al*., 2003, give a detailed summary of all these mice and their phenotypes. Therefore only the phenotype of the $LT\beta R^{-/-}$ mice is described here.

Mice deficient in the LTβR lack all lymph nodes and Peyer's patches and show reduced nasopharyngeal-associated lymphoid tissue (NALT) with an abnormal microarchitecture. Primary B cell follicles, marginal zones (MZ), germinal centers (GCs) and follicular dentritic cells (FDCs) are missing in the spleen where the segregation of T and B cells is not developed either.

Treatment of adult mice with a LTβR-Ig fusion protein which sequesters the ligands from the endogenous LTβR destroys the splenic architecture (Mackay *et al.,* 1997). This leads to the conclusion that the splenic architecture is not developmentally fixed but a question of continuous LTβR expression and activation after birth.

In contrast, the induction of lymph node genesis seems to be dependent on the expression of the LTβR during embryogenesis since in-utero treatment of mice with LTβR-Ig fusion protein inhibits the development of lymph nodes whereas an in utero treatment of $LT\alpha^{-/-}$ mice, which also lack all lymph nodes except occasionally mesenteric and cervical lymph nodes, with agonistic LTβR antibody induces the development of lymph nodes depending on the time of treatment (Rennert *et al*., 1998).

T and B cell development seems to be normal in $LT\beta R^{-/-}$ mice with one exception: mucosa patrolling $\alpha_E\beta_7^{high}$ integrin$^+$ T cells are virtually absent (Futterer *et al*., 1998). Since there are no lymph nodes to home to, T and B cells infiltrate perivascularly in multiple tissues (Locksley *et al*., 2001).

The thymocytes appear to develop normally in the $LT\beta R^{-/-}$ mice. A retention of recent thymic emigrants (RTEs) can be observed, doubling the percentage of single positive cells in the thymus, suggesting a role for the LTβR in the control of the export of mature T cells. The medullary thymic epithelial cells (mTECs) in the thymus are reduced and show aberrant differentiation due to an impaired lympho-epithelial cross-talk via the LTβR and due to the missing activation of NIK. mTECs play a crucial role in the control of autoimmunity by the promiscuous expression of tissue-specific antigens during negative selection. That impaired lympho-epithelial cross-talk was found to be associated with autoimmune phenomena suggests an unexpected role for the LTβR signaling in central tolerance induction (Boehm *et al*., 2003). This idea is supported by the detection of autoantibodies against DNA and IgG (rheumatoid factor) in five to seven-month-old $LT\beta R^{-/-}$ mice (Chin *et al*., 2003), suggesting a signaling pathway in the thymus via LTβR-NIK-IKK-NFκB which leads to the expression of AIRE, so far the only known inducer of promiscuous gene expression and key mediator of central tolerance (Boehm *et al*., 2003; Chin *et al*., 2003; Venanzi *et al*., 2004). The lympho-epithelial cross-talk also seems to be necessary for the maintenance of the three dimensional structure in the thymic medulla as injections with LTβR-Ig fusion protein lead to its rapid regression to isolated clusters of cells in mice (Boehm *et al*., 2003).

LTβR activation is also considered to be necessary for the development of natural killer cells (NK) (Ito *et al*., 1999) and dendritic cells (DC) (Wu *et al*., 1999) and their recruitment to lymphoid organs.

Lymphoid tissue chemokines like secondary lymphoid tissue chemokine (SLC), B lymphocyte chemoattractant (BLC) and Epstein Barr-virus induced molecule 1 ligand chemokine (ELC) are necessary for lymphocyte homing. Their expression in the spleen is dependent on the activation of the LTβR (Ngo *et al*., 1999).

BMMCs release certain cytokines like IL4, IL6, TNF and RANTES after activation of the LTβR with an agonistic antibody or LIGHT or in coculture with activated T cells (Stopfer *et al*., 2004).

The angiogenic CXC chemokine MIP2 is released from BFS1 cells after LTβR activation suggesting a role of the LTβR in tumor neoangiogenesis (Hehlgans *et al*., 2002).

Degli-Esposti *et al*., 1997, and Hehlgans and Mannel, 2001 found IL8 and RANTES induction, loss of adherence and growth arrest after LTβR activation in A375, a human melanoma cell line.

Interferon γ (IFNγ)-dependent growth arrest was induced after LTβR activation in some adenocarcinoma cell lines and also cell death in HT29.14S and WiDr cells (Browning *et al*., 1996; vanArsdale *et al*, 1997).

Defects observed in the mucosal immune system of mice which are deficient in LTα or LTβ motivated the examination of the importance of the LT/LTβR system in experimental colitis as a model for inflammatory bowel diseases (IBD) like Crohn's disease (CD) or ulcerative colitis (UC). A protective effect with respect to wasting and the clinical signs of colitis in three models of IBD was determined by blocking the LT mediated events with a LTβR-Ig fusion protein, pointing to the lymphotoxin pathway as a potent intervention target for the treatment of this and maybe other autoimmune diseases and chronic inflammations (Mackay *et al*., 1998; Stopfer *et al*., 2004). Many studies have followed and are in progress in order to clarify the role of the LT, LIGHT/LTβR system in autoimmune diseases like IBD, diabetes or rheumatoid arthritis driven by the intention to find a new target for the treatment of these diseases.

2. Prework and aim

In order to study the expression pattern of the LTβR, the molecular mechanisms which follow the activation of the LTβR and the role of the LT, LIGHT/LTβR system in a dextransulfate (DSS)-induced acute and chronic colitis model four monoclonal rat anti mouse LTβR antibodies and one polyclonal rabbit anti mouse LTβR serum were generated. The specificities of these antibodies were tested on several different cell lines and primary cells. NIH 3T3, L929, two mouse fibroblast cell lines, CFS1, BFS1, two mouse fibrosarcoma cell lines, and embryonal fibroblasts were known to express the LTβR. All four monoclonal antibodies (mAb) showed positive stainings in flow cytometric analyses whereas none of these antibodies bound to embryonal fibroblasts of $LT\beta R^{-/-}$ mice (Hehlgans *et al*., 2003). Since it was known that the LTβR is not expressed on lymphocytes (Force *et al*., 1995), thymocytes of C57BL/6 mice were taken as a negative control. Surprisingly two mAbs, 12H11 and 1C5, showed significant shifts in flow cytometric analyses of these cells. Both antibodies also bound to thymocytes of $LT\beta R^{-/-}$ mice excluding the possibility that the thymocyte preparation was contaminated with LTβR expressing thymic stromal cells.

HVEM, another member of the TNFR superfamily which binds $LT\alpha_3$ and LIGHT, was thought to be the cross-reacting protein because its ECD is 35% homologous to the LTβR-ECD and it is expressed on lymphocytes. Flow cytometric analyses on thymocytes of $HVEM^{-/-}$ mice still showed positive staining excluding HVEM from the list of possible candidates (Dr. P. Muller, personally communicated).

The cross reactivity of 1C5 and 12H11 with thymocytes led to the question whether there might be another LTβR-like receptor on thymocytes which would bind $LT\alpha_1\beta_2$ and/or LIGHT for further signaling.

The aim of this project was to identify and characterize this mouse LTβR-like protein on thymocytes with the following steps:

- Determination whether the unknown protein has any biological function
- Mapping the epitope to narrow down the group of probable candidates
- Discrimination of the thymocyte subpopulations which express the unknown protein
- Discrimination of the splenocyte subpopulations which express the unknown protein
- Isolation of the unknown protein from thymocytes for further sequencing
- Screening of a thymus cDNA expression library in order to clone the unknown protein

3. Material

3.1. Companies

Applied Biosystems	Darmstadt, Germany
BD Becton Dickinson Biosciences	Heidelberg, Germany
Biometra	Goettingen, Germany
Biomol	Hamburg, Germany
Biorad	Munich, Germany
Charles River	Sulzfeld, Germany
Dako	Glostrup, Denmark
GE Healthcare Bio-Sciences	Freiburg, Germany
Invitrogen	Karlsruhe, Germany
Merck	Darmstadt, Germany
Metabion	Martinsried, Germany
Millipore	Schwalbach, Germany
MoBiTec	Göttingen, Germany
NEB (New England Biolabs)	Beverly, MA, USA
PAN Biotech	Aidenbach, Germany
Promega	Mannheim, Germany
QIAGEN GmbH	Hilden, Germany
R&D Systems	Wiesbaden, Germany
Roche Applied Science	Mannheim, Germany
Carl Roth	Karlsruhe, Germany
Sigma-Aldrich Chemie	Taufkirchen, Germany
Stratagene	Amsterdam, Netherlands
The Scripps Research Institute	La Jolla, CA, USA

3.2. Chemicals and solutions

ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate(6)]	Roche Applied Science
Agarose, electrophoresis grade	Invitrogen
Agarose, for routine use (Phage library)	Sigma-Aldrich Chemie
Ampicillin, >98%	Sigma-Aldrich Chemie
APS (Ammoniumpersulfat)	Biorad
BamHI	Roche Applied Science
BSA, Bovine serum albumin, fraction V	Biomol
Complete™ EDTA-free	Roche Applied Science
Deoxynucleotides, PCR grade	Roche Applied Science
DMSO (Dimethylsulfoxide), ACS reagent	Sigma-Aldrich Chemie
DNA-Molecular weight standards, X and XIV	Roche Applied Science
EcoRI	NEB
FCS (Fetal calf serum, heat inactivated)	PAN Biotech
Glycerol, ultrapure	Gibco / Invitrogen
Guanidiniumhydrochloride, ultra	Sigma-Aldrich Chemie
Hi-Di Formamide, Genetic Analysis Grade	Applied Biosystems
H_2O	Milli Q UF Plus system
Ionomycin (1mg/ml ethanol)	Sigma-Aldrich Chemie
IPTG (Isopropyl-β-D-thiogalactoside)	Biomol
Kanamycin	Gibco / Invitrogen
KpnI	Roche Applied Science
Low fat dry milk powder	Supermarket
Methanol, technical grade	Merck
Ni-NTA (Nitrilotriacetic acid) Agarose	QIAGEN
Nowa Solution A+B (ECL)	MoBiTec
PAA, Rotiphorese® Gel30 (37,5:1)	Carl Roth
Phenol/chloroform, for DNA purification	Carl Roth

PMA (Phorbol 12-myristate 13-acetate) (1mg/ml ethanol)	Sigma-Aldrich Chemie
RNAse A, DNAse free	Roche Applied Science
RPMI-1640	Sigma-Aldrich Chemie
SalI	NEB
SDS-PAGE Molecular weight standard, Broad range	Biorad
Shrimp alkaline phosphatase, SAP	Roche Applied Science
Sigma *FAST*™ BCIP/NBT (5-Bromo-4-chloro-3-indolylphosphate/Nitroblue tetrazolium)	Sigma-Aldrich Chemie
T4 DNA ligase	NEB
Taq Polymerase	Roche Applied Science
TEMED (N, N, N, N-Tetramethyl-ethylenediamine)	Biorad
Triton-X100, plus one	Pharmacia / GE Healthcare Bio-Sciences
Tryptone	Difco / BD Biosciences
TSR (Template suppression reagent)	Applied Biosystems
Tween20, p.S.	Merck
Urea, ultrapure	USB / GE-Healthcare Bio-Sciences
all other chemicals and solutions of analytical grade	Sigma-Aldrich Chemie or Merck

3.3. Antibodies and sera

Blocking antibody/serum	
Rat anti mouse Fcy RIII/II, $IgG_{2b}κ$; clone 2G4.2	Institute of Immunology; University of Regensburg
Normal goat serum	Dako
Primary antibodies	
Rabbit anti mouse LTβR, polyclonal serum	Dr. P. Mueller, Institute of Immunology; University of Regensburg
Rat anti mouse LTβR, IgG_{2a}; clones 1C5, 12H11, 5G11b, $5D12a_2$ see chapter 3.3.1	Dr. P. Mueller, Institute of Immunology; University of Regensburg

Rat anti mouse LTβR-FITC, IgG_{2a}; clone 1C5	Dr. P. Mueller, Institute of Immunology; University of Regensburg
Rat anti mouse CD3-PE, IgG_{2b}, κ; clone 17A2	Pharmingen / BD Biosciences
Rat anti mouse CD4-PE, IgG_{2a}, κ; clone RM4-5	Pharmingen / BD Biosciences
Rat anti mouse CD8a-APC, IgG_{2a}, κ; clone 53-6.7	Pharmingen / BD Biosciences
Rat anti mouse CD45R/B220-PE, IgG_{2a}, κ; clone RA3-6B2	Pharmingen / BD Biosciences
Armenian hamster anti mouse CD69-PerCP-CY5.5, IgG_1, λ; clone H1.2F3	Pharmingen / BD Biosciences
Rat anti mouse CD117-PE, IgG_{2b}, κ; clone ACK45	Pharmingen / BD Biosciences
Mouse anti RGS-His_4, IgG_1	QIAGEN
Isotype controls	
Armenian hamster IgG_1, λ -PerCP-CY5.5; clone A19-3	Pharmingen / BD Biosciences
Rat-IgG_{2a}, κ; unlabeled or -FITC, -PE, -APC; clone R35-95	Pharmingen / BD Biosciences
Secondary antibodies:	
Goat anti mouse IgG-HRP	Sigma-Aldrich Chemie
Goat anti rabbit Ig-HRP	Sigma-Aldrich Chemie
Goat anti rat IgG	Sigma-Aldrich Chemie
Goat anti rat IgG-AP	Sigma-Aldrich Chemie
Goat anti rat IgG-HRP	Sigma-Aldrich Chemie
Rabbit anti rat Ig, FITC	Dako

3.3.1. Rat anti mouse LTβR

Clone	Purification-Date	Experiment-Name	OD280 (mg/ml)	Fraction	DC-Protein-Assay (mg/ml)	FCA on thymocytes	FCA on L929	Western pro-/eukaryotic	Comment
1C5	08.11.2001	P71	0.16		0.197	++	+++	n.d.	
			0.2		0.319	+++	+++	n.d.	to Dr. Walczak
			0.3		1.11	++	++	n.d.	
			1.5		3.05	++	++	n.d.	
			2.1		3.9	++	++	n.d.	
			2.9		6.4	+	++	n.d.	
	30.03.2001	P19	2	F 1	6	++	+++	n.d.	
			1	F 2	2	+	+++	+ (1:1000)	until 01/04
			1.3	F 3	2.74	+++	+++	+ (1:1300)	until 07/04; FITC-labeled; to Dr. Wang
			0.6	F 4	1.16	+++	+++	n.d.	
	26.06.2003			E1.1	0.486	+	+	n.d.	
				E1.2	1.7	+	+	n.d.	
				E1.3	2.91	++	+	n.d.	
				E1.4	3.81	+++	+	n.d.	
				E1.5	2.23	+++	+	n.d.	
				E2.1	3.94	++	+	n.d.	
				E2.2	3.13	+++	+	+ (1:3000)	up from 08/04
				E2.3	0.55	+++	+	n.d.	

Clone	Purification-Date	Experiment-name	OD280 (mg/ml)	Fraction	DC-Protein-Assay (mg/ml)	FCA on thymocytes	FCA on L929	Western pro-/eukaryotic	Comment
12H11	08.11.2001	P72	0.7	F 3+4	1.85	-	++	n.d.	
			0.17	F 5+6	0.38	-	++	n.d.	
	10.02.2003		0.16	D 1.0	0	nd	nd	n.d.	
			0.18	D 2.0	0	nd	nd	n.d.	
			2.21	E 1.1	2.1	-	+	n.d.	
			1.06	E 1.2	0.67	-	++	n.d.	
			1.27	E 2.1	1.7	-	++	n.d.	
			1.24	E 3.1	1.77	-	++	n.d.	
			0.995	E 4.1	0.8	-	+++	n.d.	
	03.03.2003		2.61	E 1.2	4.1	-	++	n.d.	
			0.201	E 2.1	0.08	-	+++	n.d.	
	15.07.2003	27.12.2002			supern.	-	+	n.d.	
	16.07.2003	PM06.99			supern.	-	+	n.d.	
	14.10.2003				1.14	-	+	+ (1:530)	in use
					0.77	-	+	n.d.	
					1.69	-	+	n.d.	
					0.26	-	-	n.d.	

Clone	Purification-Date	Experiment-name	OD280 (mg/ml)	Fraction	DC-Protein-Assay (mg/ml)	FCA on thymocytes	FCA on L929	Western pro-/eukaryotic	Comment
5G11b	16.11.2001	P74	3	F4/1 (?)	6.4	-	+	+ (1:3000)	until 04/04
			4.1	F2/2 (?)	10.9	-	+	n.d.	
			0.3	F1/1 v F5/2(?)	0.47	-	++	+ (1:240)	until 02/04
			5.26	F3/1 (?)	12	-	+	n.d.	
			1.3	F3/2 (?)	2.5	-	++	+ (1:1333)	until 01/04
	17.07.2003	"Standkultur"			supern.	n.d.	+	n.d.	
		"Miniperm"		1.harvest	supern.	n.d.	+	n.d.	
		"Miniperm"		2.harvest	supern.	n.d.	+	n.d.	
		"Miniperm"		3.harvest	supern.	n.d.	+	n.d.	
	10.09.2003			E1.0	1.13	n.d.	+	+ (1:266)	up from 05/04
				E2.0	0.36	n.d.	+	n.d.	
	25.09.2003			E1.1	0.39	n.d.	+	n.d.	
				E1.2	1.14	n.d.	+	n.d.	
				E1.3	1.55	n.d.	+	n.d.	
				E1.4	0.92	n.d.	+	n.d.	
5D12a2	16.11.2001	P75	0.3			n.d.	n.d.	n.d.	
			0.4			n.d.	n.d.	n.d.	
			0.5			n.d.	n.d.	n.d.	
			1			n.d.	n.d.	n.d.	
			1.1			n.d.	n.d.	n.d.	
			1.5			n.d.	n.d.	+ (1:1000)	in use
			2			n.d.	n.d.	n.d.	
			3.7			n.d.	n.d.	n.d.	
			4.5			n.d.	n.d.	n.d.	

3.4. Kits

ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit	Applied Biosystems
Dc Protein Assay	Biorad
Giga®III Gold Packaging Extract	Stratagene
IL2 ELISA "Duo Set"	R&D Systems
pBluescript®II SK(-) Phagemid Kit	Stratagene
QIAGEN Plasmid Maxi Kit	QIAGEN
TOPO TA Cloning® Kit	Invitrogen
Uni-ZAP®XR Vector Kit	Stratagene
Wizard® *Plus* Miniprep DNA Purification System	Promega

3.5. Organisms

3.5.1. Prokaryotic cells

Epicurian coli® XL1-Blue MRF'	Stratagene
Ex-Assist® interference resistant helper phage	Stratagene
M15 [pREP4]	QIAGEN
SOLR™	Stratagene
TOP10 F'	Invitrogen

3.5.2. Eukaryotic cells

L929 cells, mouse fibroblast cell line	ATCC-No.: CCL-1

3.5.3. Animals

C57BL/6 wt mice	Charles River
LTβR$^{-/-}$ mice	Generated by Prof. K. Pfeffer, University of Duesseldorf; bred in individually ventilated cages (IVC) in the animal facility of the University of Regensburg; background C57BL/6

p55-TNFRI$^{-/-}$	Generated by Prof. K. Pfeffer, University of Duesseldorf
p75-TNFRII$^{-/-}$	Generated by Dr. H. Bluethmann, Hoffmann-La Roche, Basel; both TNFR$^{-/-}$ strains are bred in the animal facility of the University of Regensburg; background C57BL/6

3.6. Vectors

Mouse thymus cDNA expression library/Uni ZAP II	Prof. Schwaeble / University of Leicester
pBluescript®II SK(-) phagemid	Stratagene
pCR®2.1-TOPO®	Invitrogen
pQE32	QIAGEN
Uni-ZAP®XR	Stratagene

3.7. Primers

(Metabion)

LTBR-ZAP 3'SAL	CCC CCG TCG ACT CAG AGG TCT TGG CAT CCT AG
mLTßR-ECD(-D2)-KpnI	CCC GGT ACC GCA AGT CTT GCA AAC CGT GTC TTG G
mLTßR-ECD(-D3)-KpnI	CCC GGT ACC GCG GCA CTC GGC TTT CCG ATC G
mLTßR-ECD(-D4)-KpnI	CCC GGT ACC GGG GAC ACA GTT GAC GTC AGT ATC
mLTßR-ECD-3'KpnI	CCC GGT ACC TGG AGC TGC CTC CAC
mLTßR-ECD-5'BamHI	CCC GGA TCC TCA GCG GGC TTC TGG TGG CC
mLTßR-ECD-5'BamHI-20aa	CCC GGA TCC CTT GCT GGG ACC AGG ACA AGG
mLTßR-ECD-5'BamHI-42aa	CCC GGA TCC CTT GTC CCC CAG GCG AGT TTG TC

mLTßR-ECD-5'BamHI-61aa	CCC GGA TCC CCC ATA ATT CCT ATA ATG AAC AC
mLTßR-EcoRI-5'	AAA CGA ATT CCA TGC GCC TGC CCC GGG CCT CC
pQE-3'Seq (pQE32-sequencing)	GAT GGA GTT CTG AGG TCA TTA CTG G ("high")
pQE-5'Seq (pQE32-sequencing)	TAA TAG ATT CAA TTG TGA GCG GAT AAC ("high")
rev-pBS (pCR®2.1-TOPO® and pBluescript®II SK(-) sequencing)	GGA AAC AGC TAT GAC CAT GAT ("normal")
uni-pBS (pCR®2.1-TOPO® and pBluescript®II SK(-) sequencing)	TTG TAA AAC GAC GGC CAG TG ("normal")

3.8. Proteins

hLTβR-ECD-Ig, S2-expressed	Dr. P. Mueller, Institute of Immunology, Regensburg
mLTβR-ECD-Ig, S2-expressed	Dr. P. Mueller, Institute of Immunology, Regensburg
mLTβR-whole ECD	cloned by Agnes Fuetterer, University of Munich; Institute of Microbiology, Hygiene and Immunology; expressed in this work
mLTβR-ECD-ΔD4	this work
mLTβR-ECD-ΔD4,3	this work
mLTβR-ECD-ΔD4,3,2	this work
mLTβR-ECD-(-61aa)	this work
mLTβR-ECD-(-42aa)	this work
mLTβR-ECD-(-20aa)	this work

3.9. Constructs

This work:

Name	Vector	Insert	Size (kb)	Restriction-sites	Host-strain	Resistance
mLTßR-cDNA clone 5	pBS-SK(-)	mLTßR-cDNA (Ex1-10)	3.0+1.256	EcoRI/SalI	XL1-MRF'	Amp-200
mLTßR-cDNA clone 1	pCR2.1-TOPO	mLTßR-cDNA (Ex1-10)	3.9+1.256	EcoRI/SalI	TOP 10 F'	Amp-50/Kan-50
mLTßR-cDNA-"library"	Uni ZAPII-XR	mLTßR-cDNA (Ex1-10)	41+1.256	EcoRI/SalI	XL1-MRF'	none
mLTßR-ECD clone 1	pQE32	mLTßR-cDNA (Ex1-6)	3.4+0.621	EcoRI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD (-D2) clone1	pQE32	mLTßR-ECD (-D2,3,4)	3.4+0.194	BamHI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD (-D2) clone6	pCR2.1-TOPO	mLTßR-ECD (-D2,3,4)	3.9+0.194	BamHI/KpnI	XL1-MRF'	Amp-100/ Kan-50
mLTßR-ECD (-D3) clone 1	pQE32	mLTßR-ECD (-D3,4)	3.4+0.320	BamHI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD (-D3) clone 3	pCR2.1-TOPO	mLTßR-ECD (-D3,4)	3.9+0.320	BamHI/KpnI	TOP 10 F'	Amp-50/ Kan-50
mLTßR-ECD (-D4) clone 1	pQE32	mLTßR-ECD(-D4)	3.4+0.458	BamHI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD-20aa clone2	pQE32	mLTßR-ECD-20aa	3.4+0.491	BamHI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD-20aa clone 1	pCR2.1-TOPO	mLTßR-ECD-20aa	3.9+0.491	BamHI/KpnI	XL1-MRF'	Amp-100/ Kan-50
mLTßR-ECD-42aa clone 1	pQE32	mLTßR-ECD-42aa	3.4+0.434	BamHI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD-42aa clone 1	pCR2.1-TOPO	mLTßR-ECD-42aa	3.9+0.434	BamHI/KpnI	XL1-MRF'	Amp-100/ Kan-50
mLTßR-ECD-61aa clone 4	pQE32	mLTßR-ECD-61aa	3.4+0.365	BamHI/KpnI	M15	Amp-100/ Kan-25
mLTßR-ECD-61aa clone 4	pCR2.1-TOPO	mLTßR-ECD-61aa	3.9+0.365	BamHI/KpnI	XL1-MRF'	Amp-100/ Kan-50

