THE ESSENTIAL ROLE OF THE DEUBIQUITINATING ENZYME UBPY IN T CELL DEVELOPMENT AND FUNCTION

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Statement

Herewith, I declare that I prepared the presented dissertation myself and without the use of illegitimate aids. I used no other but the indicated sources and accessories. Further, I insure that this dissertation has not before been submitted to any other faculty for examination.

Berlin, April 29, 2008

(Agnes Kisser)
To Charlotte
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Abbreviations

AMP ............ Adenosine monophosphate
AMSH ........... Associated molecule with the SH3 domain of STAM
ATP ............ Adenosine triphosphate
B7-2 ........... CD86 molecule
BFA ............ Brefeldin A
BrdU ........... Bromodeoxyuridine
cbl ........... Casitas B-lineage lymphoma
CD ............ Cluster of differentiation
CFTR ........... Cystic fibrosis transmembrane conductance regulator
CHMP ........... Charged MVB proteins
CLASP ........... Clathrin-coat-associated sorting protein
CP ............. Core particle (26S ribosome)
CX ............. Cycloheximide
CXCR4 ........... Chemokine (C-X-C motif) receptor 4
CYLD ........... Cylindromatosis gene product
DN ............ Double negative, CD4-CD8-
DNA .......... Deoxyribonucleic acid
DP ............ Double positive, CD4+CD8+
DUB ........... Deubiquitinating enzyme
DUBA .......... Deubiquitinating Enzyme A
EGFR .......... Epidermal growth factor receptor
eIF3 .......... Eukaryotic initiation factor 3
ELISA .......... Enzyme-linked immunosorbent assay
ER ............. Endoplasmatic reticulum
ErbB3, ErbB4 .... v-erb-b2 erythroblastic leukemia viral oncogene homolog 3, 4 (avian)
Abbreviations

<table>
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<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead Box P3</td>
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<td>GADS</td>
<td>Grb2-Related Adaptor Downstream of She</td>
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<tr>
<td>GRAIL</td>
<td>Gene related to anergy in lymphocytes</td>
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<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
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<td>HECT</td>
<td>Homologous to the E6-AP Carboxyl Terminus</td>
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<td>Hepatocyte growth factor receptor</td>
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<td>Hrs</td>
<td>Hepatocyte growth factor regulated tyrosine kinase substrate</td>
</tr>
<tr>
<td>HSA</td>
<td>Heat shock antigen</td>
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<tr>
<td>IκB</td>
<td>Inhibitor of NFκB</td>
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<td>Inflammatory bowel disease</td>
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<td>Interleukin</td>
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<tr>
<td>IL2R</td>
<td>Interleukin 2 receptor</td>
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<tr>
<td>JAMM motif</td>
<td>JAB1/MPN/Mov34 metalloenzyme motif</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LAT</td>
<td>Linker for activation of T cells</td>
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<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
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<tr>
<td>loxP</td>
<td>locus of crossing over</td>
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<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIT</td>
<td>Microtubule interacting and transport</td>
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<tr>
<td>MJD</td>
<td>Machado-Joseph-disease protein domain proteases</td>
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<tr>
<td>MLN</td>
<td>Mesenterial lymph nodes</td>
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<tr>
<td>MVB</td>
<td>Multivesicular body</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<td>Nrdp1</td>
<td>Neuregulin receptor degradation protein-1</td>
</tr>
<tr>
<td>OTU</td>
<td>ovarian tumor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PDGF(R)</td>
<td>Platelet-derived growth factor (receptor)</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PKC(\theta)</td>
<td>Protein kinase C(\theta)</td>
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<tr>
<td>PLC(\gamma_1)</td>
<td>Phospholipase C(\gamma_1)</td>
</tr>
<tr>
<td>rasGRF1</td>
<td>Ras protein-specific guanine nucleotide-releasing factor 1</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RP</td>
<td>Regulatory particle (26S ribosome)</td>
</tr>
<tr>
<td>Rpn10</td>
<td>Non-ATPase base subunit of the 19S regulatory particle (RP) of the 26S proteasome</td>
</tr>
<tr>
<td>Rpn11</td>
<td>Metalloprotease subunit of the 19S regulatory particle of the 26S proteasome lid</td>
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<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive, CD4+CD8- or CD4-CD8+</td>
</tr>
<tr>
<td>STAM</td>
<td>Signal transducing adapter molecule</td>
</tr>
<tr>
<td>Stat</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>UBPY</td>
<td>Ubiquitin binding protease Y</td>
</tr>
<tr>
<td>UCH</td>
<td>Ubiquitin carboxy-terminal hydrolases</td>
</tr>
<tr>
<td>UIM</td>
<td>Ubiquitin interacting motif</td>
</tr>
<tr>
<td>USP</td>
<td>Ubiquitin-specific processing proteases</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>Zap70</td>
<td>Zeta-chain-associated protein kinase 70</td>
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Chapter 1