3.10. Prepared buffers and solutions

3.10.1. Working with cells; flow cytometry

6x ACK-buffer	0.155 M	NH_4Cl
	0.1 M	$KHCO_3$
	0.1 mM	EDTA pH8.0
	pH should be 7.4; adjust with NaOH or HCl	
	sterilize by filtration; store at room temperature	
DNase free RNase A	100 mg	Pancreatic RNase A
	10 ml	0,01 M CH_3COONa pH 5.2
	disolve well; heat for 15min at 100°C	
	slowly cool down to room temperature	
	adjust pH with 0.1 volumes 1M Tris.Cl pH 7.4	
	store 1ml aliquots at -20°C	
PBS pH7.4	137 mM	NaCl
	2.7 mM	KCl
	8.1 mM	Na_2HPO_4
	1.5 mM	KH_2PO_4
Trypan blue	1.5 mg/ml	Trypan blue
	0.9 %	NaCl

3.10.2. Molecular biology techniques

6x DNA Loading buffer III	0.25 %	Bromphenolblue
	0.25 %	Xylene Cyanol FF
	30 %	Glycerol
SM-buffer	100 mM	NaCl
	10 mM	$MgSO_4.7H_2O$
	50 mM	Tris.Cl pH 7.5
	autoclave; store at room temperature	
3M Sodiumacetate, pH 5.2 or pH 4.6	81,6 g	$CH_3COONa.2H_2O$
	ad 400 ml	H_2O
	adjust pH to 5.2 or 4.6 with glacial acetic acid	
	ad 500 ml	H_2O
	autoclave; store at room temperature	
50x TAE-buffer	242 g/l	Tris
	57.1 ml/l	Glacial acetic acid
	100 ml/l	0.5M EDTA pH8.0

10x TBE-buffer	0.89 M Tris 0.89 M Boric acid 2 mM EDTA pH8.0
TE pH8.0	10 mM Tris.Cl pH 8.0 1 mM EDTA pH 8.0 autoclave; store at room temperature

3.10.3. Media and antibiotics

LB-medium and plates	10 g/l Tryptone 5 g/l Yeast-extract 10 g/l NaCl adjust pH to 7.0 with 5N NaOH autoclave; store at room temperature plates: add 1.5% agar-agar before autoclaving
NZY-plates and top agarose	5 g/l NaCl 5 g/l Yeast-extract 10 g/l NZ-amine from bovine milk 2 g/l $MgSO_4.7H_2O$ 0.7% Agarose or 1.5% agar-agar adjust pH to ~7.5 with 10 N NaOH autoclave; store at room temperature
SOC medium	20 g Tryptone 5 g Yeast extract 0.5 g NaCl disolve in 950ml H_2O 10 ml 250 mM KCl adjust pH to 7.0 with 5N NaOH 5 ml 2 M $MgCl_2$ ad 1000 ml H_2O autoclave; store at room temperature right before use add glucose to 20mM final concentration

	stock solution	working solution
Ampicillin	200 mg/ml H_2O	100 or 200µg/ml
Kanamycin	50 mg/ml H_2O	50 µg/ml
	store in aliquots at -20°C	

3.10.4. Expression and purification of His-tagged proteins

all lysis, wash and elution buffers should be prepared right before use

Lysis buffer A	100 mM	NaH_2PO_4
	10 mM	Tris·Cl pH8.0
	6 M	Guanidiniumhydrochloride
	0.1%	Triton-X100
	10%	Glycerol
	adjust pH to 8.0 with NaOH	
Wash buffer C	100 mM	NaH_2PO_4
	10 mM	Tris·Cl pH8.0
	8 M	Urea
	20 mM	Imidazole
	0.1%	Triton-X100
	10%	Glycerol
	adjust pH to 8.0 with NaOH	
Elution buffer D	100 mM	NaH_2PO_4
	10 mM	Tris·Cl pH8.0
	8 M	Urea
	10%	Glycerol
	adjust pH to 5.9 with HCl.	
Elution buffer E	100 mM	NaH_2PO_4
	10 mM	Tris·Cl pH8.0
	8 M	Urea
	10%	Glycerol
	adjust pH to 4.5 with HCl	
RIPA buffer	50 mM	Tris.Cl pH7.4
	150 mM	NaCl
	1 mM	EDTA
	0.25%	Sodiumdesoxycholat
	1%	Triton-X100
	1%	NP40
“complete” (Roche):	1 mM	PMSF
	each 1 µg/ml	Aprotinine, Leupeptine and Pepstatine
	1 mM	Na_3VO_4
	1 mM	NaF
	store in aliquots at -20°C	

3.10.5. SDS-PAGE and western blots

4x Laemmli loading buffer	250 mM	Tris
	4%	SDS
	40%	Glycerol
	4%	β-Mercaptoethanol
	pH should be ~6.8	
17% SDS PAGE		
- Stacking gel	4%	PAA
	125 mM	Tris.Cl pH6.8
	0.1 %	SDS
	0.1 %	TEMED
	0.1 %	APS
- Resolving gel	17%	PAA
	375 mM	Tris.Cl pH8.8
	0.1 %	SDS
	0.075 %	TEMED
	0.075 %	APS
5x SDS-PAGE running buffer	120 mM	Tris
	950 mM	Glycine
	0.5 %	SDS
Western blot buffer A	300 mM	Tris
	10%	Methanol
	adjust pH to 10.4	
Western blot buffer B	25 mM	Tris
	10%	Methanol
	adjust pH to 10.4	
Western blot buffer C	25 mM	Tris
	40 mM	6-amino-n-capronic acid
	10%	Methanol
	adjust pH to 9.4	
TBS pH7.4	1.5 M	NaCl
	27 mM	KCl
	250 mM	Tris
Coomassie-Staining		
Staining	10%	Glacial acetic acid
	40%	Methanol
	0.2 %	Coomassie brillant blue R250
	filter before use	
Destaining	10%	Glacial acetic acid
	40%	Methanol

Silver Staining

Fixation	10%	Glacial acetic acid
	40%	Ethanol
Sensitization	0.02%	Sodiumthiosulfate
Staining	0.2%	Silvernitrate
	0.02%	Formaldehyde
Developing	3%	Sodiumcarbonate
	0.05%	Formaldehyde
Stop solution	0.5%	Glycine
Solution for air-drying	5%	Glycerol
	35%	Ethanol

4. Methods

4.1. Working with thymocytes and splenocytes

4.1.1. Isolation of thymocytes

Thymi were taken from six to thirteen-week-old mice and strained through a 0.4µm mesh with 10ml 10% FCS/RPMI. Aggregates needed to be disrupted by pipetting up and down. The following washing step was performed in 40ml 10% FCS/RPMI and with centrifugation for 10 min at 200*g*. The clear supernatant was decanted and the cells were resuspended in 10ml 10% FCS/RPMI. A 1:10 dilution with 5mg/ml Trypan blue was optimal for counting in a Neubauer chamber. Approximately 10^8 thymocytes were obtained in this way. All the cells needed for the following experiments were centrifuged again and resuspended in 5% FCS/PBS or 10% FCS/RPMI to a final concentration of $2x10^6$ cells/ml.
The whole procedure was performed on ice.

4.1.2. Isolation of splenocytes

Simultaneously, spleens were taken from the mice above. Any adhering adipose or connective tissue was cut off before opening the spleen on one end and carefully scraping the hematopoietic cells out of it. The cells were harvested in 10ml 10% FCS/RPMI avoiding aggregates by vigorously pipetting up and down. In suspension the splenocytes were passed through a 0.4µm mesh and washed as described for the thymocytes. The pellet was resuspended in 1ml 10% FCS/PBS and erythrocytes were lysed with 4ml of 1x ACK buffer for 4 min with occasional shaking. Lysis was stopped with 5ml 10% FCS/RPMI and the cells were resuspended and passed through a mesh again. After an additional washing step the cells were resuspended in 10ml 10% FCS/RPMI and diluted 1:20 with Trypan blue for counting. 1-1.5x 10^8 splenocytes were obtained in this way. Like the thymocytes the splenocytes were adjusted to $2x10^6$ cells/ml for further experiments after additional centrifuging.
The whole procedure was performed on ice.

4.1.3. Cultivation of thymocytes

Thymocytes were isolated under sterile conditions and distributed into a 48 well plate with $1x10^6$ cells/500µl 10%FCS/RPMI/well.

PMA (phorbol 12-myristate 13-acetate)/ionomycin (2µg/ml each) treatment was chosen as a positive control for thymocyte activation whereas the treatment with Rat-IgG_{2a} (10µg/ml) served as a negative control for the treatment with the monoclonal rat anti mouse LTβR antibody 1C5 (10µg/ml). Rat-IgG_{2a} and 1C5 were also crosslinked with a secondary antibody, goat anti rat-IgG (10µg/ml), in order to mimic oligomerization of ligands before binding their receptors.

After 24 hours the cells were harvested, washed twice with ice-cold PBS and fixed in ice-cold 70% methanol for propidium iodide (PI) staining and flow cytometric analysis.

For the IL2 ELISA the plates were centrifuged after 24h for 2 min at 200*g* and cell free supernatants were taken.

4.2. Flow cytometric analysis

4.2.1. Determination of the DNA profile with propidium iodide

The DNA content of cells differs depending on the cell cycle. By staining the DNA for example with propidium iodide (PI) for further measurement by flow cytometry it is possible to conclude whether cells are resting or proliferating or maybe dying.
Therefore 10^6 cells/tube were washed twice in ice-cold PBS and fixed/permeabilized in 1ml ice-cold 70% methanol for a minimum of one hour up to several days in the cold. After sufficient fixation the cells were washed twice with ice-cold PBS before destroying the RNA by adding 475µl of DNase-free RNase A (100µg/ml) for 20 min at 37°C. In order to stain the DNA 25µl PI (1mg/ml) were added. Immediately the cells were measured by flow cytometry on a FACSCalibur applying CellQuest software (both Becton Dickinson) with the following instrument settings:
FSC E-1 log; SSC 340 log; FL3 485 lin 1.64; FL3A lin 1.43; FL3W lin 1.81; treshold FSC 52.
30,000 events with ~300 events/sec were measured and finally analysed with WinMDI 2.8. (The Scripps Research Institute).

4.2.2. Testing of all fractions of three different anti mLTβR antibodies

Altogether four different monoclonal rat antibodies against the ECD of the mLTβR were generated (Hehlgans *et al*., 2003). Since then several people have worked with these antibodies and have purified a lot of different fractions from each clone. As first observations led to the conclusion that the different batches of each antibody had different avidities to its antigen it was necessary to test all of them for their binding behaviour.
Therefore L929 cells, a mouse fibroblast cell line bearing the mLTβR on its surface, and, in the case of the cross-reacting antibody 1C5, thymocytes, were chosen for testing all fractions of the three monoclonal antibodies 1C5, 12H11 and 5G11b. The fourth antibody 5D12a_2 was of minor interest at this point. All antibody concentrations were determined with the D_C Protein Assay, a modified Lowry, according to the manufacturer's protocol.

L929 cells were harvested by mild trypsinisation, washed, counted and adjusted to $1x10^6$ cells/ml/tube. Thymocytes were prepared as described above and adjusted

to the same concentration. After centrifugation for 10 min at 200*g* the pellets were resuspended in 100µl of blocking solution containing 1% normal rabbit serum in 10% FCS/PBS and incubated for 20min on ice. The blocking solution was removed before the addition of the different fractions of the primary antibodies 1C5, 12H11 or 5G11b (10µg/ml in 10% FCS/PBS) or the isotype control, rat IgG_{2a} (10µg/ml) for one hour on ice. In the case there were no purified antibodies available but rather supernatants, 100µl of the supernatants were taken as the primary antibody. Three washing steps followed, each included the addition of 1ml 5% FCS/PBS, mixing and centrifuging for 10min at 200*g*. For the detection of the bound primary antibodies, 100µl of the secondary antibody, rabbit anti rat Ig, FITC-labeled, with a concentration of 4µg/ml was added for an additional hour on ice followed by three washing steps as above. The cells were resuspended in 500µl 5% FCS/PBS after the last washing step and measured as soon as possible by flow cytometry.

Instrument settings for the FACSCalibur:

FSC E-1 log; SSC 260 log; FL1 750 (thymocytes) or 475 (L929); treshold FSC 52. 50,000 events with ~1000 events/sec were measured with CellQuest and finally analysed with WinMDI 2.8.

For further flow cytometric analysis and western blots the antibodies which showed the strongest shift were chosen (see chapter 3.3.1).

4.2.3. Four colour flow cytometric analysis

The method chosen to differentiate the thymocytes and the splenocytes expressing the unknown protein was a simultaneous staining of the cells with four different antibodies, each labeled with another dye, and analysis by flow cytometry. The prerequisite for a multicoclor analysis is the optimal concentration of each antibody and the right compensation of each colour/channel at the flow cytometer. Therefore it was necessary first to perform single color staining with each antibody.

Thymocytes were prepared as described above. $2x10^6$ cells per tube were incubated on ice for 15min with 50µl of rat anti mouse Fcγ RIII/II, 10µg/ml in 10% FCS/PBS, to block any unspecific binding sites. The labeled primary antibodies were added in a 50µl volume leading to a final concentration of 1.0, 2.5, 5.0 and 10.0µg/ml in 10% FCS/PBS. As primary antibodies the following clones were chosen, all generated in rats except anti-CD69 (armenian hamster):

Antibody-Colour	Clone	Channel
1C5-FITC	F3	FL1
Anti-CD3-PE	17A2	FL2
Anti-CD4-PE	RM4-5	FL2
Anti-CD8a-APC	53-6.7	FL4
Anti-CD45R/B220-PE	RA3-6B2	FL2
Anti-CD69-PerCP-CY5.5	H1.2F3	FL3
Anti-CD117-PE	ack45	FL2

The individual isotype controls were used at 5µg/ml. After one hour of incubation on ice the cells were washed three times as described above and resuspended in 500µl 5% FCS/PBS.

Cells were measured at the FACSCalibur with CellQuest and the following settings:

FSC E-1 log; SSC 260 log; FL1 750 log; FL2 690 log; FL3 937 log; FL4 850 log; threshold FSC 230.

20,000 events in the lymphocyte gate were counted with ~500 events/sec.

To obtain the optimal concentration for each antibody the mean fluorescence intensities were plotted against the applied concentrations. The concentration leading to the last point before the curve reached the plateau was chosen as the optimal concentration for four colour staining. The optimal concentration for each antibody was 5µg/ml, except for anti-CD117 which should be used with 10µg/ml.

With these optimised concentrations the compensation for each channel at the flow cytometer was performed with thymocytes and splenocytes.

The staining protocol was the same as above. This time 30,000 events were counted in the lymphocyte gate with the following instrument settings:

FSC E00 lin 1.8; SSC 447 lin 1.49; FL1 750 log; FL2 722 log; FL3 937 log; FL4 850 log; threshold FSC 52. The linear scale for the forward and the sideward scatter was chosen because it was more convenient for distinguishing the lymphocytes from the other cells especially in the spleen. FL2 needed a little correction in the PMT voltage.

Viewing the neighbouring channels on parallel dot plots provided the chance to compensate fluorescence overspills.

The following compensations were determined:

FL1- 2.0% FL2
FL2-17.4% FL1
FL2- 2.1% FL3
FL3-12.5% FL2
FL3- 2.4% FL4
FL4- 2.5% FL3

In the thymus 1C5-FITC in FL1 needed a stronger compensation (2.5%) against CD45R/B220-PE and CD117-PE in FL2.

With these optimized concentrations, compensations and instrument settings, four color analysis could finally be performed following the protocol described above. All four antibodies were added to the cells at the same time with these combinations:
1C5-FITC + CD69-PerCP-CY5.5 + CD8a-APC + either CD3-PE, CD4-PE, CD45R/B220-PE or CD117-PE. The isotype control was a combination of rat-IgG_{2a}-FITC, -PE, -APC and armenian hamster-IgG1-PerCP-CY5.5, each with 5µg/ml.
In order to get enough cells for analysis even in the subpopulations with very few cells, 100,000 cells were measured in the lymphocyte gate. This meant to measuring ~ 140,000 thymocytes and 220,000 splenocytes.
To exclude dead cells PI was added to the autofluorescence control. PI stains necrotic and late apoptotic cells but not living cells if they are not fixed/permeabilized. There were only up to five percent dead cells equally distributed over all populations so they could be neglected for the analysis in WinMDI 2.8.
Regions and quadrants separating positive from negative cells were set according to the autofluorescence, isotype-controls and single-staining patterns.

4.3. Molecular biology techniques

4.3.1. Cloning of the expression constructs

4.3.1.1. PCR

Saiki *et al.,* 1985

For the epitope mapping it was necessary to clone the truncated extracellular domain (ECD)-cDNAs of the mLTβR into a prokaryotic expression vector. In a first step these cDNAs needed to be amplified via PCR with the addition of restriction sites for the following ligation into a vector. The cDNA of the whole ECD, previously cloned into pcDNA3, was taken as the template for all PCRs. The truncation of the ECD starting from the 3' end and deleting the fourth (ΔD4), the third and the fourth (ΔD4,3) or the second, third and fourth cysteine rich domains (ΔD4,3,2) was performed with one 5' primer (mLTßR-ECD-5'BamHI) and three different 3' primers (mLTßR-ECD(-D4)-KpnI or mLTßR-ECD(-D3)-KpnI or mLTßR-ECD(-D2)-KpnI). For the truncation of the ECD from the 5' end, in order to delete 20 amino acids (-20aa), 39 amino acids (-42aa) or 61amino acids (-61aa), one common 3' primer (mLTßR-ECD-3'KpnI) and three different 5' primers (mLTßR-ECD-5'BamHI-20aa or mLTßR-ECD-5'BamHI-42aa or mLTßR-ECD-5'BamHI-61aa) were combined. The cDNAs were amplified in a 50µl volume under the following conditions:

	Final concentration
Template DNA:	300ng
Reaction buffer:	1x, incl. 1.5mM Mg^{2+}
5' primer:	1.0µM
3' primer:	1.0µM
dNTPs:	0.2mM each
Taq polymerase:	0.1U/µl

Cycling conditions:

2min 96°C → 45 cycles : 30sec 96°C

30sec 65°C (5'truncation) or 68°C (3'truncation)

1min 72°C → 30min 72°C

Water was substituted for the template DNA in the case of a negative control for each reaction.

To check the PCRs, 5µl of each reaction product and its water control were loaded on a 1% agarose/1xTAE gel and separated at 10V/cm according to their size. DNA was visible after ethidiumbromide staining and exposure to UV light (254nm).

4.3.1.2. Purification of DNA from PAA gels

The PCR products were purified from a PAA gel according to Sambrook's and Russells' (2001) suggestion for DNAs with less than 1000 bp. For the purification of the PCR products the residual 45µl were mixed with 15µl 6x loading buffer and loaded on a 5% PAA/1xTBE gel, separated with constant current of 30mA and cut out under UV light control (366nm) after ethidiumbromide staining. The cut fragments were ground with a pestle and vigorously shaken for 2h in 1ml 0.1x TE buffer for eluting the DNA from the PAA. The volume was reduced with butanol to ~400µl for further precipitation of the DNA with 1/10 volumes of 3M sodiumacetate, pH5.2 and 2 volumes of 100% ethanol. After centrifuging for 30min the pellets were washed with 500µl 70% ethanol, dried and disolved in 10µl water.

In order to check the purity and the concentration of the PCR products 2µl of the purified DNA were separated on a 1% agarose gel again.

4.3.1.3. Cloning into pCR®2.1-TOPO®

An intermediary step with cloning the PCR products into pCR®2.1-TOPO® with the TOPO TA Cloning® kit before insertion into the expression vector was chosen because the restriction enzymes do not cut right at the beginning of the PCR products depending on the secondary structure given by the DNA sequence. PCR products can be cloned directly into pCR®2.1-TOPO® using the property of the Taq polymerase of adding an adenine to the 3' end of each product with its terminal transferase activity. The pCR®2.1-TOPO® vector carries an overhanging thymidine at its 5' end. Cloning was performed according to the manufacturer's instructions with some changes.

The DNA concentration of the purified PCR products was estimated according to the molecular weight standard XIV. Approximately 2.5-6.0ng DNA, depending on the length of the PCR product, were added to 10ng of pCR®2.1-TOPO® vector DNA together with the recommended salt solution (final concentrations: 0.2M NaCl, 6mM $MgCl_2$) in a final volume of 6µl. After 30min at room temperature the mixture was cooled down on ice and 200µl of competent XL1-Blue MRF' or One

Shot®TOP10F' *E.coli* instead of One Shot®TOP10 *E.coli* were added. XL1-Blue MRF' and One Shot®TOP10F' were chosen because the smallest construct seemed to be toxic. Therefore competent cells with the *lac* repressor (*lac*I^q gene) were needed to avoid any expression of the construct during the cloning procedure. One Shot®TOP10F' were bought from Invitrogen whereas XL1-Blue MRF' were made competent with $CaCl_2$, $MnCl_2$ and $CoCl_2$ according to Hanahan, 1983. After incubation on ice for 30min the bacteria were heat shocked for 90sec at 42°C to further open their membrane for an easier import of the plasmid and immediately cooled down on ice again. With the addition of three volumes SOC medium and incubation for 1h at 37°C with gentle shaking the bacteria started expressing the resistance gene which is needed for the selection of the plasmid carrying cells. For this selection the transformed bacteria were spread on LB plates with each 50µg/ml ampicillin and kanamycin (One Shot®TOP10F') or 100µg/ml ampicillin and 50µg/ml kanamycin (XL1-Blue MRF') and let grow overnight at 37°C. The next day five colonies per clone were inoculated into 3ml LB medium containing ampicillin and kanamycin and again let grow overnight at 37°C.

4.3.1.4. Small scale plasmid DNA preparation

Plasmid DNA was extracted from the whole 3ml overnight culture with the Wizard® *Plus* Minipreps DNA Purification System from Promega according to the manufacturer's instruction. DNA was eluted from the column with 50µl 10mM Tris·Cl pH8.0 instead of 100µl water. Five µl of each plasmid were taken for a restriction enzyme digest to check whether the plasmids were the right ones. In the pCR®2.1-TOPO® vector two EcoRI sites 5' and 3' flanking the cloning region allow to cut out the insert. As there are no BamHI or KpnI sites in pCR®2.1-TOPO® it was also possible to check the plasmids for the added restriction sites with these two enzymes. The restriction digests were performed in a total volume of 10µl with 1x buffer and 5 units of each enzyme for 2h at 37°C. After the separation on a 1% agarose gel with 10V/cm all clones with the right restriction patterns were sequenced before further subcloning into the expression vector.

4.3.1.5. Sequencing

Sequencing was performed with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems which combines the original didesoxynucleotide sequencing technique Sanger established in 1977 with

the use of fluorescent labeled terminators and the PCR. The concentration of the plasmid DNA was estimated from the gel according to the molecular weight standards X and XIV or, in case of large scale prepared DNA, measured at 260nm in a photometer. 700ng of plasmid DNA were added to 6.4pmol sequencing primer and 8µl premix from the kit. The volume was filled with water to 20µl. Depending on the G/C content of the primer one of the three established sequencing-programs was let run.

"normal":	15sec	96°C	"low":	15sec	96°C	"high":	15sec	96°C
	15sec	55°C		15sec	50°C		15sec	60°C
	4min	60°C		4min	60°C		4min	62°C

Each cycle was repeated 35 times. The sequencing reactions were purified by the addition of 1/10 volume of 3M sodiumacetate pH4.6 and 2 volumes of 100% ethanol. After precipitation at room temperature for 20min in the dark the samples were centrifuged for 30min at 16000*g*, washed with 500µl 70% ethanol, dried and dissolved in 25µl template suppression reagent (TSR) for the ABI310 Genetic Analyzer or 12µl Hi-Di Formamide, Genetic Analysis Grade for the ABI3100-Avant Genetic Analyzer. Both machines separate the sequencing products on a polymer in a capillary. The received sequence data were compared with the published mLTβR sequence, accession number NM_010736. Those clones without any mutation were used for further work and stored long term as a 25% glycerol stock at -80°C.

4.3.1.6. Cloning into the expression vector pQE32

The pQE32 vector from QIAGEN was chosen for expressing the constructs because it was also used for generating the original clone expressing the whole ECD of mLTβR, which was used for immunization. This vector adds a 6x His tag at the amino terminus of each expressed protein. Such a 6x His tag can be used for further enrichment or purification and detection of the expressed proteins. pQE32 consists of a phage T5 promoter which is recognized by the *E. coli* RNA polymerase. The host strain M15[pREP4] expresses a *lac* repressor from the *lac*Iq gene on its pREP4 plasmid. The *lac* repressor blocks the two *lac* operator sequences in the vector until IPTG is added to induce the expression of the inserted cDNA. With this system cDNAs which would be transcribed and

translated into toxic proteins can also be cloned. Transformed bacteria are resistant to 100µg/ml ampicillin (pQE32) and 25µg/ml kanamycin (pREP4).

4.3.1.6.1. Preparation of the vector

pQE32-DNA was bought from QIAGEN, transformed into *E.coli* and prepared in a large scale for further applications. As a BamHI- and a KpnI-site were added to the truncated ECDs during PCR for further cloning, the vector needed to be cut with these two enzymes. To avoid any religation of the vector caused by insufficient digestion it was also dephosphorylated. For the digestion with BamHI and KpnI 15µg vector DNA were cut overnight at 37°C in 100µl of the following reaction mixture: 1x buffer A + 1mg/ml BSA + 0.5u/µl of each enzyme.

The digested DNA was separated on a 1% agarose gel. The fragment representing the digested vector DNA was cut out under UV-light control (366nm) and purified with the QIAEXII Gel Extraction Kit from QIAGEN according to the manufacturer's instructions. After elution of the DNA with 2x 50µl 10mM Tris·Cl ph8.0 an additional purification step was performed. Residual proteins were extracted once with phenol/chloroform and once with chloroform. The DNA was precipitated as described in 4.3.1.2 and resuspended in 20µl water.

The dephosphorylation of the digested and purified DNA was performed in a 100µl reaction mixture of the following composition: 1x SAP buffer + 0.02u/µl shrimp alkaline phosphatase + 20µl purified vector DNA. After 90min at 37°C the dephosphorylation was stopped for 15min at 65°C. A double phenol/chloroform and chloroform extraction and a precipitation were performed as described above. After disolving in 20µl water 2µl of the DNA were checked on a 1% agarose gel for quality and quantity.

4.3.1.6.2. Preparation of the inserts

For cloning the truncated ECDs from pCR®2.1-TOPO® into pQE32, the TOPO® clones were cut overnight with BamHI/KpnI. For this process 35µl DNA from the small scale preparation were incubated with 1x buffer A + 1mg/ml BSA + 0.8u/µl of each enzyme in a total volume of 75µl. The fragments were separated on a 5% PAA gel and purified as described in 4.3.1.2. Four µl of the purified 10µl were separated on a 1% agarose gel to estimate the DNA concentration for the following ligation.

4.3.1.6.3. Ligation of the truncated ECDs into pQE32

For the ligation a molar ratio of vector:insert of 1:3-5 and a volume as small as possible were chosen according to Sambrook and Russell, 2001. Before ligating both, vector and insert were precipitated together in a total volume of 100µl.
The dried pellet was disolved in 8µl water before adding 1x ligation buffer and 400U T4 DNA ligase in a total volume of 10µl. Ligation ran over night at 16°C.

4.3.1.6.4. Transformation ito M15[pREP4] E. coli

M15 *E. coli* were made competent with $CaCl_2$, $MnCl_2$ and $CoCl_2$ according to Hanahan, 1983. The whole ligation reaction was added to 200µl of competent M15 on ice. Transformation followed the same procedure used for TOPO TA® cloning and clones were selected using 100µg/ml ampicillin and 25µg/ml kanamycin.
Plasmid DNAs of the pQE32 clones were prepared in a small scale and checked as described above for the TOPO® clones with restriction enzymes and sequencing. For long term storage large scale preparations were performed with the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions. All bacterial clones with the right restriction pattern and a sequence in frame without any mistakes were stored as 25% glycerol stocks at -80°C.

4.3.2. Screening of a mouse thymus cDNA expression library

A mouse thymus cDNA expression library was available as a gift from Prof. Schwaeble, University of Leicester. Poly $(A)^+$-mRNA was extracted from thymi of three newborn mice, transcribed into cDNA and cloned into λZAPII by scinet. A first round of screening was performed according to the instructions of Stratagene with some variations.
The maltose receptors of XL1MRF' *E.coli* were induced overnight with 0.2% maltose and 10mM $MgSO_4$ in 50ml LB medium for uptaking the phages. After intensive washing with 10mM $MgSO_4$ to get rid of the maltose and adjusting the bacteria to an optical density OD_{600}=0.6 (~$5x10^8$/ml), a serial ten fold dilution of the phages with SM buffer, starting with estimated 10^3 pfu (plaque forming unit) per plate, was performed in order to find the actual titer of the library. Infected bacteria (200µl) were incubated for 15min at 37°C before being mixed with 3ml NZY 0.7% top agarose (48°C), poured on prewarmed 96mm NZY plates and let grow overnight at 37°C. In a first round of screening 20 plates (132mm) were prepared each with calculated $8x10^3$ phages in 600µl induced XL1MRF' and 8ml top

agarose. After five hours at 42°C the first small plaques were visible. For the induction of the expression nitrocellulose filters, soaked in 20mM IPTG, were laid on the plaques for 4h at 37°C. These filters, marked for the right orientation, were carefully lifted after the induction and washed in 0.05% Tween-TBS for 5min and for 30min with gentle shaking. For blocking of unspecific binding the filters were incubated over night with 1% low fat dry milk powder/0.05% Tween-TBS at 8°C.

To avoid false positive plaques the plates were screened a second time. Again the plaques were covered with filters for 6h at 37°C. The filters were processed like the first ones.

The prerequisite an antibody which is used for library screens has to fulfill is a sensitivity high enough to detect down to 50-100pg of its antigen. Therefore dot blots were performed titrating the S2-expressed mLTβR-Ig-V5 fusion protein from 100ng to 16pg. These dot blots were incubated with the cross-reacting antibody 1C5 in concentrations of 2µg/ml and 3µg/ml. With the detection system alkaline phosphatase-NBT/BCIP amounts of protein down to 160pg with both antibody concentrations were visible. Controls with another mLTβR antibody, 5G11b, with anti-V5 or rat-IgG_{2a} instead of 1C5 and with the prokaryotically expressed mLTβR-ECD proved the sensitivity and specificity of this system.

To detect phages expressing the unknown protein the filters were incubated with 1C5 (2µg/ml) in 1% low fat dry milk powder/0.05% Tween-TBS for 3h at room temperature. After three intensive washing steps, each lasting 15min, the filters were incubated with the detection antibody goat anti rat IgG-alkaline phosphatase coupled (AP) (1.2µg/ml total protein) for 90min at room temperature followed by intensive washing again. NBT/BCIP is a substrate for AP which turns from white to violet and would make the positive plaques on the filter visible. Incubation with the substrate Sigma *FAST*™ BCIP/NBT (1 tablet/10ml water) was performed over night although first signals were seen after 30min on the dot blots. As there were no positive plaques visible this screen needs to be repeated with a minimum 10^6 pfu.

4.3.3. Preparation of a cDNA library only expressing the mLTβR

The screening of an expression library is a useful tool in order to find a protein with which an antibody cross-reacts. As there was no positive control for checking the technique itself, it was decided to prepare a library only expressing the mLTβR. Therefore the whole cDNA of the mLTβR was amplified via PCR as described in 4.3.1.1. Depending on the G/C content of the primers the annealing temperature needed to be changed to 62°C. With the primers mLTβR-EcoRI-5' and LTβR-ZAP-3'SaI an EcoRI site was added at the 5' end and a SalI site at the 3' end for further cloning into Uni-ZAP® XR, the arms of λZAPII, the phage in which the mouse thymus cDNA library also was cloned. The PCRs were purified and cloned into pCR®2.1-TOPO® as described above. The TOPO® clones with the right restriction patterns and a sequence with no flaws were subcloned into pBluescript®II SK(-) phagemid (pBS SK(-)) as a control for the right orientation because pBS-SK (-) is a part of the later used phage Uni-ZAP® XR. The pBS-SK(-) was bought from Stratagene and prepared like the pQE32 vector before it was used for ligation. Ligation into the EcoRI/XhoI-cut pBS-SK(-) and transformation into XL1MRF' were performed as described above in 4.3.1.6.3 and 4.3.1.6.4. Clones with the right restriction patterns, orientation and frame were prepared for long term storage in a large scale and as a glycerol stock and used for further subcloning into the phage arms Uni-ZAP® XR. Before ligation into the phage arms, 15µg of the pBS-SK(-) containing the whole mLTβR-cDNA were cut with EcoRI/SalI and purified as described above in 4.3.1.2. Overhanging ends after SalI digest are compatible with XhoI sites. Ligation of the mLTβR-EcoRI/SalI fragment into the phage arms Uni-ZAP® XR was performed in a 5µl volume containing 1x ligation buffer, 200U T4 DNA ligase, ~30ng of the fragment and 1µg of the bought phage arms at 16°C overnight. Equal molar ratios of insert and phage arms were suggested to avoid multiple inserts. Control ligations with or without control plasmid DNA were performed in the same way. After the ligation the phages were packed with the use of the Gigapack® III Gold Packaging Extract from Stratagene. The packaging of the phages and the induction of the maltose receptors of XL1MRF' for uptaking the packed phages were performed according to the manufacturer's instructions (the latter described in detail in 4.3.2). The titer of the produced library was determined by plating the phages in NZY top agarose on NZY plates without any antibiotics. For long term storage at -80°C the library was

amplified and the titer was checked again. Amplification was performed by plating 20x $5.5x10^4$ pfu for 7h at 37°C. Eight ml SM buffer were added per plate and shaken for 16h at 8°C. Phages and part of the bacteria were harvested in the SM buffer and incubated with 5% chloroform for 15min at room temperature in order to lyse the bacteria. After centrifuging for 10min at 500*g* the supernatants were combined. Three tubes were taken for long term storage at -80°C in 7% DMSO. The remaining supernatants were stored at 8°C after adding 0.3% chloroform.

Uni-ZAP® XR phages have the advantage of enabling the extraction of the cDNA of a positive plaque directly as a plasmid with a technique called “in vivo excision”. Getting a plasmid containing the whole mLTβR-cDNA was a good quality control. Therefore another *E.coli* strain, SOLR™, was induced and infected with $5x10^8$ phages together with 10^7 pfu of the Ex-Assist® interference-resistant helper phage. According to the manufacturer’s instructions, 200µl of the final bacteria suspension were plated on LB plates with 50µg/ml ampicillin. After an overnight incubation at 37°C colonies appeared, were inoculated and their DNA was extracted on a small scale as described above in 4.3.1.4. Checking for the restriction pattern and the sequence led to the conclusion that the library was fine. The last control will be the expression screen with the cross-reacting antibody 1C5, with the mLTβR-specific antibody 5G11b and with rat-IgG_{2a} as a negative control.

4.4. Protein chemistry techniques

4.4.1. IL2 ELISA

Activated T cells secrete IL2 in order to activate their own IL2 receptor for further proliferation. The IL2 ELISA "Duo Set" from R&D was used for measuring the supernatants of cultivated thymocytes after 24h of stimulation with PMA/ionomycin or the cross-reacting antibody 1C5. The procedure was performed in triplicates of 10% of the supernatants according to the manufacturer's protocol. The IL2 antibody complexes were detected with streptavidin-horseradish peroxidase (HRP) and its substrate ABTS followed by measurement at 405nm in an ELISA reader. Optical densities of the supernatants were compared to those of a serial two fold diluted standard.

4.4.2. Protein assay

The concentrations of the purified anti mLTβR-antibody fractions were determined by measuring their optical density at 280nm and by the D_C Protein Assay from Biorad, a modified Lowry assay. According to the manufacturer's instructions a serial two fold diluted albumin standard or the antibodies were mixed with reagent A and B and measured at 650nm in an ELISA reader after 15min.

4.4.3. Prokaryotic expression and enrichment of His tagged proteins

All truncated ECDs of mLTβR were cloned into the expression vector pQE32 and M15[pREP4] *E. coli* from QIAGEN. For the enrichment of His tagged proteins Ni-NTA technology was applied. Nitrilotriacetic acid (NTA) occupies four of the six ligand binding sites of the nickel ion leaving two sites free to interact with the 6x His tag. The Ni-NTA complex, coupled to Sepharose® CL-6B, called "Ni-NTA Agarose" (QIAGEN), was chosen for the enrichment of the expressed truncated constructs, performed according to a procedure which is based on the manufacturer's, Frenzel *et al* 2003 and Harrison 1991.

Three colonies of each construct, fresh spread from glycerol stocks on selection plates, were inoculated in 10ml LB medium with ampicillin/kanamycin. After being gently shaken overnight at 30°C, 2.5ml of each culture were transferred into 50ml LB medium with ampicillin/kanamycin. Approximately 90min later the cultures reached an OD_{600} of 0.5 which was optimal for the expression induction. Therefore 1mM IPTG was added for 1h at 30°C. Expression was stopped on ice for 10min

before the cells were centrifuged at 3000*g* for 20min at 4°C. The pellets were resuspended in 5ml lysis buffer A and shaken for 1-2h at 8°C or until the suspensions became clear. After centrifuging for 40min the supernatants were transferred to a 15ml tube and incubated with 1ml of Ni-NTA Agarose overnight at 8°C with rotation. To get rid of all non bound proteins the suspension was centrifuged for 5min at 3000*g*. The collected Agarose with the bound His tagged proteins was washed twice with wash buffer B and finally transferred to 1.5ml cups. Elution of the proteins from the Agarose was performed with 2x 300µl elution buffer D and 2x 300µl elution buffer E. To separate the proteins on a SDS-PAGE 200µl 4x Laemmli buffer were added to the eluted proteins and 175µl to the residual Agarose.

4.4.4. Preparation of the mLTβR from L929 cells for western blots

Approximately $2x10^8$ L929 cells were trypsinized, centrifuged for 10min at 200g and lysed in 6ml RIPA buffer. After one hour of shaking at room temperature the cell debris was centrifuged for 20min at 3000g. The supernatant was stored at -20°C and the pellet was resuspended in 100µl 4x Laemmli buffer, denatured for 5min at 95°C and subjected to an SDS-PAGE.

4.4.5. SDS-PAGE

Laemmli, 1970

SDS binds proteins in a constant charge to mass ratio which allows the separation of the proteins in an electric field approximately according to their molecular weight. To separate the truncated proteins with a size between 9kD and 30kD 17% SDS-PAA gels were let run in 1x SDS-PAGE running buffer with a constant current of 30mA in the stacking gel and 40mA in the resolving gel. The sizes of the fragments were compared with the broad range molecular weight standard of Biorad (20µl). Either 40µl of the residual resin or 100µl of the eluted proteins each in 1x Laemmli buffer were loaded after a denaturing step of 5min at 95°C and cooling down.

4.4.6. Silver staining of the proteins after SDS-PAGE

Proteins can be made visible after electrophoresis either for example with Coomassie or with silver. In the case of the truncated proteins the more sensitive silver staining was the method of choice because of the small amounts of extracted proteins. The staining procedure followed a procedure published by

Blum *et al* 1987, adapted by Prof. Goerg/University of Weihenstephan (personal communication).

PAA gels were fixed in 10% acetic acid/40% ethanol overnight at 8°C with gentle shaking. Three washing steps for 20min each were performed at room temperature, twice with 30% ethanol and once with water. Gels were sensitized with 0.02% sodiumthiosulfate and washed three times for 20sec with water. Staining was performed in a filtered 0.2% silvernitrate/0.02% formaldehyde solution at 8°C for 20min. Three 20sec washing steps with water followed before the silver grains were developed in 3% sodiumcarbonat/0.05% formaldehyde at room temperature until the right intensity appeared. The gels were washed three times in water for 20 sec and the color development was stopped with 0.5% glycine for 10min at room temperature. Gels were finally washed in water for three 10min periods and scanned for documentation. In order to store the gels they were soaked in 5% glycerol/35% ethanol for 1h, wrapped with cellophane also soaked in the mixture and fixed in an easy breeze gel drying frame for air drying overnight at room temperature.

4.4.7. Western blots

Towbin *et al*., 1979

Proteins were transferred from a gel to a PVDF membrane by a semi dry blotting system. The manufacturers of the blot machine (Biometra) and of the PVDF membrane Immobilon P (Millipore) suggest a three buffer system. Accordingly, each three pieces of 3MM Chr chromatography paper the same size as the gel were soaked in blot buffer A, B or C. The gel was washed and fixed for 15min in blot buffer C and the membrane needed to be rehydrated in methanol for 15sec, washed in water for 2min and prepared for the transfer for 5min in blot buffer B. Three papers from blot buffer A were laid on the anode without any air bubbles in between. Three papers from blot buffer B were laid down before the membrane was added. The gel was placed on the membrane and marked for later identification and orientation. Three papers from blot buffer C provided the contact to the cathode. The transfer ran at 0.4mA/cm^2 for 2h. The current was half of the normally used current and the transfer time was doubled due to the small molecular weight of the proteins.

4.4.8. Immunodetection of proteins on a membrane with enhanced chemiluminiscence ECL

Proteins were detected on the membranes with indirect immunoperoxidase technique. For this the membranes were incubated overnight at 8°C with a blocking solution containing 1% low fat dry milk powder in 0.05% Tween-TBS. In the case of the polyclonal serum an additional blocking step with 0.1% normal goat serum in blocking solution was performed for 1h. The first antibody, specific for the protein, or the isotype control Ig, were added for 2h at room temperature with a concentration of 2µg/ml in blocking solution. The polyclonal serum was used in a 1:10000 dilution in blocking solution. If the antibody anti-RGS-His_4 (mouse IgG1) was used, blocking was performed in 3% BSA-fractionV/0.05% Tween-TBS and the antibody was diluted to 0.1µg/ml in BSA blocking solution. After three 10min washing steps with 0.05% Tween/TBS a secondary antibody, detecting the isotype of the first antibody (i.e. goat anti rat IgG, goat anti rabbit Ig, or goat anti mouse IgG), coupled with horseradish-peroxidase (HRP), was added for another hour at room temperature, diluted 1:1000 in blocking solution(~0.5µg/ml total protein). Three intensive washing steps were needed again before adding the substrate. The substrate is a 1+1 mixture of solution A and B from Nowa. HRP oxidizes luminol (B) in the presence of hydrogen peroxide (A) and alkaline pH. Oxidized and activated luminol emitts light for a short time. After being rinsed for ~1min with the substrate solutions, the blots were placed between two foils in a radiography cassette and exposed to a special autoradiography film (Hyperfilm) for 15sec to 30min depending on the signal intensities.

4.4.9. Coomassie staining of the membrane

In order to see which proteins the signals belong to, the membranes were stained with Coomassie. For this the membranes were soaked in 0.2% Coomassie Brillant Blue R250 for a few seconds and destained with 10% acetic acid/40% methanol. Before drying the membranes they were washed intensively with water to get rid of the acetic acid.

5. Results

5.1. Biological function of the unknown protein

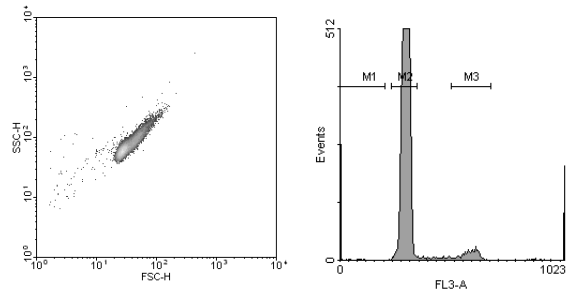
In earlier studies BFS1-cells, a mouse fibrosarcoma cell line, showed MIP2-release as a sign for activation after treatment with the antibody 1C5 (Muller, 2001). Therefore it was interesting to find out whether thymocytes also can be activated with 1C5.

To study this question thymocytes from C57Bl/6-wt and LTβR-deficient ($LT\beta R^{-/-}$) mice were treated for 24h with PMA/ionomycin as a positive control for activation or with the antibody 1C5. As a read-out for activated thymocytes IL2 production was measured.

Despite expectations no IL2 was found in the supernatants (data not shown). The explanation for this was found after staining the cells with PI for analysis by flow cytometry. The thymocytes became apoptotic instead of being activated after 1C5 treatment.

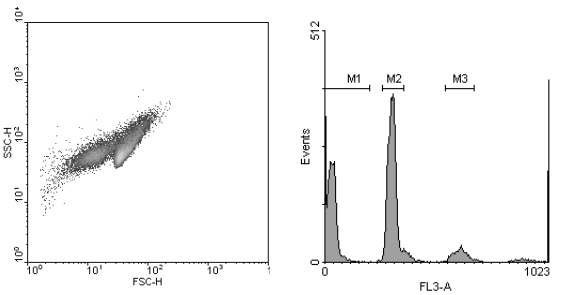
Results, representative of two experiments, are shown in the following density plots showing size and granularity of the thymocytes and in the histograms showing the DNA content of the cells in each cell cycle.

Thymocytes become apoptotic when they are held in single cell culture without any kind of activation (Anderson 1996). This can be seen in the histogram of the untreated cells. An additional so-called “pre G1-peak” appears. This pre G1-peak is also found in the negative controls where the thymocytes were treated with either rat IgG, goat anti rat alone or rat-IgG, crosslinked with goat anti rat.



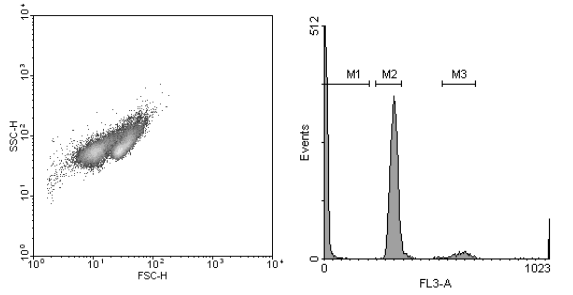
File: Data.003 Sample: fresh thymocytes

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	318.57
1	0, 207	1088	3.63	3.63	14.15
2	236, 353	26168	87.23	87.23	301.71
3	506, 687	1626	5.42	5.42	592.39



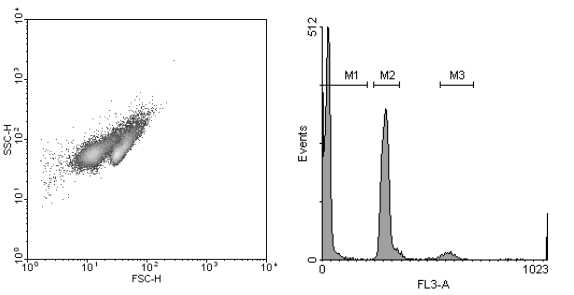
File: Data.004 Sample: untreated thymocytes

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	243.98
1	0, 208	11815	39.38	39.38	31.23
2	266, 365	14280	47.60	47.60	308.38
3	550, 682	1966	6.55	6.55	617.52



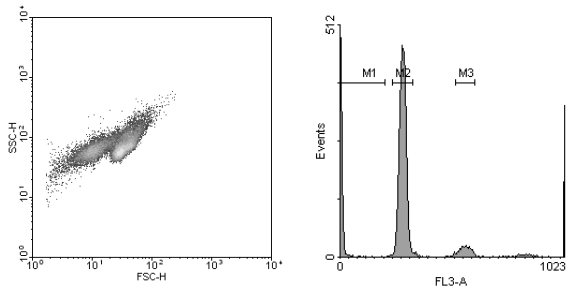
File: Data.012 Sample: Rat-IgG-treated

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	172.85
1	0, 207	15625	52.08	52.08	6.98
2	235, 355	12608	42.03	42.03	317.93
3	535, 687	997	3.32	3.32	621.68



File: Data.018 Sample: Rat-IgG+2nd ab-treat.

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	167.69
1	0, 207	15778	52.59	52.59	24.04
2	236, 353	12423	41.41	41.41	289.16
3	534, 686	958	3.19	3.19	583.00



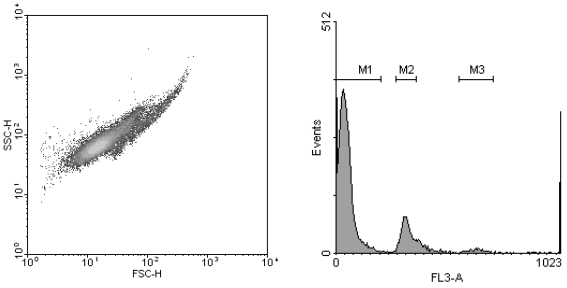
File: Data.024 Sample: only 2nd ab-treated

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	204.25
1	0, 208	12189	40.63	40.63	3.97
2	240, 334	15213	50.71	50.71	287.28
3	528, 619	1293	4.31	4.31	572.00

Figure 5:

Density plots of thymocytes, fresh, untreated and treated with control antibodies for 24h, showing the size (FSC) and the granularity (SSC) of the cells. The histograms show the DNA profile (FL3A) of these thymocytes together with the statistics for each cell cycle stage.

The treatment with PMA/ionomycin was too strong. Most of the cells were found in the apoptotic pre G1-peak instead of in the S-phase.



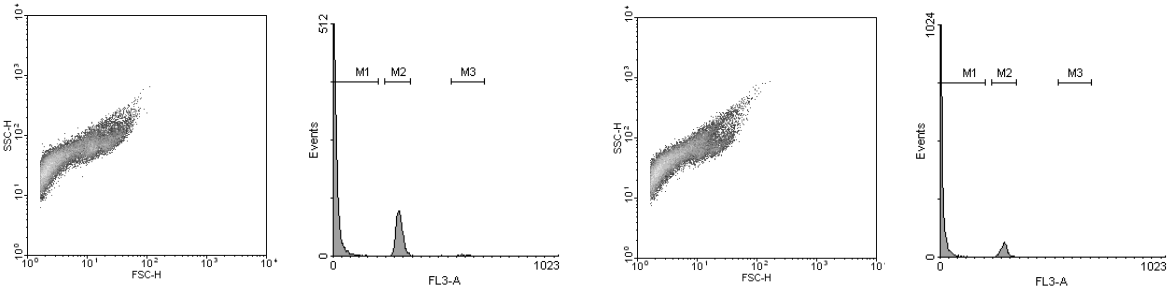
File: Data.008 Sample: PMA/Iono.-treated

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	130.02
1	0, 208	23328	77.76	77.76	43.91
2	272, 368	3911	13.04	13.04	322.34
3	560, 720	661	2.20	2.20	638.47

Figure 6:

Density plots of PMA/ionomycin treated thymocytes after 24h, showing the size (FSC) and the granularity (SSC) of the cells. The histogram shows the DNA profile (FL3A) of these thymocytes together with the statistics for each cell cycle stage.

Compared to the negative controls treatment of thymocytes with the antibody 1C5 results in a stronger pre G1-peak. Crosslinking 1C5 with a secondary antibody, goat anti rat, increases this shift into apoptosis.



File: Data.013 Sample: 1C5-treated

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	40.77
1	0, 208	26693	88.98	88.98	6.31
2	236, 355	3073	10.24	10.24	302.35
3	535, 687	132	0.44	0.44	593.85

File: Data.020 Sample: 1C5+2nd ab-treated

M	Low,High	Events	%Total	%Gated	GMean
0	0,1023	30000	100.00	100.00	26.67
1	0, 208	27976	93.25	93.25	6.41
2	236, 350	1884	6.28	6.28	291.64
3	535, 686	55	0.18	0.18	585.82

Figure 7:

Density plots, histograms and statistics of thymocytes treated with 1C5 or crosslinked 1C5.

These experiments gave a first hint, that the unknown protein on thymocytes might play a role in apoptotic events which are the central processes during thymocyte development.

To prove these data 1C5-negative cells e.g. the later found “subpopulation” of 1C5-negative CD4 SP thymocytes should be tested in the same way.

5.2. Exclusion of members of the TNFR superfamily

Since the members of the TNFR superfamily share a high homology in their ECDs, it was probable that one of the members would cross-react with the antibody 1C5. The easiest way to check this was to investigate thymocytes of mice deficient in certain members of the TNFR superfamily by flow cytometry.

Both TNFRs, TNFRI (p55) and TNFRII (p75), share very high homologies with the mLTβR (Force 1995). Therefore they were the first receptors to be checked to determine whether they were the unknown protein.

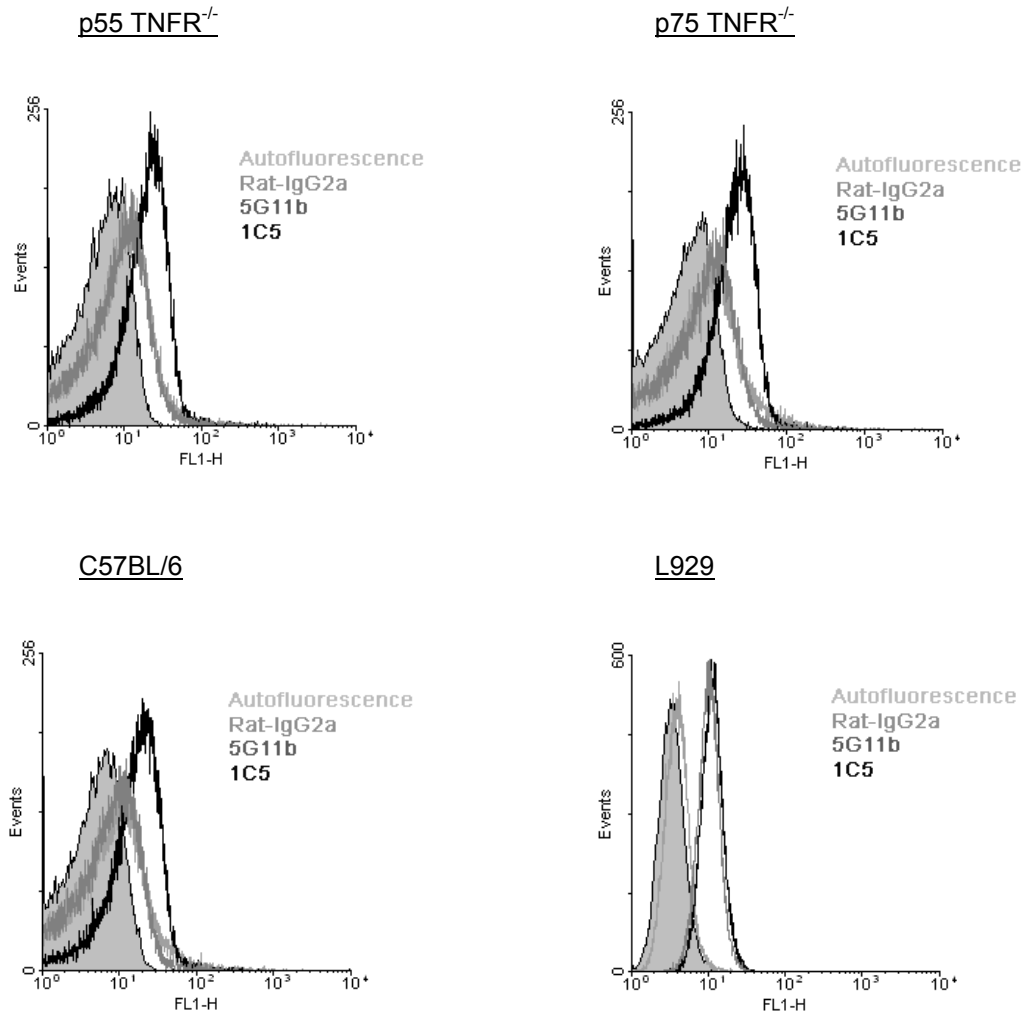


Figure 8:

Histograms of thymocytes from wt C57BL/6 mice and from mice deficient in p55 TNFR or p75 TNFR after staining with rat-IgG_{2a} for the isotype control, 5G11b as a mLTβR-specific antibody and 1C5 for the unknown protein. Interestingly and in contrast to the literature a very small amount of mLTβR was detected on the thymocytes whereas a significant shift with 1C5 appeared. L929 cells, naturally expressing mLTβR on their surface, served as a positive control for both antibodies.

As shown in figure 8 thymocytes from mice deficient in p55 TNFR or p75 TNFR also reacted with the antibody 1C5, excluding these two TNFRs as the unknown protein.
HVEM and DcR3 share not only a sequence homology but also their common ligand LIGHT with the LTβR which raised the question as to whether one of these two receptors could be the unknown protein. Earlier flow cytometric analysis with 1C5 on thymocytes of $HVEM^{-/-}$ mice showed positive staining (personal communication Dr. P. Mueller) removing HVEM from the list of probable candidates. DcR3 did not need to be checked because it is a soluble receptor which is not detectable on the surface of cells.

According to Force, 1995, CD40 is very homologous to the membrane proximal part of the mLTβR-ECD. As CD40 is known to be not expressed on T cells it can be excluded as the unknown protein without any experiment.

As the biological function of the unknown protein was determined to be a role in apoptosis, members of the TNFR superfamily with a so-called “death domain”, DD, which is responsible for the apoptotic process in the receptor bearing cell, were the next candidates to be investigated. Therefore similar analyses on thymocytes of mice deficient in TRAMP/DR3, TRAIL-R and of MRL lpr/lpr mice, which natively carry a mutated Fas gene, were performed. These thymocytes also showed positive staining, excluding TRAMP/DR3, TRAIL-R and Fas as the unknown protein. The studies on MRL lpr/lpr mice and $TRAILR^{-/-}$ mice were performed in the lab of Dr. H. Walczak at the German Cancer Research Center in Heidelberg, shown in figure 10. Dr. E. Wang at the University of Cardiff communicated personally that he determined the positivity of thymocytes of $TRAMP/DR3^{-/-}$ mice for 1C5.

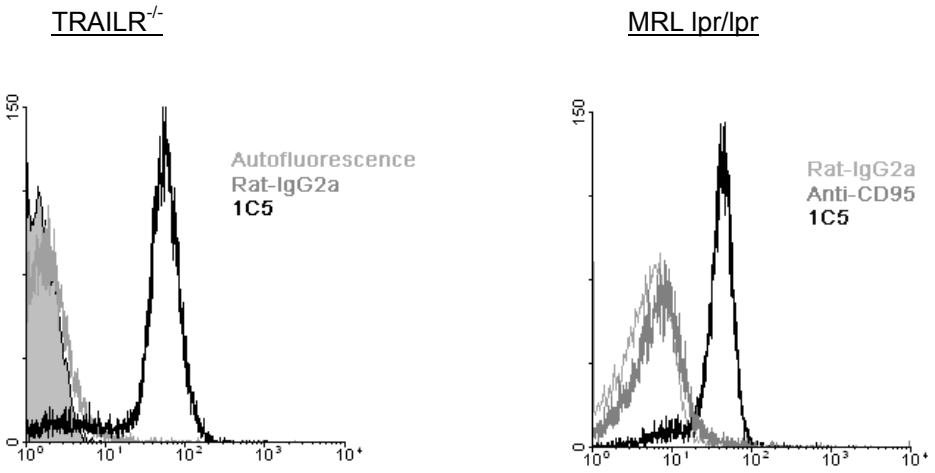


Figure 10:
Histograms of thymocytes from mice deficient in TRAIL-R or mutated in the Fas gene after staining with rat-IgG_{2a} for the isotype control and 1C5 for the unknown protein. Again 1C5 showed a shift on thymocytes of both mice. On MRL lpr/lpr anti-CD95 was stained to prove the mutation in the fas gene.

Finally OPG, another death receptor (DR) of the TNFR superfamily, can be excluded as the unknown protein because it is a soluble receptor like DcR3.

Taken together the following TNFR superfamily members can be excluded as the unknown protein: p55 TNFRI, p75 TNFRII, HVEM, TRAIL-R, FAS, TRAMP, CD40 OPG and DcR3.

5.3. Epitope mapping

One possibility to more closely define an unknown protein for which an antibody exists is to map the epitope on the protein which was used for immunization.

For the generation of monoclonal antibodies against mLTβR a rat was immunized twice with the prokaryotically expressed ECD of the mLTβR and once with the S2-expressed ECD-hIg fusion protein. The construct for the prokaryotic expression in *E. coli* is shown in figure 11. In order to map the epitope it was necessary to truncate the ECD and find the smallest peptide to which the antibody 1C5 still binds.

It was known that the antibody 1C5 was able to detect the prokaryotically expressed mLTβR-ECD on a western blot. Therefore constructs were cloned deleting regions from the carboxy terminus and in a second round of cloning deleting regions from the amino terminus for expression in *E.coli* and analyses by western blots.

```
  1 CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
    GAGCTCTTTA GTATTTTTTA AATAAACGAA ACACTCGCCT ATTGTTAATA

                                            EcoRI
 51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
    TTATCTAAGT TAACACTCGC CTATTGTTAA AGTGTGTCTT AAGTAATTTC

                                                BamHI/BglII
101 AGGAGAAATT AACTATGAGA GGATCTCACC ATCACCATCA CCATGGGATC
    TCCTCTTTAA TTGATACTCT CCTAGAGTGG TAGTGGTAGT GGTACCCTAG
                  M  R   G  S  H  H   H  H  H   H  G  I

151 AATTCGGCAC GAGGCGGGCT TCTGGTGGCC TCTCAGCCCC AGCTGGTGCC
    TTAAGCCGTG CTCCGCCCGA AGACCACCGG AGAGTCGGGG TCGACCACGG
    N  S  A  R   G  G  L   L  V  A   S  Q  P  Q   L  V  P

201 CCCTTATCGC ATAGAAAACC AGACTTGCTG GGACCAGGAC AAGGAATACT
    GGGAATAGCG TATCTTTTGG TCTGAACGAC CCTGGTCCTG TTCCTTATGA
     P  Y  R   I  E  N  Q   T  C  W   D  Q  D   K  E  Y  Y

251 ACGAGCCCAT GCACGACGTC TGCTGCTCCC GCTGTCCCCC AGGCGAGTTT
    TGCTCGGGTA CGTGCTGCAG ACGACGAGGG CGACAGGGGG TCCGCTCAAA
      E  P  M   H  D  V   C  C  S  R   C  P  P   G  E  F

301 GTCTTTGCGG TATGCAGCCG CAGCCAAGAC ACGGTTTGCA AGACTTGCCC
    CAGAAACGCC ATACGTCGGC GTCGGTTCTG TGCCAAACGT TCTGAACGGG
    V  F  A  V   C  S  R   S  Q  D   T  V  C  K   T  C  P
```

```
351 CCATAATTCC TATAATGAAC ACTGGAACCA TCTCTCCACC TGCCAGCTGT
    GGTATTAAGG ATATTACTTG TGACCTTGGT AGAGAGGTGG ACGGTCGACA
     H  N  S   Y  N  E  H   W  N  H   L  S  T   C  Q  L  C

401 GCCGCCCCTG TGACATTGTG CTGGGCTTTG AGGAGGTTGC CCCTTGCACC
    CGGCGGGGAC ACTGTAACAC GACCCGAAAC TCCTCCAACG GGGAACGTGG
      R  P  C   D  I  V   L  G  F  E   E  V  A   P  C  T

451 AGCGATCGGA AAGCCGAGTG CCGCTGTCAG CCGGGGATGT CCTGTGTGTA
    TCGCTAGCCT TTCGGCTCAC GGCGACAGTC GGCCCCTACA GGACACACAT
    S  D  R  K   A  E  C   R  C  Q   P  G  M  S   C  V  Y

501 TCTGGACAAT GAGTGTGTGC ACTGTGAGGA GGAGCGGCTT GTACTCTGCC
    AGACCTGTTA CTCACACACG TGACACTCCT CCTCGCCGAA CATGAGACGG
     L  D  N   E  C  V  H   C  E  E   E  R  L   V  L  C  Q

551 AGCCTGGCAC AGAAGCCGAG GTCACAGATG AAATTATGGA TACTGACGTC
    TCGGACCGTG TCTTCGGCTC CAGTGTCTAC TTTAATACCT ATGACTGCAG
      P  G  T   E  A  E   V  T  D  E   I  M  D   T  D  V

601 AACTGTGTCC CCTGTAAGCC GGGACACTTC CAGAACACTT CCTCCCCTCG
    TTGACACAGG GGACATTCGG CCCTGTGAAG GTCTTGTGAA GGAGGGGAGC
    N  C  V  P   C  K  P   G  H  F   Q  N  T  S   S  P  R

651 AGCCCGCTGT CAACCCCATA CCAGATGTGA GATCCAGGGC CTGGTGGAGG
    TCGGGCGACA GTTGGGGTAT GGTCTACACT CTAGGTCCCG GACCACCTCC
     A  R  C   Q  P  H  T   R  C  E   I  Q  G   L  V  E  A

            KpnI
701 CAGCTCCAGG TACCCCGGGT CGACCTGCAG CCAAGCTTAA TTAGCTGAGC
    GTCGAGGTCC ATGGGGCCCA GCTGGACGTC GGTTCGAATT AATCGACTCG
      A  P  G   T  P  G   R  P  A  A   K  L  N   *
```

Figure 11:

Sequence of the ECD of mLTβR (accession number NM_010736) in the expression vector pQE32 The translation start site (115), the stop codon (742) and the 6x His tag (127) are part of the vector. The insert is cloned with BamHI/BglII (146) and KpnI (709). The last 8 amino acids of the signal peptide and the following 12 amino acids, which are called ”spacer” in this work, start at position 166 followed by the four cysteine rich regions starting at positions 226 (D1), 349 (D2), 475 (D3) and 613 (D4).

5.3.1. Cloning of the truncated ECDs

For the first round of cloning the following primers were designed. The 5' primer, introducing a BamHI site at position +66 of the mLTβR-ECD cDNA, leaving only 24 bp of the 90 bp for the signal peptide, was the same for all three new constructs. The 3' primers, adding a KpnI-site, start at different bases in the cDNA so that finally products were amplified without the fourth (ΔD4), the third and the fourth (ΔD4,3), and the second, third and fourth cysteine-rich domains (CRDs) (ΔD4,3,2). As a template for the PCRs the pcDNA3 plasmid containing the whole mLTβR was chosen.

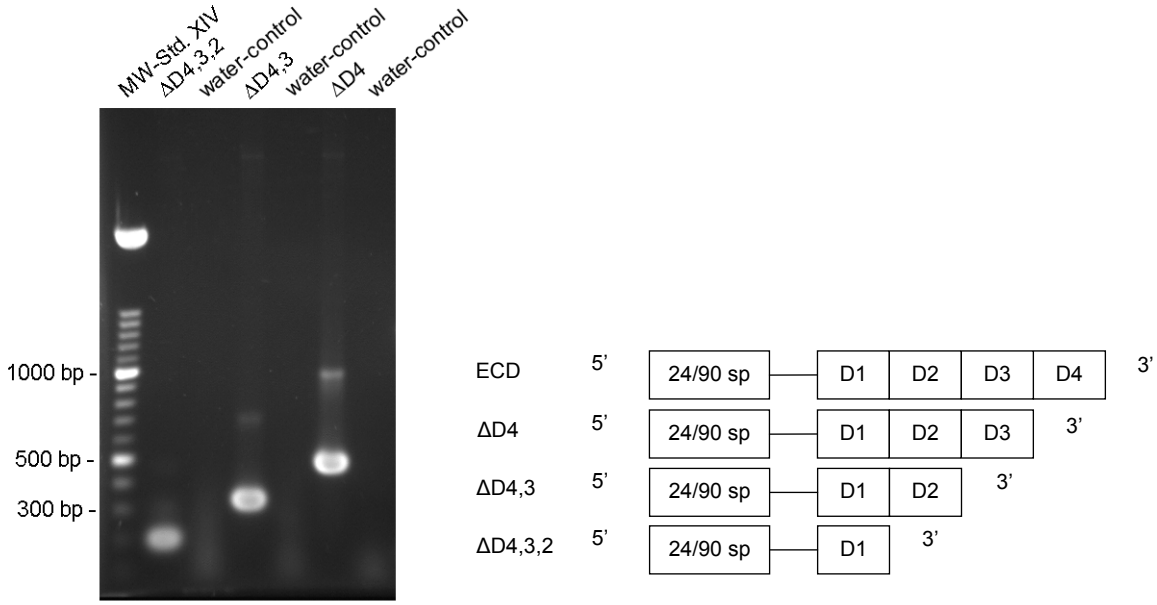


Figure 12:
PCR for cloning the truncated proteins; round I
The PCR products and their individual water controls (each 5µl) were checked on a 1% agarose gel before cloning into pCR®2.1-TOPO®. Molecular weight standard XIV: 1µg.

For the second round of cloning the 3' primer, using the KpnI-site 45 bp upstream of the carboxy terminus of the ECD, was the same for all three constructs. Coming from the amino terminus primers were designed in order to delete the first 20 amino acids (-20aa), the first 39 amino acids (-42aa) or the first 61 amino acids (-61aa). Each primer added a BamHI-site to the 5' of each PCR product.

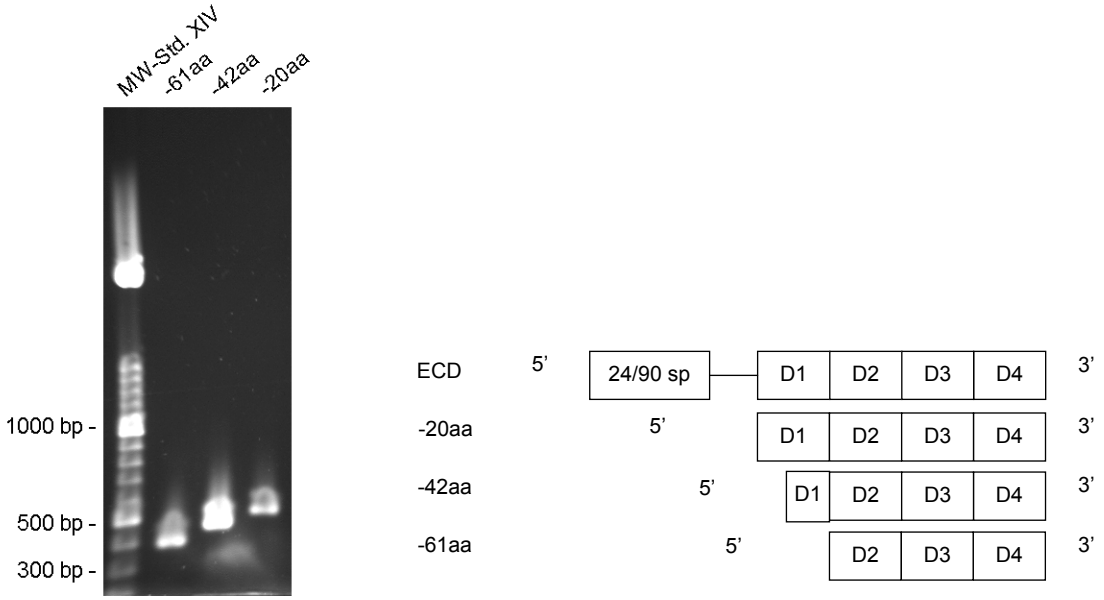
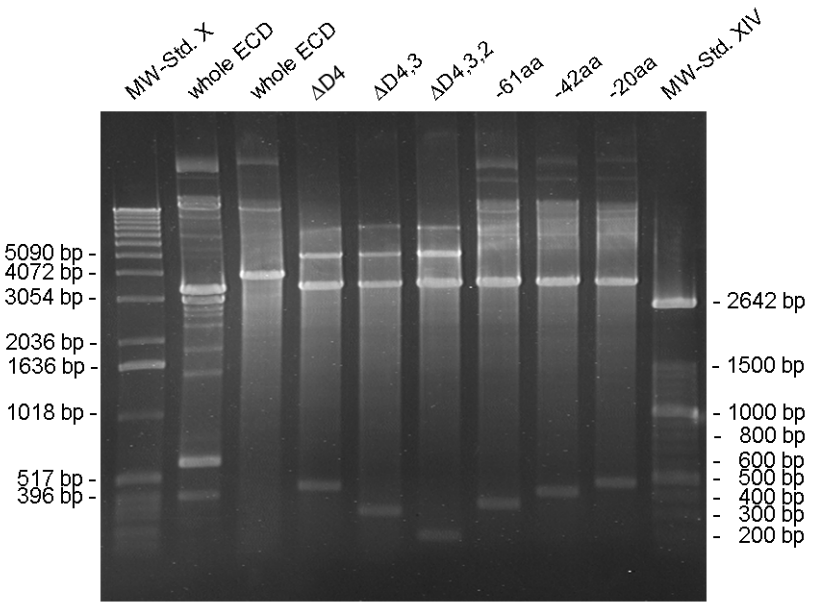


Figure 13:

PCR for cloning the truncated proteins; round II

After purifying the PCR products they were checked on a 1% agarose gel (each 2µl) before cloning into pCR®2.1-TOPO®. Molecular weight standard XIV: 1µg.

The TOPO TA Cloning® system was used for cloning these PCR-products before finally inserting them into the expression vector pQE32. As a last control, sequencing of all clones with the right restriction pattern was performed. Only the ones in frame and without any mutation were chosen for further work.



construct	size
ECD	563 bp
ΔD4	458 bp
ΔD4,3	320 bp
ΔD4,3,2	194 bp
-61aa	365 bp
-42aa	434 bp
-20aa	491 bp

Figure 14:

1% agarose gel of all constructs cloned into pQE32 for the expression of the truncated proteins. Restriction digest of 2µg of each DNA was performed with BamHI/KpnI (lanes 3-9). The construct with the whole ECD was also cut with EcoRI/KpnI (lane 2). The size of each construct is listed. Molecular weight standards X and XIV: each 1µg.

All clones were cut with the enzymes used for cloning, BamHI and KpnI. Each plasmid showed a band at 3.4 kb for the vector and an insert of the expected size. Only the original clone with the whole ECD could not be cut with these two enzymes because the BamHI-site was destroyed during BamHI/BglII-cloning. EcoRI/KpnI digest was used as an additional control resulting in the release of a 621 bp fragment. The additional bands might be due to EcoRI star activity because the restriction buffer was not optimal for the EcoRI- but necessary for the KpnI-digest.

5.3.2. Expression of the truncated proteins

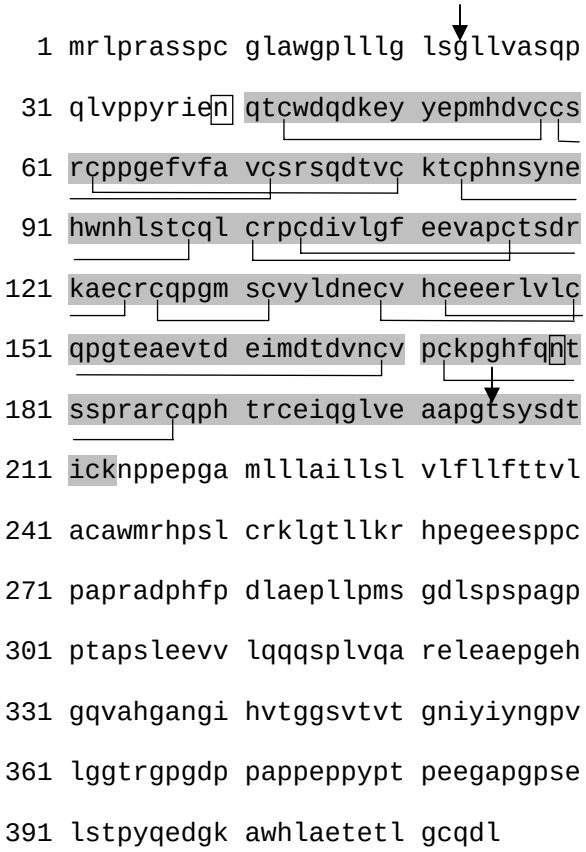


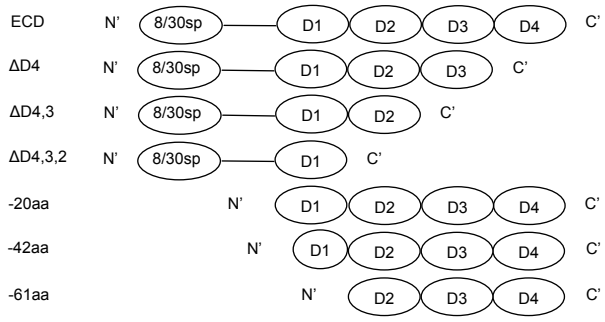
Figure 15:

Protein sequence of the mLTβR

The mLTβR consists of a signal peptide sp (aa1-30), an ECD (aa 31-223), a TMD (aa224-244) and an ICD (aa245-415). Its ECD is divided into four cysteine rich domains (gray shaded):

D1 (aa42-81), D2 (aa82-124), D3 (aa125-170) and D4 (aa171-213). In the ECD there are 10 disulfide bonds ⌊——⌋ and two glycosylation sites □ (accession number P50284).

The arrows mark the amino terminus (aa25) and the carboxy terminus (aa205) of the protein which was used for immunization.



	Length [aa]	Calcul. MW [kD]	Position SDS-PAA gel
ECD	209	23.4	24-30
ΔD4	174	19.6	24
ΔD4,3	128	14.5	16
ΔD4,3,2	86	9.7	9
-61aa	143	16.0	21
-42aa	166	18.4	24
-20aa	185	20.8	27

Figure 16:
Schematic view of the truncated proteins, expressed in *E. coli*

All clones were expressed in M15 *E. coli* and enriched with Ni-NTA technology. Figure 17 shows all expressed truncated proteins on a 17% silver stained SDS-PAA gel.

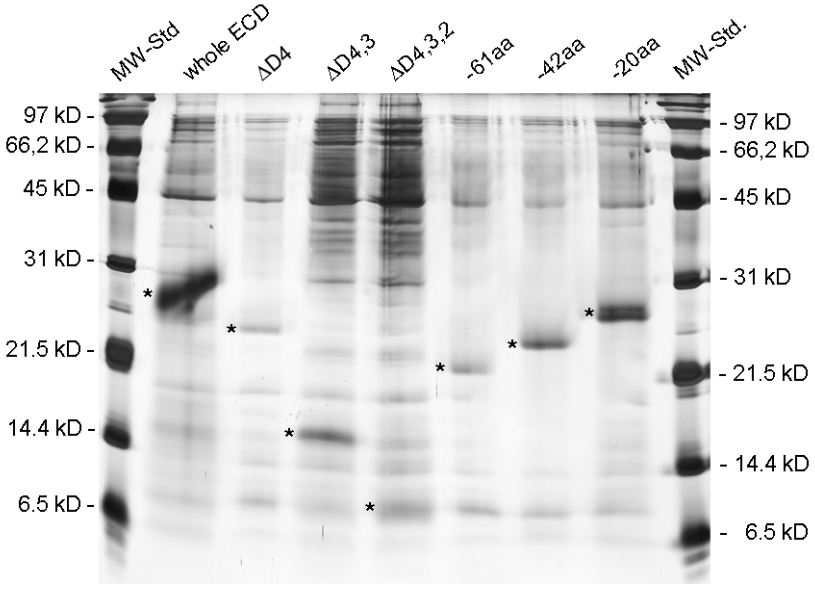


Figure 17:
17% silver stained SDS-PAA gel showing all truncated ECDs (*) and the whole ECD (*) after enrichment with Ni-NTA technology; 100µl of each eluted protein and 20µl of the broad range molecular weight standard were subjected to the gel.

5.3.3. Western blots and immunodetection of the truncated proteins

The experiments to check whether all proteins could be blotted and detected on the membrane were performed with an anti-RGS-His_4 or with a polyclonal anti mLTβR serum. As shown in figure 18 all proteins were detectable in this way.

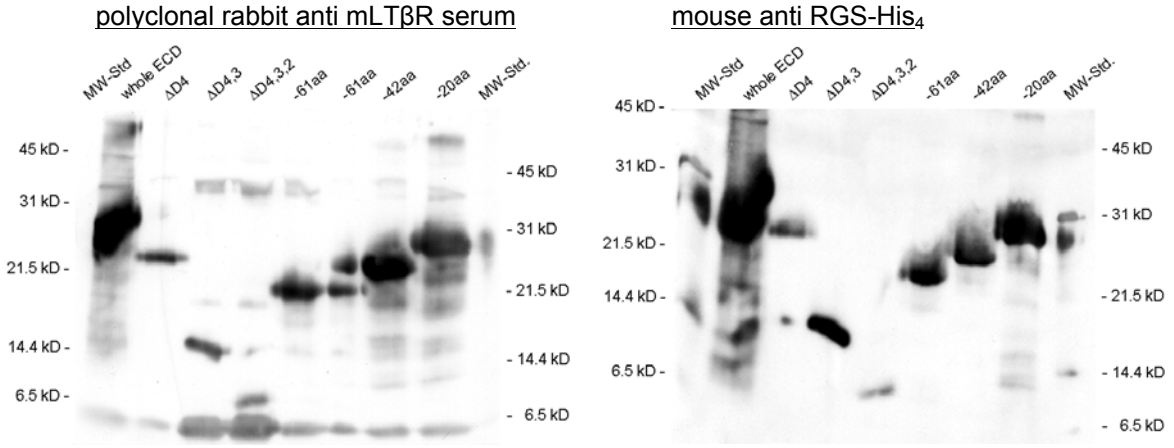


Figure 18:

Western blots of all truncated ECDs of mLTβR, detected with polyclonal rabbit anti mLTβR serum (left) and mouse anti RGS-His_4 (right). The same amounts of proteins were loaded like on the silver stained gel.

Since four different monoclonal rat anti mLTβR antibodies had been generated, it was interesting to map the epitopes of all of them.

From earlier competition assays (Muller, 2001) three different epitopes were expected for the four monoclonal antibodies. This was supported by the epitope mapping.

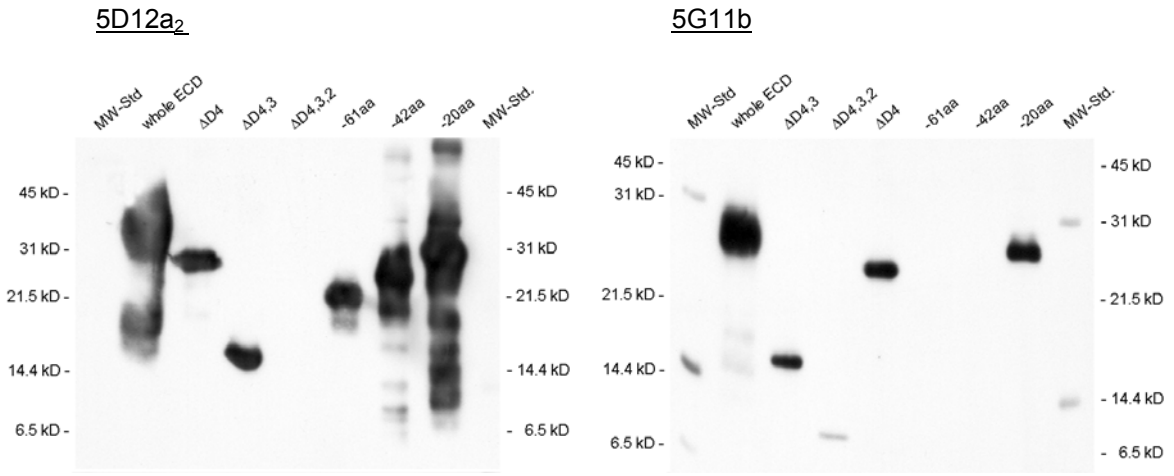


Figure 19:
Western blots of all truncated ECDs of mLTβR, detected with the mLTβR specific rat monoclonal antibodies 5D12a$_2$ (left) and 5G11b (right). The same amounts of proteins were loaded like on the silver stained gel.

5D12a$_2$ does not bind the ECD of mLTβR anymore if the second domain is deleted indicating that the epitope of 5D12a$_2$ is located in the second domain which is part of the ligand binding domain (Force *et al*., 1995).
As 5G11b cannot detect the protein if the first 39 amino acids are missing ("-42aa") but still binds if the first 20 amino acids are deleted, it is clear that the antibody binds the first 19 amino acids of the first cysteine rich region.

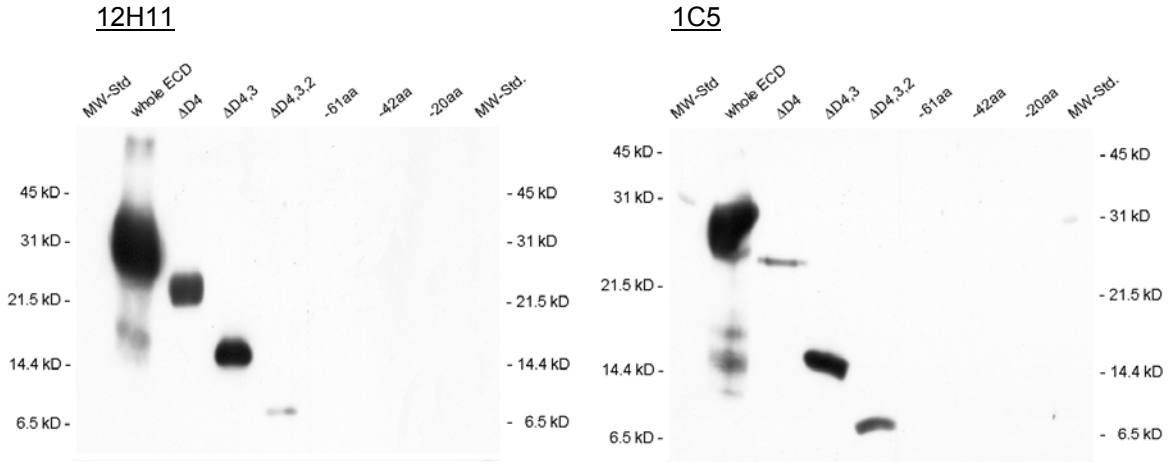


Figure 20:
Western blots of all truncated ECDs of mLTβR, detected with the monoclonal rat antibody 12H11 (left) and the cross-reacting monoclonal rat antibody 1C5 (right). The same amounts of proteins were loaded like on the silver stained gel.

The antibodies 12H11 and 1C5 had competed with each other and showed binding to the same epitope on the membrane. Both bind the first 20 amino acids of the ECD of mLTβR. They bind every construct which is deleted from the carboxy terminus down to the smallest one which represents only a part of the signal peptide (8aa), the “spacer” of 12 amino acids between the signal peptide and the cysteine rich regions and the first cysteine rich domain. Binding was lost when the first 20 amino acids from the amino terminus were deleted. This indicates that the epitope of 12H11 and 1C5 is localized upstream of the conserved cysteine rich regions. Therefore the possibility exists that the unknown protein might not be a member of the TNFR superfamily.

Eight amino acids of the smallest construct to which 1C5 binds, ΔD4,3,2, belong to the signal peptide of the mLTβR. The mature mLTβR no longer has the signal peptide but can be detected with 1C5 in flow cytometric analysis. Thus the antibody 1C5 must bind to the “spacer” of 12 amino acids.

In addition 12H11 and 1C5 bind the same epitope of the mLTβR whereas only 1C5 cross-reacts with an antigen on the surface of thymocytes; 12H11 has lost its cross-reactivity with the unknown protein on thymocytes. This might be due to an initially lower affinity of 12H11 which decreased further down with the storage of the antibody. Testing the affinity of both antibodies with titration curves will help to find an explanation for this phenomenon.

Taken together the epitope mapping supported the findings of the competition assays. There are three different epitopes recognized by the four monoclonal antibodies and only 12H11 and 1C5 detect the same epitope of the mLTβR and compete with each other.

In order to further narrow down the epitope its 12 amino acids were compared to the human LTβR. There is a homology of 60% between human and mouse LTβR for the whole protein and of 75% regarding these 12 amino acids.

Q L VPPY RI ENQT mLTβR
Q A VPPY AS ENQT hLTβR

To check whether 1C5 also binds the hLTβR a western blot with the S2 expressed hLTβR-Ig fusion protein was performed.

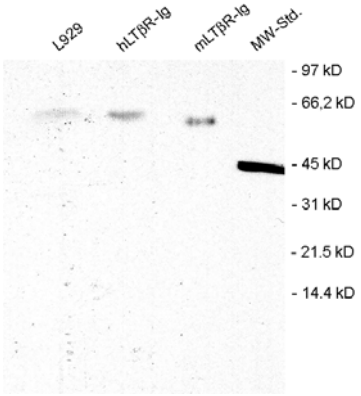


Figure 21:
Western blot of mouse and human LTβR-Ig fusion protein and mLTβR, prepared from L929 cells, detected with the antibody 1C5.
(The “band” in the lane with the standard is not a protein band but a scratch on the membrane).

Figure 21 shows that 1C5 can bind the hLTβR-hIg fusion protein as well as the mLTβR. The mLTβR was expressed in S2 cells as a hIg fusion protein or prepared from the membrane fraction of L929 cells, a mouse fibroblast cell line. Ware (1995) observed a mass of 61kd for the human LTβR. The mature human LTβR protein has a calculated mass of 44kD for its 405aa. The difference between the calculated and the observed molecular weight (MW) of the human LTβR might be due to glycosylation at the two N-glycosylation sites in the ECD. The calculated mass of the mature mouse LTβR protein is 42kD for its 385aa. As the mouse LTβR also has two N-glycosylation sites its true MW is ~ 60kD like it was seen in the western blot.
It needs to be mentioned here that residues of proteinG were found in the proteinG-purified antibody 1C5. Binding of proteinG to the Ig-tail of the fusion proteins of mLTβR and hLTβR needs to be excluded. Therefore tests with the untagged ECD of the hLTβR need to be performed in order to prove the binding of 1C5 to the hLTβR.

With this epitope homology searches with the NCBI protein BLAST (http:/www.ncbi.nih.gov/BLAST/)(blastp for short, nearly exact matches) were carried out. Nearly 70 proteins (50% are unnamed, RIKEN or EST products) matched over a minimum of four amino acids. None of the known proteins was a member of the TNFR superfamily again pointing to a protein which does not

belong to this group of receptors. With rpsblast no conserved domain was detectable for these 12 amino acids although they are shared by so many different proteins. The next step will be to find out which of the matching proteins is expressed on the surface of thymocytes.

Alignments with swissprotplus on Bioccellerator applying Smith-Waterman algorithm (performed by Dr. A. Hotz-Wagenblatt, HUSAR Bioinformatics Lab, German Cancer Research Center) revealed numerous prokaryotic proteins, human and mouse LTβR and chicken TGFβ3 to be homologous to the found 1C5-epitope. Chicken and mouse TGFβ3 have a 84% identity. Therefore the epitope was compared to mTGFβ3 and showed a 88% similarity and a 33% identity. Beside the other 70 matching proteins now TGFβ3 is the main candidate to be the protein with which 1C5 cross-reacts.

5.4. Differentiation of thymocytes and splenocytes expressing the unknown protein

5.4.1. Thymocytes

Four colour flow cytometric analysis was performed in order to find out if the unknown protein with which the antibody 1C5 cross-reacts is expressed on all thymocytes or only on a certain subpopulation depending on the stage of maturation/development. Defining a maturation-dependent expression would make this unknown protein again very interesting and could involve its biological function, such as a role in apoptosis, in the thymocyte development where apoptosis is a major event.

The data are representative of four different experiments.

Thymocytes from C57BL/6 mice and from mice deficient in the LTβR, excluding all possible binding of 1C5 to the LTβR on thymic stromal cells, were stained with 1C5, CD4 and CD8a for a first survey. Thymocytes of LTβR$^{-/-}$ mice exhibited a small difference in their distribution of CD4 SP and CD8 SP cells compared to wt mice. Recent thymic emigrants (RTE), the most mature single-positive thymocytes (SP), accumulate in the thymi of LTβR$^{-/-}$ mice and their numbers double (Boehm et al. 2003).

For flow cytometric analysis the thymocyte population was determined in region 1 (R1) according to the size (FSC) and the granularity (SSC) of the thymocytes. That way 77% of all cells in the wt thymus and 79.5% of all cells in the LTβR$^{-/-}$ thymus were chosen.

C57BL/6 LTβR$^{-/-}$

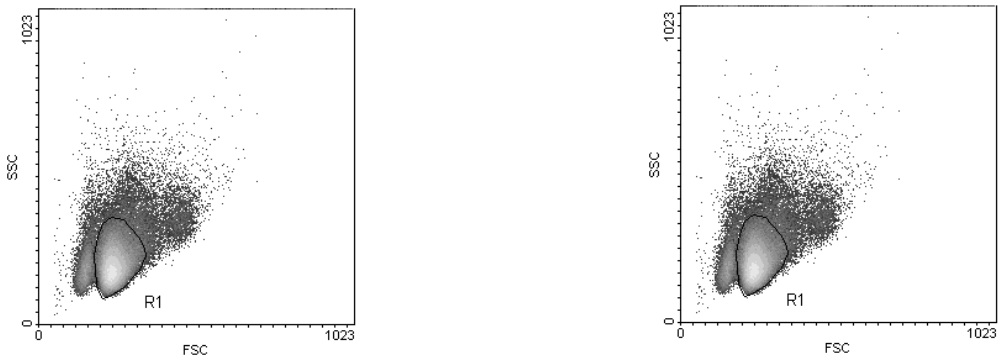


Figure 22:
Density plots of isolated thymocytes from wt and LTβR$^{-/-}$ mice for the determination of region 1 (R1) , including all single thymocytes, excluding all other cell types and doublettes.

The antibody 1C5 bound to 85% of thymocytes of LTβR-deficient mice and 90% of thymocytes from wt mice.

C57BL/ 6 LTβR$^{-/-}$

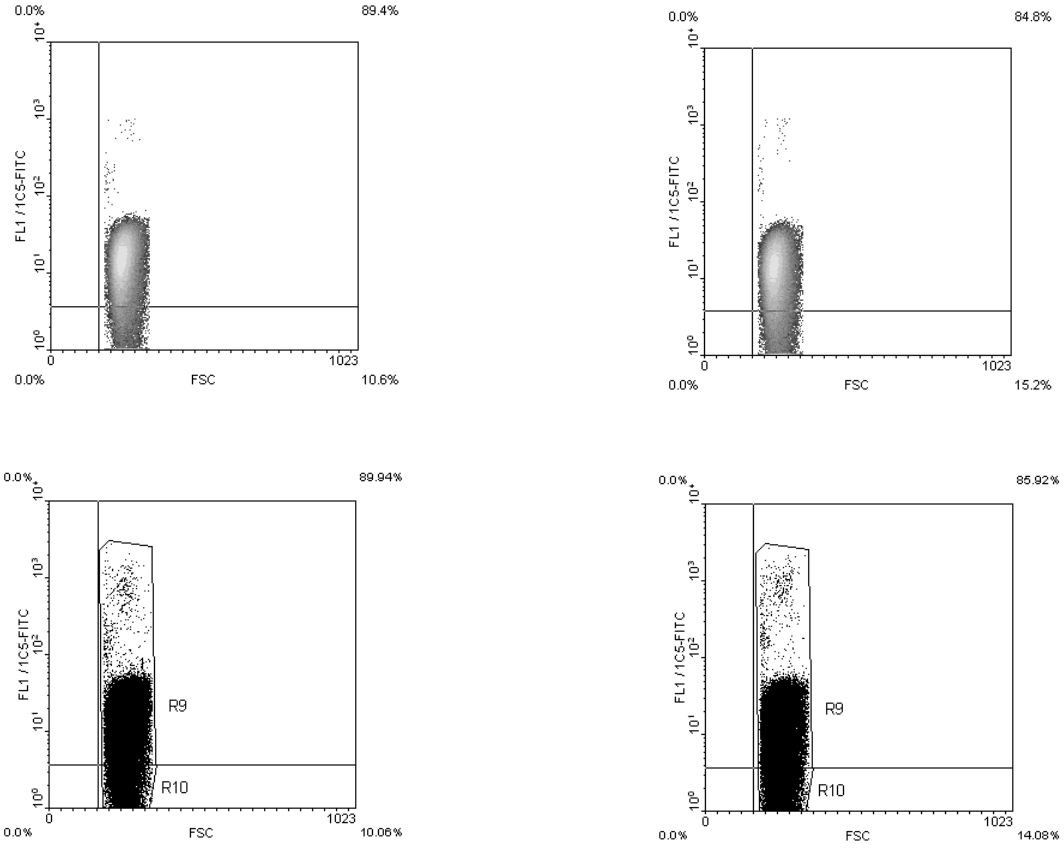
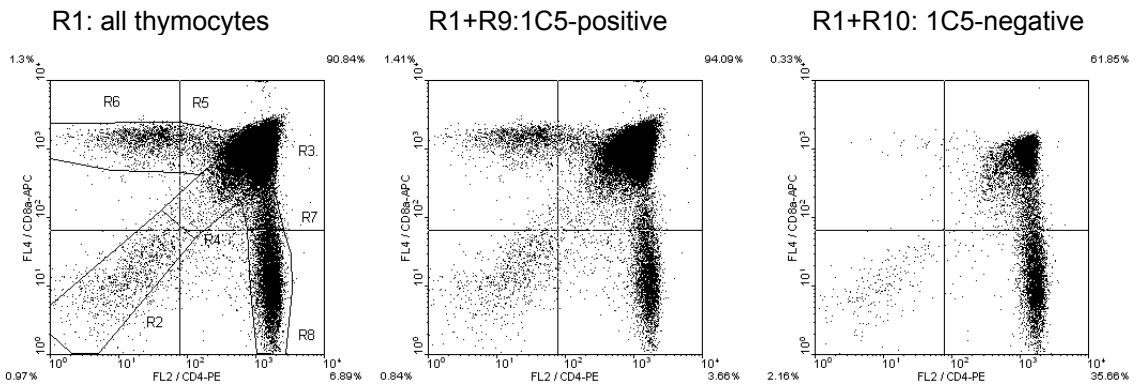


Figure 23:
Density plots and dot plots of thymocytes from wt and LTβR$^{-/-}$ mice, gated for region 1 (R1), showing the staining with the antibody 1C5 and the determination of the 1C5-positive (R9) and

1C5-negative (R10) populations. 1C5-positivity was determined with regard to the single staining pattern, the autofluorescence and the isotype controls. Dot plots were chosen for the following figures because they show every single event which is necessary for some very small subpopulations of the thymocytes.

To obtain the first overview regions were set for 1C5-positive and 1C5-negative thymocytes looking for their expression of CD4 and CD8a. Figure 24 shows that there might be a difference in the maturation of CD4 single-positive and CD8 single-positive thymocytes in regard to their expression of the unknown protein. There were nearly no CD8 single-positive thymocytes which did not express the unknown protein whereas the amount of 1C5-positive and 1C5-negative CD4 single-positive thymocytes seemed to be same.

C57BL/ 6



LTβR$^{-/-}$

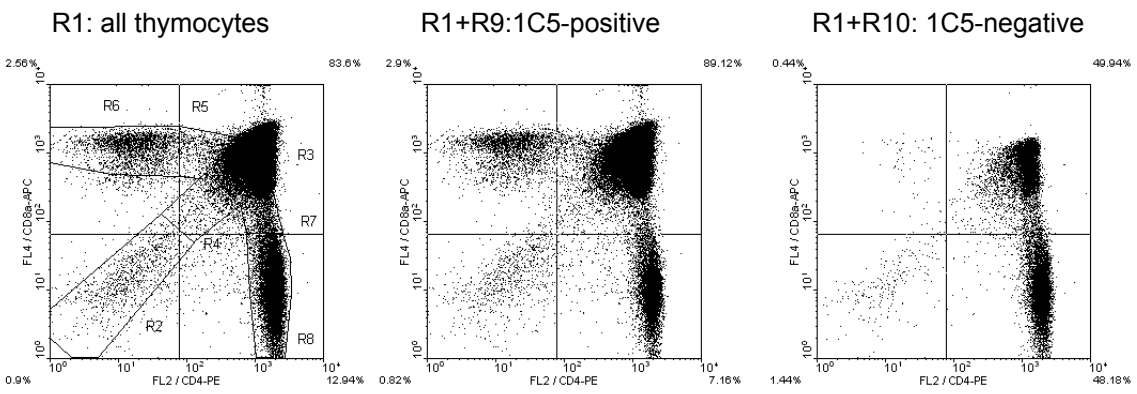


Figure 24:

Dot plots of thymocytes from wt and LTβR$^{-/-}$ mice, gated for region 1 (R1), showing the CD4/CD8 expression determining the different thymocyte subpopulations (R2-R8). Application of region 9 (R9), representing the 1C5-positive cells, and region 10 (R10), representing the 1C5-negative cells shows a difference in the CD4/CD8 expression between 1C5-positive and 1C5-negative thymocytes for both mouse strains.

The maturation of thymocytes can be followed by the observation of the expression of CD4 and CD8. Sant'Angelo *et al* (1998) suggested a model to better understand the thymocyte maturation. According to this model regions were chosen to further investigate the expression of the unknown protein during the thymocyte maturation.

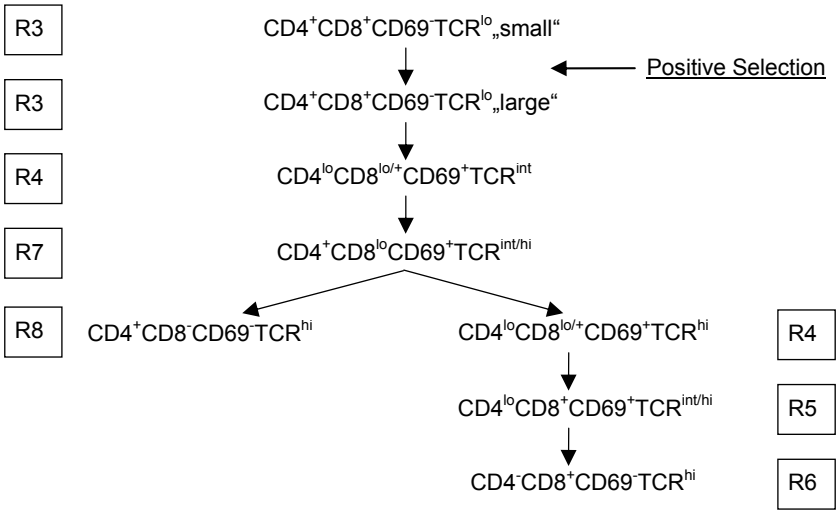


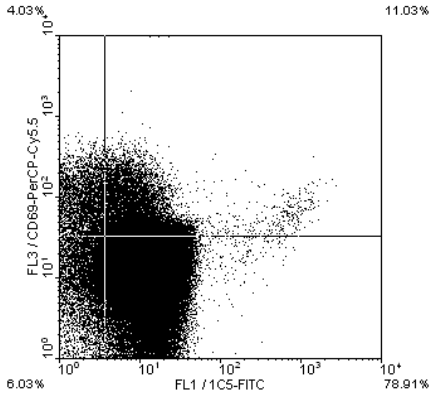
Figure 25:
Model of thymocyte maturation according to Sant'Angelo *et al*, 1998.
Regions R3-R8 were set in figure 24 for further analysis.

During thymocyte maturation CD69, activation inducer molecule (AIM), a C-type lectin, is upregulated after positive selection in the double-positive stage (DP) and downregulated right before the single-positive cells leave the thymus as RTE. Therefore CD69/AIM was used as an additional differentiation marker for thymocyte maturation. The expression of the unknown protein was compared with this marker to investigate whether the unknown protein might be regulated during thymocyte maturation.

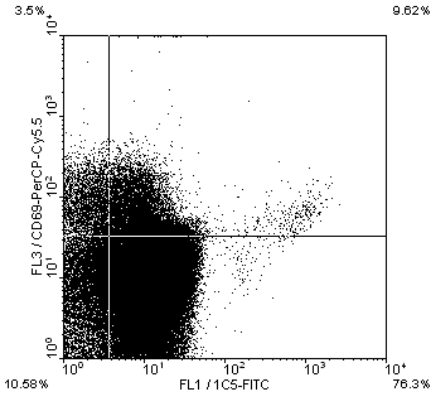
Figure 26: (next two pages)
Expression of the unknown protein, stained with 1C5 (x-axis) in comparison to CD69 (y-axis) in the different stages (R2-R8) of thymocyte maturation of wt and LTβR$^{-/-}$ mice.
The regions were set in figures 22 and 24 .

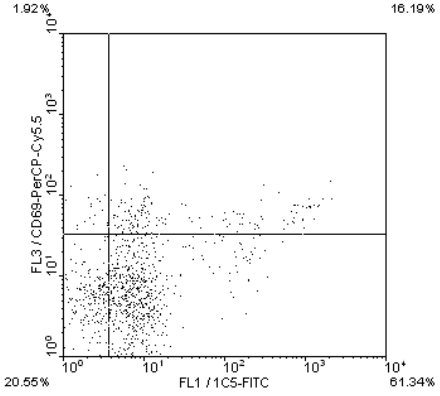
C57BL/6

LTβR$^{-/-}$

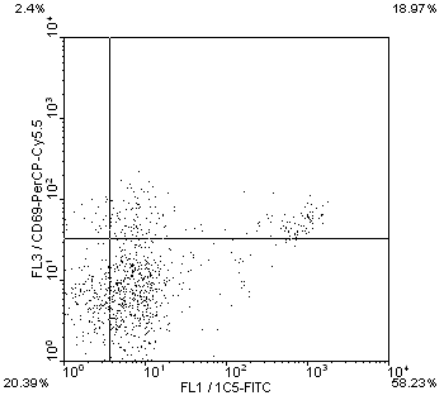


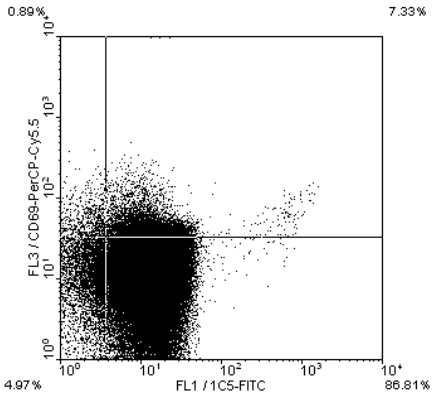
R1



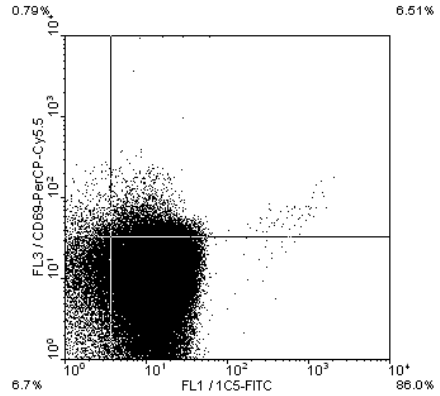


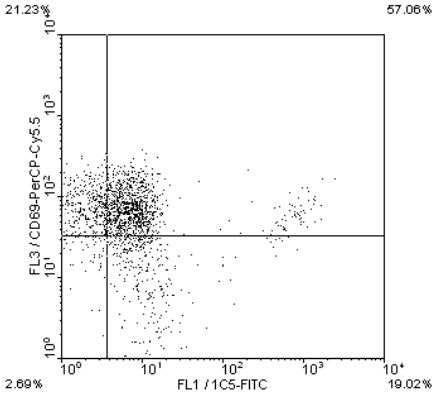
R1+R2



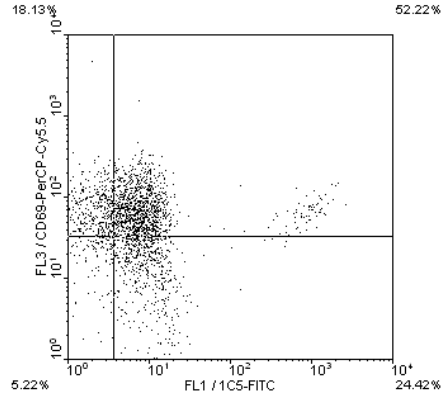


R1+R3



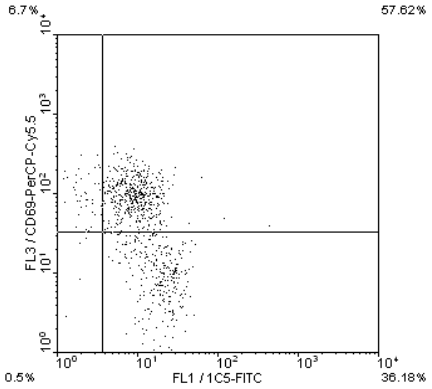


R1+R4

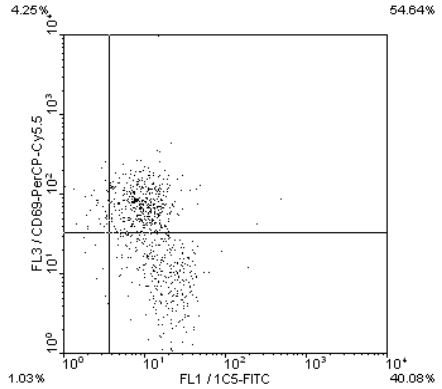


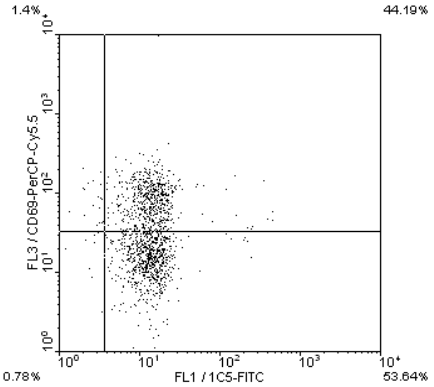
C57BL/ 6

LTβR$^{-/-}$

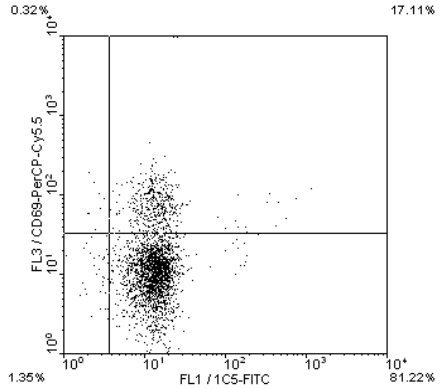


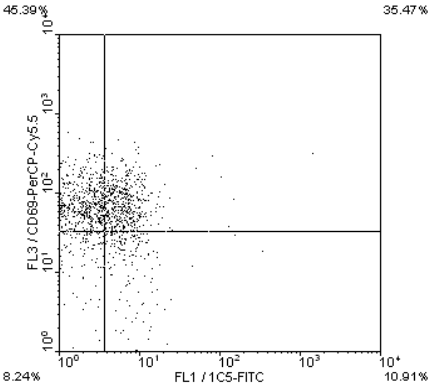
R1+R5



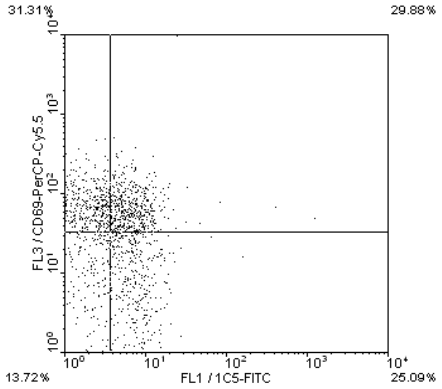


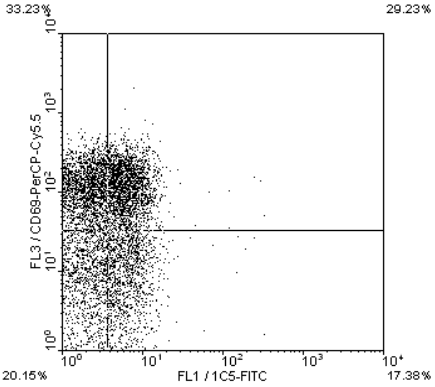
R1+R6



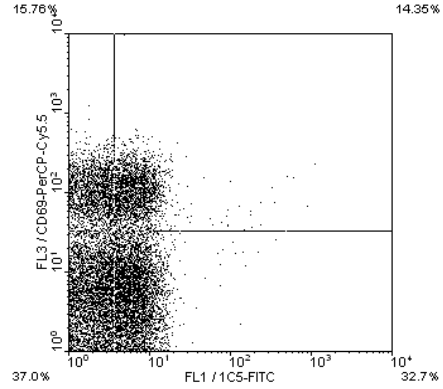


R1+R7



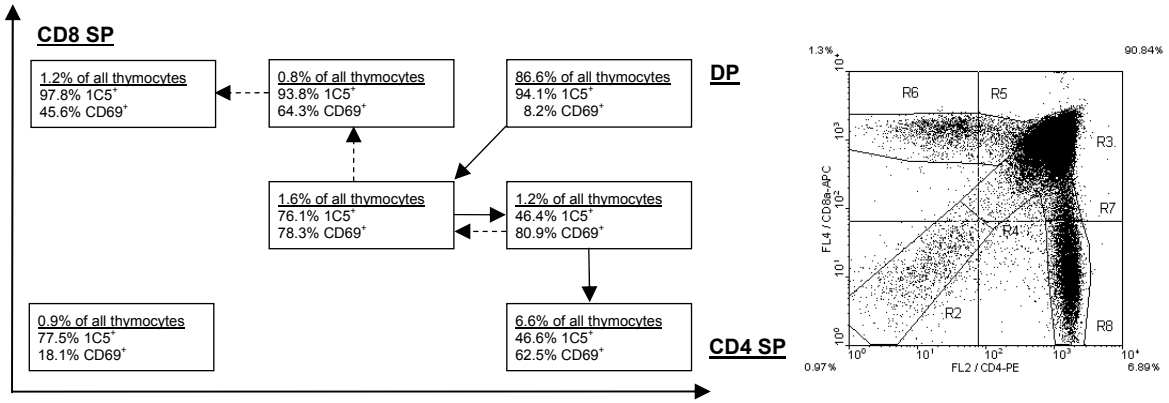


R1+R8



For a better understanding all the information of the flow cytometric analyses above are delineated in the following two diagrams.

C57BL/6



LTβR$^{-/-}$

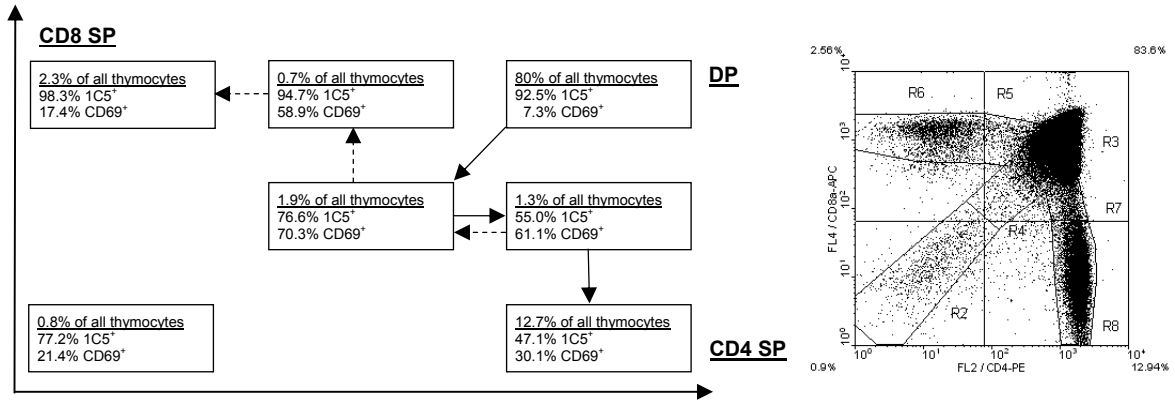


Figure 27:
Summary of the analyses shown in figure 26 with the use of Sant'Angelo's model.

In accordance with Sant'Angelo's model CD69 was upregulated in wt mice after the double-positive stage (from 8% to 78%) and downregulated in the most mature single-positive thymocytes (63% for CD4 and 46% for CD8). In LTβR$^{-/-}$ mice a lower percentage of CD69-positive cells was found in the single-positive stage (30% for CD4 and 17% for CD8). This might be explained by the accumulation of CD69-negative RTEs in the LTβR$^{-/-}$ mice.

Compared to the CD69 expression, the expression of the unknown protein also seemed to be regulated, but in a different way. The expression is independent of

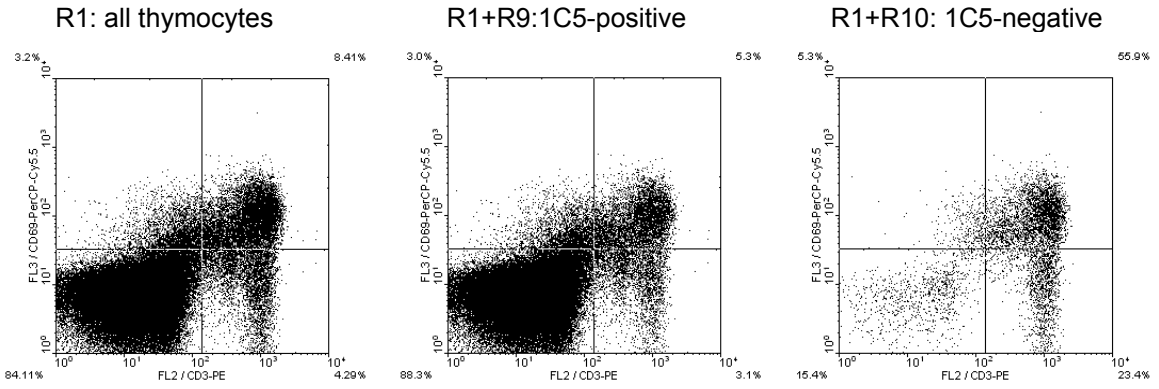
the LTβR since the percentage of 1C5-positive cells in the different thymocyte subpopulations did not differ between wt and LTβR$^{-/-}$ mice.

During the double-positive stage (R3) nearly all thymocytes (94%) were 1C5-positive. With the next step, where cells become “double dull” (CD4^{lo}CD8^{lo}) (R4), the unknown protein was downregulated to 76% while the expression of CD69 increased. In the following CD4^{hi}CD8^{lo} stage (R7) the percentage of 1C5-positive cells dropped and the number of CD69-positive cells remained nearly stable. Cells becoming mature CD4 single-positive thymocytes (R8) decreased their CD69 expression and 47% of them maintained the expression of the unknown protein.

The pathways to develop into either CD4 single-positive or CD8 single-positive thymocytes bifurcate in the CD4^{hi}CD8^{lo} stage (R7). The CD4 single-positive thymocytes need only one last step before leaving the thymus whereas the CD8 single-positive thymocytes have to become “double dull” (R4) again before finally abrogating their CD4 expression. From “double dull” to CD4^{lo}CD8^{hi} (R5) the thymocytes decreased their CD69 expression and strongly increased the expression of the unknown protein up to 94%. Before leaving the thymus as CD8 single-positive T cells (R6) the expression of the unknown protein was further increased to 98% while CD69 expression was further lowered.

Additional analysis with 1C5, CD3, and CD69 supported these findings. Only 10% of the wt thymocytes and 14% of the LTβR$^{-/-}$ thymocytes were 1C5-negative. Approximately 80% of these cells are CD3^{hi}CD69$^{+/-}$ defining these cells as the most mature thymocytes. These mature thymocytes probably constitute the CD4 single-positive population because 53% of CD4 single-positive thymocytes were 1C5-negative whereas only 2% of the CD8 single-positive thymocytes were 1C5-negative.

C57BL/ 6



LTβR$^{-/-}$

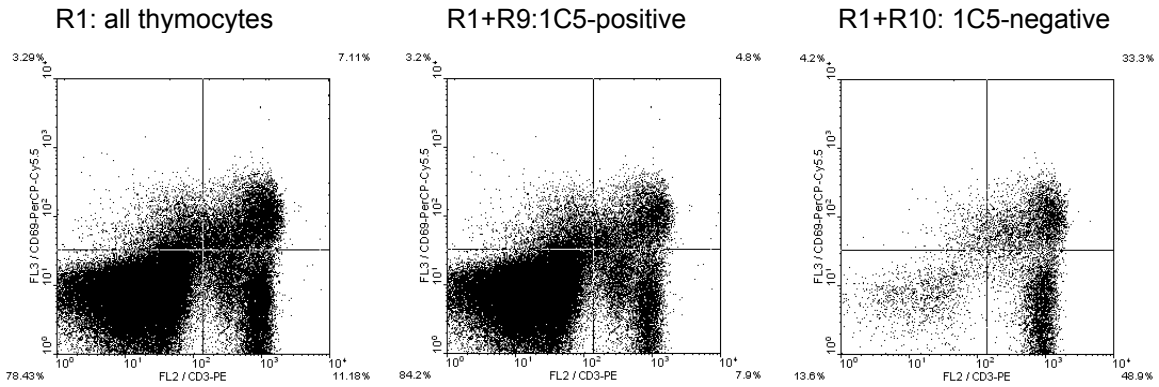


Figure 28:

Dot plots of thymocytes from wt and LTβR$^{-/-}$ mice, gated for region 1 (R1), showing the CD3/CD69 expression of all thymocytes. Application of region 9 (R9), representing the 1C5-positive cells, and region 10 (R10), representing the 1C5-negative cells, shows a difference in the CD3/CD69 expression between 1C5-positive and 1C5-negative thymocytes for both mouse strains.

In order to detect the very early thymocytes, the CD4^{-}CD8^{-} double-negative cells (DN) which are part of region 2 (R2), staining of the thymocytes with anti-CD117 was performed. Less than 0.2 % of all thymocytes were CD117-positive cells. With these few cells no clear result could be obtained concerning the expression of the unknown protein in the very early thymocyte population.

The results of the flow cytometric analyses with 1C5, CD3, CD4, CD8a, and CD69 hinted at an association of the unknown protein to the T cell development and maybe also to the CD4/CD8 lineage commitment.

5.4.2. Splenocytes

As there was a difference observed between the percentage of 1C5-positive cells in the CD4 single-positive and the CD8 single-positive thymocytes it became important to know not only whether mature peripheral lymphocytes express the unknown protein but also whether there is a difference between CD4-positive and CD8-positive T cells. Therefore similar analyses were again performed with anti-CD3, anti-CD4, anti-CD8a, anti-CD45R/B220, anti-CD69, and 1C5, this time on splenocytes of C57BL/6 mice and of LTβR$^{-/-}$ mice. The following data represent the results of four different experiments.

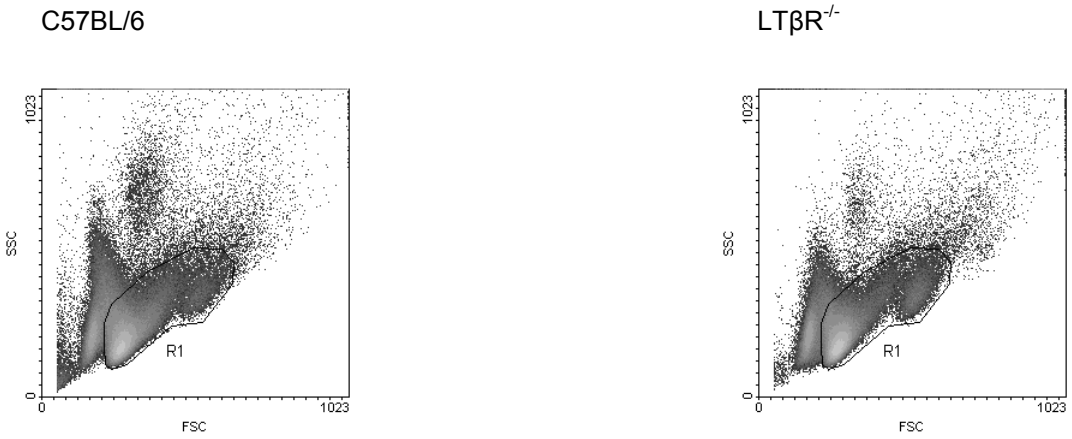


Figure 29:
Density plots of isolated splenocytes from wt and LTβR$^{-/-}$ mice for the determination of region 1 (R1), including all lymphocytes.

Region 1 (R1) was chosen to represent all lymphocytes. 63% and 68% of all cells were lymphocytes in wt and in LTβR$^{-/-}$ mice respectively.

The percentage of 1C5-positive cells in the spleen was much lower than in the thymus and nearly the same for LTβR$^{-/-}$ mice and wt mice with 5.3% and 4.7% respectively, shown in figure 30.

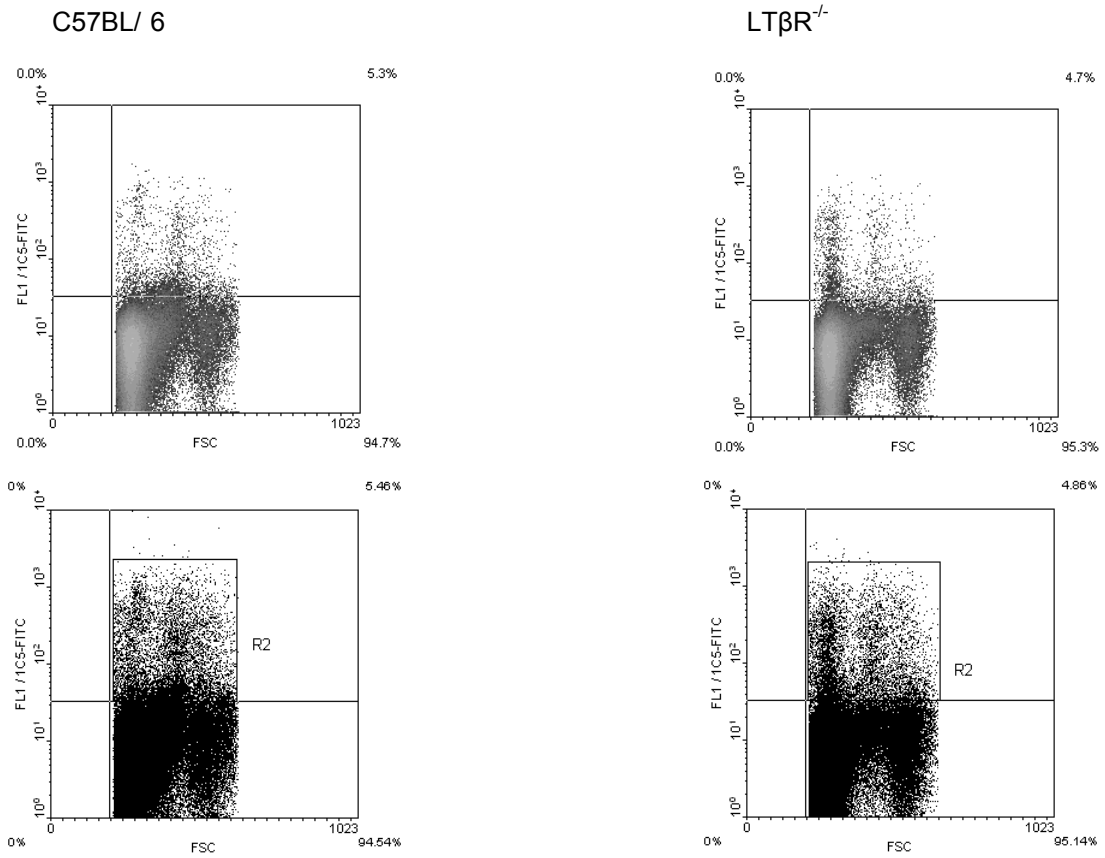


Figure 30:

Density plots and dot plots of isolated splenocytes from wt and LTβR$^{-/-}$ mice for the determination of region 2 (R2) representing 1C5-positive cells.

In the T cell population the percentage of 1C5-positive cells was nearly the same in wt and LTβR$^{-/-}$ mice with 1.4% 1C5^{+} T cells in wt and 1.3% 1C5^{+} T cells in the LTβR$^{-/-}$ mice. Regarding the CD4^{+} and the CD8^{+} subpopulations again no difference was found between both mouse strains and, in contrast to the thymus, between the subpopulations themselves either.

These results are plotted in figure 31 for the LTβR$^{-/-}$ mice and summarized in table 1. Only the plots of the LTβR$^{-/-}$ splenocytes are shown as an example because of the similar results of the wt mice.

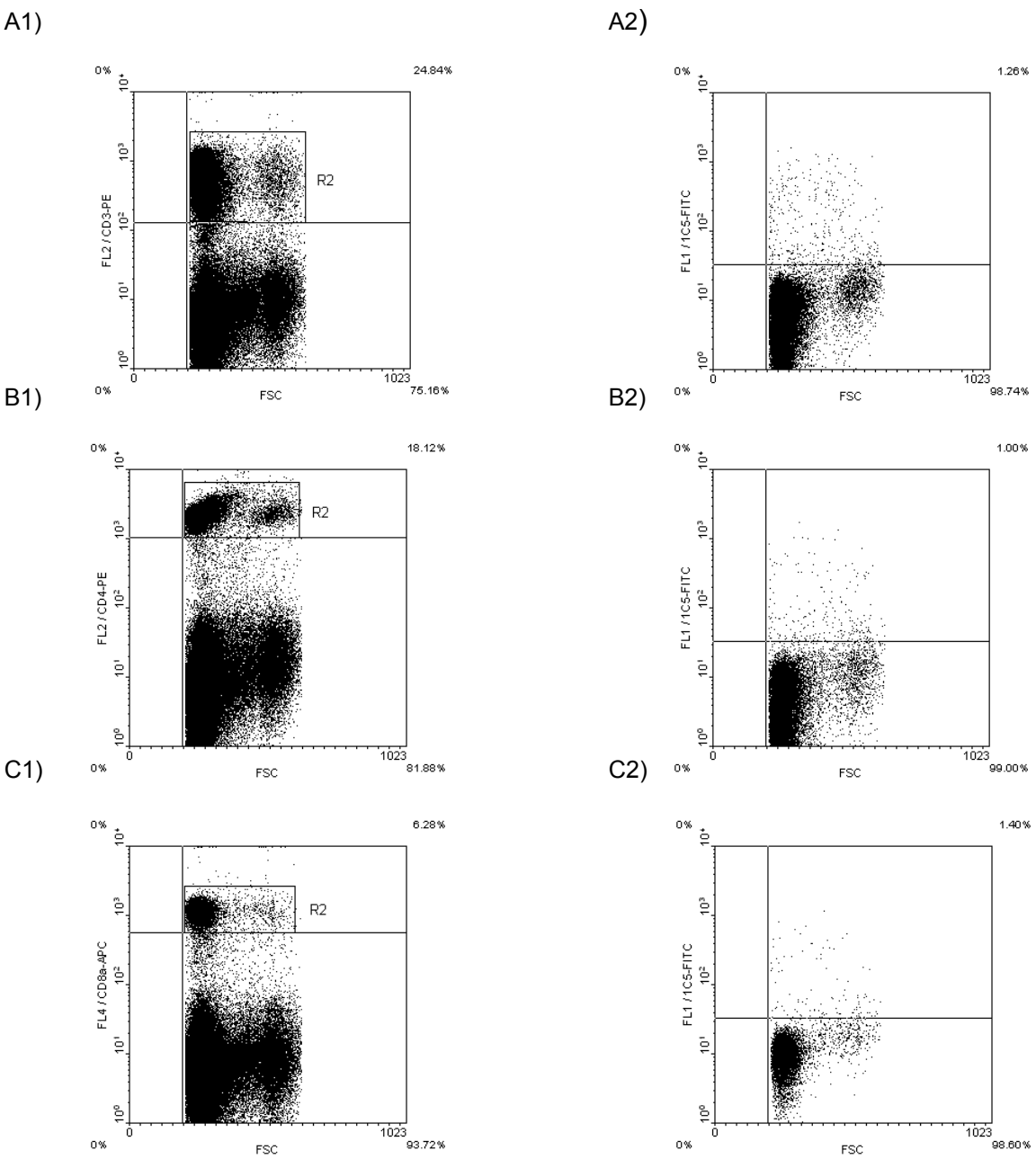


Figure 31:

Dot plots of isolated splenocytes from LTβR$^{-/-}$ mice , gated for region 1 (R1), stained with anti-CD3 (A1), anti-CD4 (B1) or anti-CD8a (C1). Region 2 (R2) was set for the positive cells of each staining which were further analysed for their expression of the unknown protein (A2, B2, C2).

In the thymus less than 0.2% of all lymphocytes were CD45R/B220-positive B-cells. It was impossible to determine the expression of the unknown protein in these few thymic B cells. However there was a clear subpopulation of B cells in the spleen also expressing the unknown protein (6.4% in wt, 5.7% in LTβR$^{-/-}$ mice).

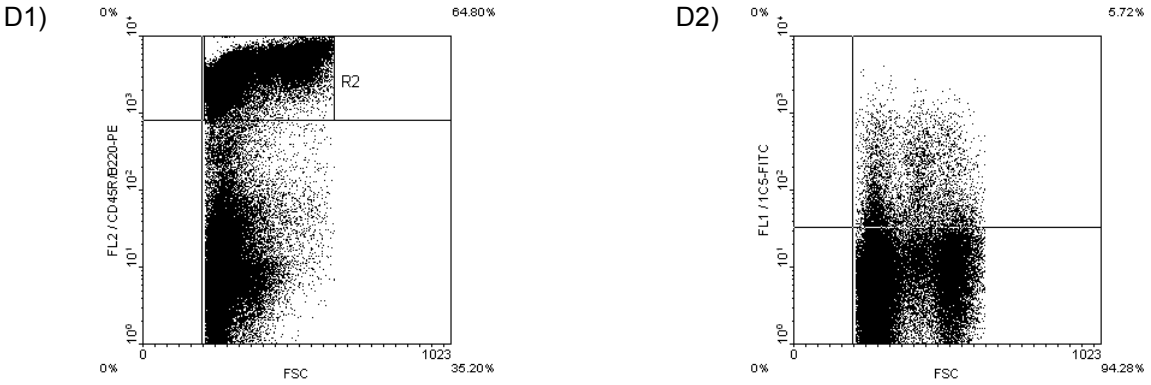


Figure 32:

Dot plots of isolated splenocytes from LTβR$^{-/-}$ mice, gated for region 1 (R1), stained with anti-CD45R/B220 (D1). Region 2 (R2) was set for the CD45R/B220-positive B cells, which were further analysed for their expression of the unknown protein (D2).

	Wt % of 1C5$^+$ cells	LTβR$^{-/-}$ % of 1C5$^+$ cells
CD4	0.81	1.0
CD8	1.43	1.4
CD3	1.43	1.26
CD45R/B220	6.37	5.72

Table 1:

Percentage of 1C5-positive cells in the different subpopulations of mature T and B cells of both mouse strains.

These results identified B cells as the major mature lymphocytes bearing the unknown protein in the spleen. The difference between CD4 SP and CD8 SP thymocytes did not continue in the mature T cells.

CD69 is a marker not only for developing thymocytes but also for activated peripheral leukocytes. For example, the CD69 expression on T cells increases immediately after PMA treatment reaching a maximum level after 12 hours. After 24 hours the expression has declined partially and persists for at least 48 hours. CD69-mRNA becomes detectable after 30-60 minutes, reaches its maximum level by 6 hours, and then vanishes (López-Cabrera *et al.*,1993).

Nearly 7% of all wt lymphocytes and 8% of all LTβR$^{-/-}$ lymphocytes were CD69-positive. In wt mice 32% of all CD69-positive cells were 1C5-positive whereas in LTβR$^{-/-}$ mice 25% of the activated lymphocytes expressed the unknown protein.

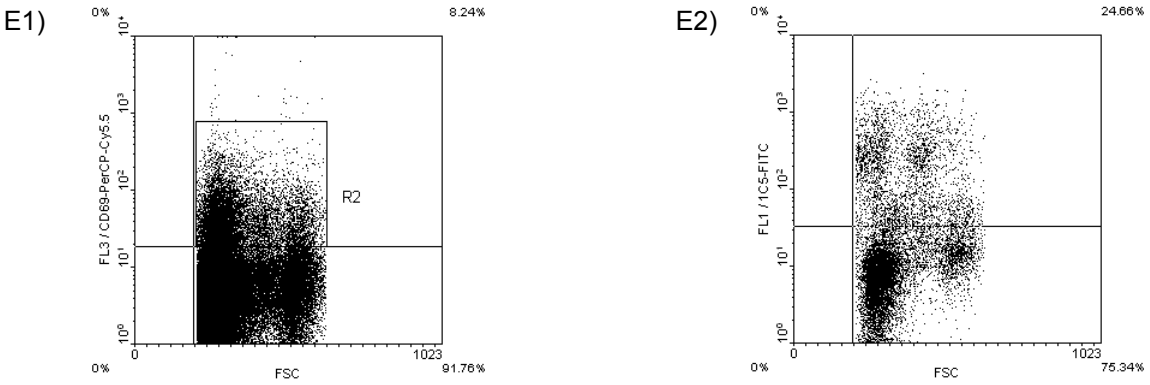


Figure 33:

Dot plots of isolated splenocytes from LTβR$^{-/-}$ mice, gated for region 1 (R1), stained with anti-CD69 (E1). Region 2 (R2) was set for the CD69-positive lymphocytes which were further analysed for their expression of the unknown protein (E2).

These results indicated a possible role for the unknown protein in the activation of lymphocytes. Therefore the composition of the activated lymphocytes was further investigated.

In wt mice 44% of the activated cells were T cells and 45% were B cells. LTβR$^{-/-}$ mice showed a different distribution with 36% T cells and 49% B cells (figures 34 and 38).

Activated T cells were further analysed with the cross-reacting antibody 1C5. The percentage of 1C5-positive cells in this subpopulation of T cells (7.7% and 5.6% in wt and LTβR$^{-/-}$ mice respectively) is much higher than the overall percentage of 1C5-positive cells in the whole T cell population (1.4%, 1.3%).

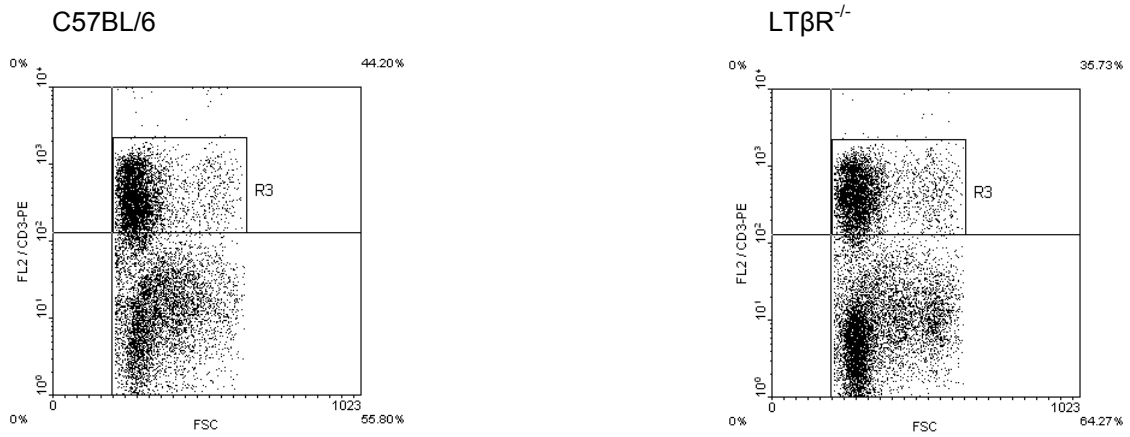


Figure 34:

Splenocytes of wt and LTβR$^{-/-}$ mice, gated for R1 (figure 30) representing all lymphocytes, and gated for R2 (figure 33), representing all activated CD69^{+} lymphocytes, showing the percentage of CD3^{+} T cells on CD69^{+} lymphocytes. Region 3 (R3) represents all activated T cells which were further analysed for their expression of the unknown protein, shown in figure 35:

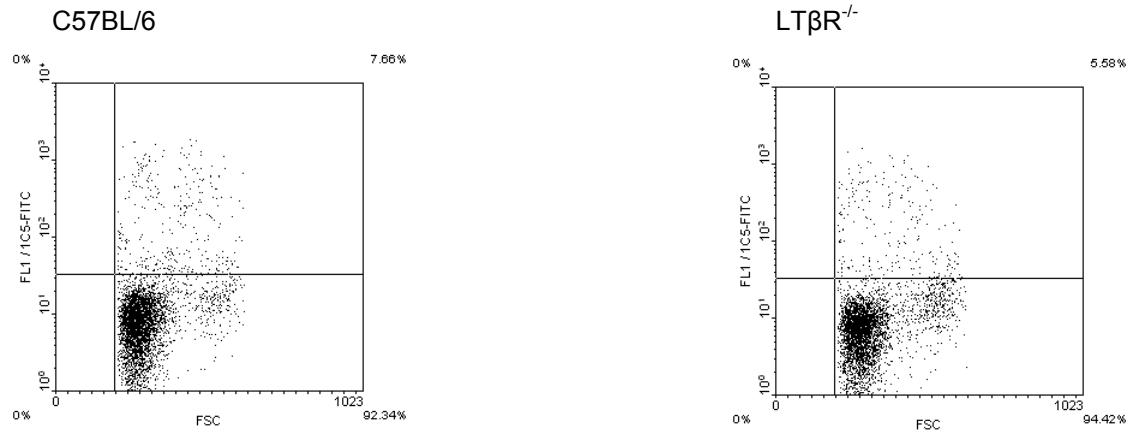


Figure 35:

Splenocytes of wt and LTβR$^{-/-}$ mice, gated for R1, R2 and R3 representing all CD69^{+} CD3^{+} T cells showing the percentage of 1C5^{+} cells in the activated T cell population.

Therefore it was interesting to see whether there was a difference between activated CD4 T cells and CD8 T cells. In wt mice 34.5% of all activated lymphocytes were CD4 T cells and 6.7% were CD8 T cells. Again the composition was different in LTβR$^{-/-}$ mice with 26% CD4 and 4.8% CD8 T cells. The percentage of 1C5 positive activated CD4 T cells was nearly the same in wt and LTβR$^{-/-}$ mice with 3.1% and 3.8% respectively. Interestingly the percentage of 1C5 positive CD8 T cells was much higher with 8.6% and 11.2%, also showing a difference between wt and LTβR$^{-/-}$ mice (summarized in table 2).

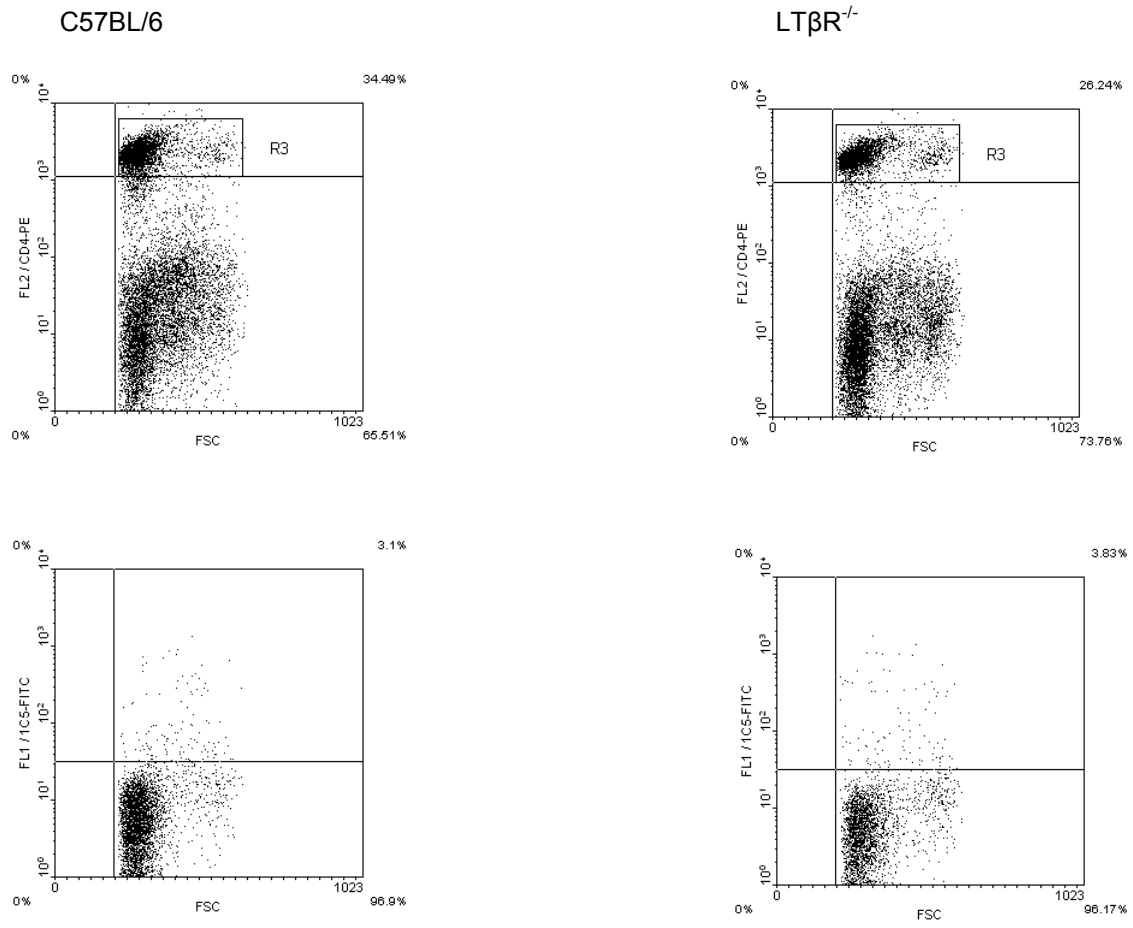


Figure 36:
Dot plots of activated lymphocytes, gated for R1 and R2, representing all CD69^{+} lymphocytes in the spleen of wt and LTβR$^{-/-}$ mice. R3 represents all activated CD4 T cells (upper panel) which were further analysed for their expression of the unknown protein (lower panel)

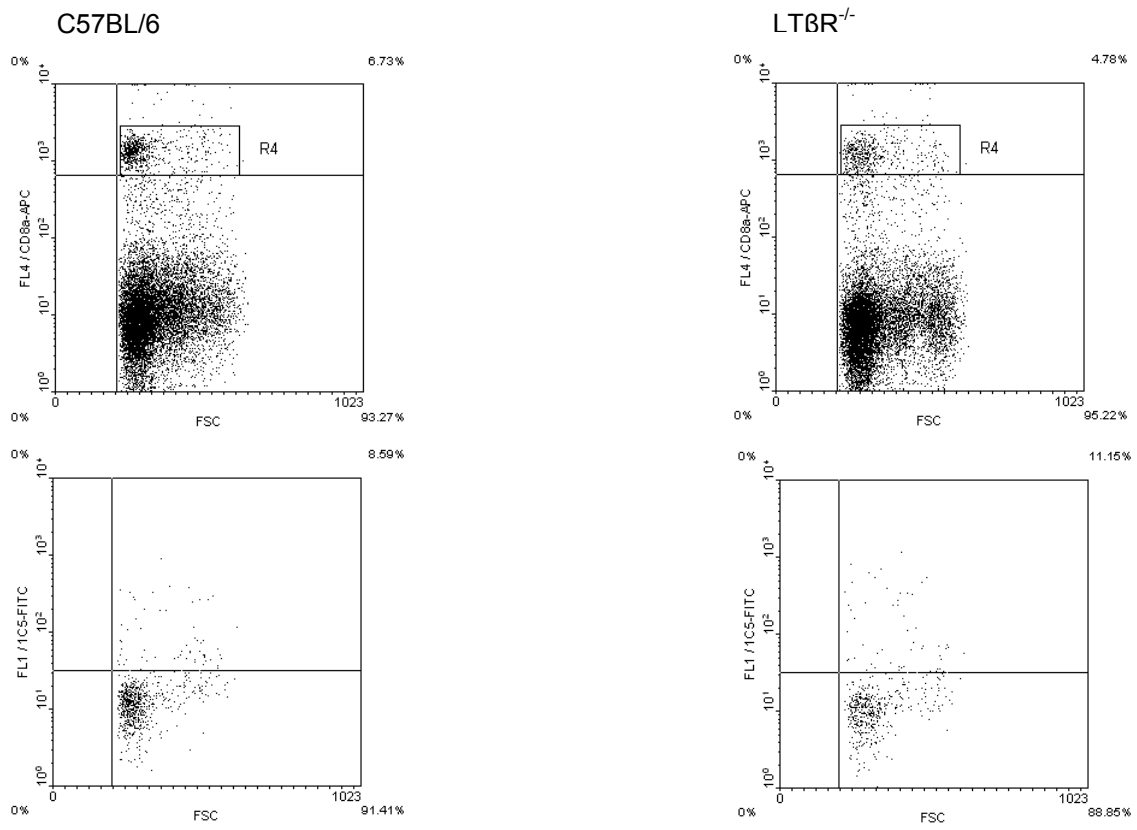


Figure 37:

Dot plots of activated lymphocytes, gated for R1 and R2, representing all $CD69^+$ lymphocytes in the spleen of wt and $LT\beta R^{-/-}$ mice. R4 represents all activated CD8 T cells (upper panel) which were further analysed for their expression of the unknown protein (lower panel)

	wt	$LT\beta R^{-/-}$
$CD69^+$	7%	8%
$\%1C5^+/CD69^+$	32%	25%
$\%CD3^+/CD69^+$	44%	36%
$\%1C5^+/CD3^+CD69+$	7.7%	5.6%
$\%CD4^+/CD69^+$	34.5%	26.2%
$\%1C5^+/CD4^+CD69^+$	3.1%	3.8%
$\%CD8^+/CD69^+$	6.7%	4.8%
$\%1C5^+/CD8^+CD69^+$	8.6%	11.2%

Table 2:

Summary of 1C5-positivity on activated T cells and its subpopulations.

In the thymus a difference was determined between CD4 SP and CD8 SP concerning the percentage of 1C5 positive cells. This difference was not found on mature T cells but appears again on activated T cells.

The CD45R/B220^{+} B cells portion of the CD69^{+} cells was also analysed for its 1C5 positivity.

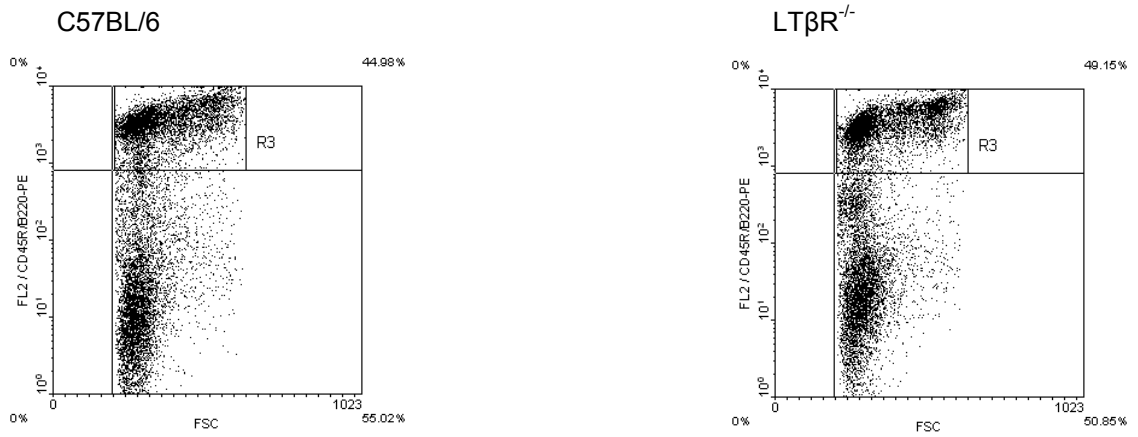


Figure 38:
Splenocytes of wt and LTβR$^{-/-}$ mice, gated for R1 representing all lymphocytes, and gated for R2, representing all activated CD69^{+} lymphocytes, showing the percentage of CD45R/B220^{+} B cells on CD69^{+} lymphocytes. Region 3 (R3) represents all activated B cells which will be further analysed for their expression of the unknown protein, shown in figure 39:

Nearly 35% of all activated CD69^{+}CD45R/B220^{+} cells in the wt mice and 23% in the LTβR$^{-/-}$ mice demonstrated the expression of the unknown protein.

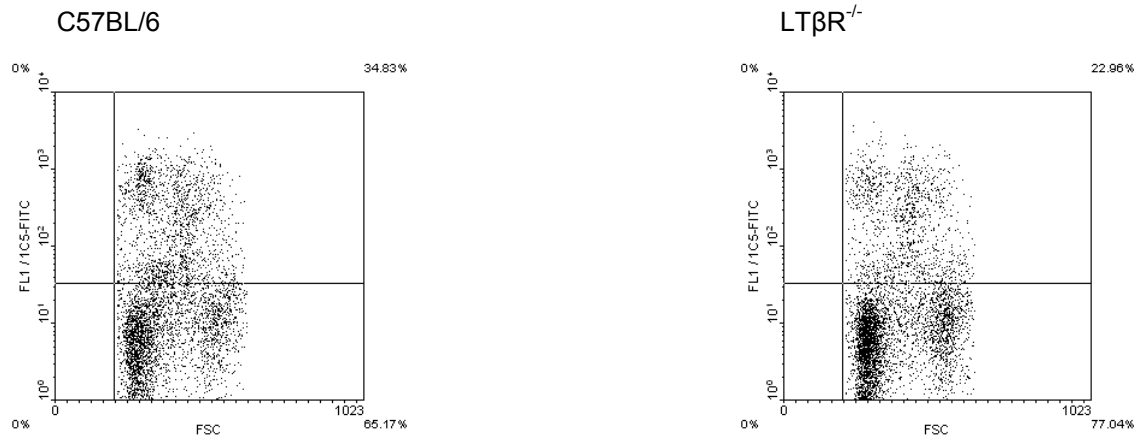


Figure 39:
Splenocytes of wt and LTβR$^{-/-}$ mice, gated for R1, R2 and R3 reprenting all CD69^{+} CD45R/B220^{+} B cells showing the percentage of 1C5^{+} cells in this population of activated B cells.

With these analyses it was clarified that the unknown protein is expressed on 35% of activated B cells in the spleen of wt mice and 23% in the spleen of LTβR$^{-/-}$ mice.

6. Discussion

The monoclonal rat anti mouse LTβR antibody 1C5 detects the mLTβR under native (flow cytometric analyses) and under denaturing conditions (western-blot). Binding to the unknown protein has only been shown for the native protein on thymocytes and activated mature B and T cells yet. It is not known whether 1C5 also detects the unknown protein in its denatured form. First attempts to isolate and blot the unknown protein from thymocytes failed due to technical problems.

The epitope of the monoclonal rat anti mouse LTβR antibody 1C5 was narrowed down to the first 12 aa of the mature protein under denaturing conditions.

Binding to the human LTβR which has a 75% homology to the mouse receptor in these 12 aa was found by western blot. Interestingly testing 1C5 on A375, a human melanoma cell line, by flow cytometric analysis under native conditions showed no binding (data not shown).

From these data it can be concluded that the epitope is necessary for binding but may not be sufficient for binding.

The secondary structure of the LTβR and / or the unknown protein might influence the binding of 1C5.

There is a slight possibility that the prokaryotically expressed proteins were not fully denatured in SDS-gels and western-blots. Binding of 1C5 to these blotted proteins might not only be due to the epitope but also to remaining secondary structures. To solve this problem binding of 1C5 to a synthetic peptide of these 12 aa should be investigated under denaturing conditions, i.e. western-blot, and under native conditions, i.e. immobilized to an ELISA-plate.

If 1C5 binds the synthetic peptide it will be clear that the epitope is sufficient for binding and homology searches may help to define the unknown protein.

Finding a conformation-dependent binding would lead to the necessity to do any experiment under native conditions. Homology searches would not lead any further.

There is a small possibility that the antigen to which 1C5 binds is not a protein at all. Observations were made of peptide-specific antibodies also cross-reacting with other structures like glycosylated proteins, DNA, or lipids. If this were the case for 1C5 it would decrease the chance to find the unknown protein dramatically.

Independent from these speculations homology searches applying NCBI protein BLAST were performed and revealed ~70 proteins which match the epitope with four or more amino acids. None of the members of the TNFR superfamily was found in this list of homologous proteins, which supports the finding that the epitope is located upstream of the conserved CRDs.
It is interesting that an antibody which binds to such a common epitope exhibits distinct staining patterns on subsets of thymocytes and splenocytes.
In order to come closer to the unknown protein the homologous proteins will be checked to determine whether they are expressed on the surface of thymocytes and activated CD8 T cells and B cells.

In 2003 Schmitz *et al*. performed gene expression analysis of thymocytes and found 244 differentially expressed DP thymocyte genes induced or repressed by TCR triggering *in vivo*. Checking whether one of these 244 proteins consists of the epitope might lead to the elucidation of the unknown protein which was found to be upregulated in the DP stage of thymocyte development.

With regard to the Smith-Waterman algorithm, TGFβ3 now seems to be the main candidate for the unknown protein. If TGFβ3 is a plausible candidate the easiest way to check this assumption will be to perform flow cytometric analysis of thymocytes of TGFβ3$^{-/-}$ mice like it was performed for the earlier excluded TNFR superfamily members. Since these mice were not available RT-PCRs were performed on thymocytes and on sorted 1C5^{+} and 1C5^{-} thymocytes to check first whether these cells express TGFβ3. Semiquantitative comparison to the expression of β-actin showed no significant difference between 1C5^{+} and 1C5^{-} thymocytes (data not shown). These analyses do not exclude TGFβ3 from the list of probable candidates although there was no difference between 1C5^{+} and 1C5^{-} thymocytes. There might be a slight difference on mRNA level which is only detectable in quantitative real-time PCR but which might lead to a very low expression on 1C5^{-} thymocytes which is under the detection level of flow cytometric analysis.
To elucidate this hypothesis quantitative real-time PCR on sorted thymocytes need to be performed.
In parallel it would be useful to transfect 1C5^{-} cells with a TGFβ3-expressing vector and look for 1C5-binding by flow cytometry.

Four colour flow cytometric analyses on thymocytes of wt and LTβR$^{-/-}$ mice led to two conclusions. First, the unknown protein is regulated during thymocyte development and second, this regulation is independent of the LTβR.
This independence was demonstrated since there was no difference in the expression of the unknown protein in the different thymocyte subpopulations between wt and LTβR$^{-/-}$ mice.

A difference was found between the development of CD4 SP and CD8 SP thymocytes. Whereas future CD4 SP thymocytes downregulate the unknown protein to 47% after positive selection, future CD8 SP cells upregulate it to 98% after leaving the shared pathway at the CD4^{hi}CD8^{lo} step. Up to now it is not clear which parameter determines the commitment and differentiation to CD4 SP or CD8 SP thymocytes. Signal strength or signal duration of the TCR-peptide-MHC complex, transcription factors, transcription repressors and members of the NOTCH family are thought to play a role in the lineage commitment but this process has not been clarified yet (Singer, 2002; Shanker *et al*., 2004). Therefore one can only speculate that the unknown protein is either an active contributor to the lineage commitment or that its different regulation in CD4 SP and CD8 SP thymocytes is the consequence of the lineage decision.

The observation that the expression of the unknown protein is upregulated between the DN (78%) and the DP (94%) stage leads to the questions whether this protein is also differentially expressed in the four different DN-stages and when the cells start to express the unknown protein. Answers to these questions would give a hint to the nature of this unknown protein. Therefore it will be interesting to investigate the DN cells by flow cytometry with 1C5 together with CD4, CD8, CD25, CD44. With these latter four antibodies the DN cells can be differentiated.

The finding that treatment of thymocytes with the cross-reacting antibody 1C5 induces apoptosis in these cells pointed to a role of the unknown protein in the selection processes during thymocyte development, i.e. death by neglect and negative selection. These selection processes occur at the DP stage and lead to the elimination of thymocytes by apoptosis. Mature T cells are also subjected to apoptosis by peripheral deletion or activation induced cell death (AICD) (Green *et*

al., 2003). Therefore it is interesting to investigate whether mature T cells, i.e. $1C5^+$ mature T cells, also die by apoptosis if they are treated with 1C5.

The expression of the unknown protein seems to be dramatically downregulated after the thymocytes have left the thymus as mature T cells. Only ~1% of all $CD3^+$ T cells or $CD4^+$ and $CD8^+$ subpopulations still express this protein. Whether this downregulation is really as dramatic as it appears now needs to be investigated in an additional experiment. Splenocytes of mice should be analysed pre- and postnatally at certain time intervals such as one week until the age of 6 weeks. $1C5^+$ T cells are expected to be found in the spleen of embryos and right after birth. These $1C5^+$ T cells might then be diluted after the cells' proliferation and remain at a level of 1% in the spleen.

It is also probable that thymocytes need the unknown protein to be allowed to stay in the thymus and cease its expression when they are mature enough to leave the thymus. This hypothesis could also be clarified with the experiment described above. One would never find more than 1% of peripheral $CD3^+$ cells being $1C5^+$.

Interestingly the unknown protein seems to be upregulated when the cells are activated with a higher level of $1C5^+$ cells in the activated $CD8^+$ subpopulation (8.6%) than in the activated $CD4^+$ subpopulation (3.1%) mirrowing the difference which was found between CD4 SP and CD8 SP thymocytes.
A small subset of activated T and B cells can re-enter the thymus. Therefore the upregulation of the unknown protein on activated B and T cells might enable them to move to and enter the thymus. This phenomenon could be monitored by injecting mice with $1C5^+$ activated, green-fluorescent protein (GFP)-transfected T and B cells. Part of these cells should be found in the thymus.

The lower percentage of $1C5^+$ cells in the $CD4^+$ subpopulation in the thymus and on activated $CD4^+$ T cells compared to $CD8^+$ cells might point to a lineage dependent expression of the unknown protein on $CD4^+$ cells. This should be checked with additional markers differentiating CD4 SP cells in the thymus (for example heat stable antigen HSA^+ versus more mature HSA^- thymocytes) and activated mature CD4 T cells in the spleen (for example T helper 1 (TH1) versus

TH2 (by intracellular cytokine staining); regulatory T cells (T_{Reg}) (by the expression of CD25, FoxP3)).

Having started with the hypothesis that 1C5 might be a marker for developing thymocytes, it was very surprising to see results exhibiting B cells with a higher percentage of $1C5^+$ cells (6%) than T cells in the spleen. Activated B cells upregulate the expression of the unknown protein to 35%, pointing to a role in the activation of mature lymphocytes, especially B cells.
In order to investigate this possible role in activation, lymphocytes should be activated either *in vitro* with PMA/ionomycin or *in vivo* in an infection model. Lymphocytes activated in this way are expected to show a higher percentage of $1C5^+$ cells in flow cytometric analysis.

Interestingly 15% of the $1C5^+$ cells in the spleen are neither B nor T cells (data not shown). It will also be important to type these cells e.g. by their surface markers in flow cytometric analyses.

The different expression of the unknown protein in the mature lymphocyte subsets of wt and $LT\beta R^{-/-}$ mice might be due to the changes and resulting problems in the immune system of adult $LT\beta R^{-/-}$ mice, described in the introduction, and might become important after determining the unknown protein.

7. Future work

All these interesting experiments described above only make sense after the unknown protein has been determined.

There are three ways to determine the unknown protein.

First, the isolation and sequencing of the protein from thymocytes, second, the cloning of the protein via screening of an expression library, and third, the generation of gene expression profiles of $1C5^+$ CD4 SP thymocytes in comparison with $1C5^-$ CD4 SP thymocytes.

RIPA buffer was used in order to isolate the unknown protein from thymocytes and the LTβR from L929 cells. The LTβR only was found in the insoluble fraction (chapter 4.4.4, figure 21) whereas the unknown protein was detectable neither in the soluble nor in the insoluble fraction (data not shown). Therefore another system should be chosen to lyse the cells and to get the unknown protein into solution.

Immunoprecipitation might help to enrich the unknown protein. Separating the proteins on SDS-PAGE and detecting the unknown protein via western blot will help to identify the right band in the gel to be cut for further sequencing.

Since it is not known at the moment whether 1C5 also detects the unknown protein if it is denatured, metabolic labeling of the cells with ^{35}S before immunoprecipitation can circumvent this problem.

Culturing cells in the presence of ^{35}S-methionine will lead to the incorporation of the isoptope into the cellular proteins. Immunoprecipitation of the labeled proteins with 1C5 bound to proteinG-sepharose can be performed under native conditions also enabeling conformation-determined binding. The eluted $1C5^+$ ^{35}S-labeled proteins can be separated on SDS-PAGE and exposed to an autoradiographic film for the detection of the bands. In case there are multiple bands it would be helpful to work with $1C5^+$ cells in comparison with $1C5^-$ cells from the same origin like for example $1C5^+$ and $1C5^-$ CD4 SP thymocytes. The differentially appearing band(s) can be isolated for further sequencing.

The screening of a prokaryotic mouse thymus cDNA expression library with the cross-reacting antibody 1C5 has failed for the first $1.6x10^5$ pfu. Now it is necessary to check the technique with the prepared library which only expresses the mLTβR.

This library can also be used to test if the sensitivity of the antibody 1C5 is really high enough as to detect a certain protein in an expression library. Knowing that the antibody 1C5 is sensitive enough and can detect the prokaryotically expressed mLTβR does not guarantee that this is also the case for the unknown protein.
If the antibody 1C5 is optimal for screening and the technique is established screening can be started again either with this prokaryotic mouse cDNA expression library or with the now available eukaryotic mouse thymocyte cDNA expression library.

The fastest way to find the unknown protein seems to be the application of "proteomics" i.e. mouse gene expression arrays hybridized with cDNA of either $1C5^+$ or $1C5^-$ CD4 SP thymocytes. Looking for the expression profile of CD8 SP thymocytes which are nearly 100% $1C5^+$ can help to verify the findings.
The upregulated genes in $1C5^+$ CD4 SP and CD8 SP thymocytes in comparison with $1C5^-$ CD4 SP thymocytes finally need to be checked for the expression of the epitope on the surface of cells.

The final prove for every found candidate in each of these experiments would be flow cytometric analysis on $1C5^-$ cells after transfection with the candidate gene in an expression vector.

8. Abstract

Silvia Seegers

Identification and Functional Characterization of a Mouse Lymphotoxin β-Receptor Like Protein

Four different monoclonal rat anti mouse lymphotoxin β-receptor (mLTβR) antibodies were generated in order to study the expression patterns and molecular mechanisms that follow the activation of this receptor which belongs to the TNFR superfamily. One of these four antibodies, namely 1C5, cross-reacts with a protein on thymocytes of wildtype and LTβR$^{-/-}$ mice. Since thymocytes do not express the LTβR a similar receptor which might complement the LTβR on these cells was expected to be found.
On the way to identify the unknown protein the epitope of the antibody 1C5 was narrowed down to the first twelve amino acids of the mature mLTβR. None of the other members of the TNFR superfamily contains this epitope. Homology searches led to a long list of matching candidates which need to be checked for plausibility.
With flow cytometric analyses and treatment of thymocytes with 1C5 the following characteristics of the unknown protein were found.

- Treatment with 1C5 induces apoptosis in thymocytes.
- The unknown protein is LTβR-independently regulated during thymocyte development with a difference between the CD4 single-positive and the CD8 single-positive pathway.
- Only a few mature T cells and B cells express the unknown protein. Its expression is upregulated when the cells are activated.

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