Introduction

Ubiquitination was discovered in the late 1970’s as an energy-dependent mechanism for intracellular protein breakdown [1, 2]. Soon it became clear that ubiquitin mediated degradation of proteins helps regulating such essential cellular mechanisms as cell cycle progression [3]. Today ubiquitination is known to be implicated in endocytosis and intracellular trafficking of proteins, in multiple signal transduction cascades and in the modulation of gene expression. This multifariousness of ubiquitin’s functions places the molecule on a level with the well known regulator phosphate, which controls the activity of thousands of proteins.

While the enzymes responsible for the conjugation of ubiquitin to substrates have already extensively been studied, the role of the deubiquitinating enzymes (DUB) is only beginning to emerge. DUB are implicated in substrate unspecific processes such as recycling of ubiquitin from proteasome-targeted proteins and processing of ubiquitin precursors. In yeast, the only ubiquitin deconjugating enzyme necessary for viability is the metalloprotease Rpn11, responsible for ubiquitin recycling at the proteasome[4, 5]. In eukaryotes, however, substrate specific deubiquitination has a more essential role. The deubiquitinating enzyme A20 for example negatively regulates NFκB signaling and mice deficient for this enzyme die prematurely due to an
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excessive inflammatory response [6]. In humans, a rare tumor of the skin appendages named cylindromatosis is caused by mutation of the ubiquitin peptidase CYLD [7].

The ubiquitin specific peptidase UBPY has first been described in 1998 as a growth-regulated isopeptidase [8]. The vital and non-redundant role of UBPY is demonstrated by the fact that the conventional knockout of UBPY is embryonically lethal [9]. Further studies with UBPY deficient cells revealed a function of UBPY in endocytic trafficking and stability of receptor tyrosine kinases [10, 11, 9]. Both mechanisms play a central role in T cell development and function. Since UBPY was also reported to interact with a major player in TCR signaling, the adaptor molecule GADS [12], and with a central regulator of T cell anergy, the E3 ligase GRAIL [13], this lead us to investigate the role of UBPY in T cells using a T cell specific knockout mouse model.

This introduction gives a general overview of the ubiquitin system in general and the deubiquitinating enzymes in detail. The present knowledge about UBPY is summarized. Finally, special emphasis is placed on the known functions of ubiquitination and deubiquitination in T cells.

1.1 Ubiquitination and deubiquitination

1.1.1 The ubiquitin molecule and the ubiquitination process

Ubiquitin is a small, heat-stable polypeptide of 76 amino acids that folds into a compact globular structure (Fig. 1.1). It can either be found in free form or covalently attached to target proteins via an isopeptide bond between the C-terminal glycine of ubiquitin and the $\epsilon$-amino group of a lysine residue in substrate proteins. The ubiquitin molecule itself contains seven lysine residues at positions 6, 11, 27, 29, 33, 48 and 63, (referred to as K6, K11, K27...) through which
ubiquitin molecules can be linked to each other, leading to the formation of ubiquitin chains. The attachment of a single ubiquitin to a lysine residue of a substrate is defined as monoubiquitination, while polyubiquitinated substrates are modified by ubiquitin chains of four or more residues. Polyubiquitination can be further categorized according to the lysine residue used for chain formation. This variability of possible ubiquitin modifications explains how ubiquitination can regulate such a great diversity of processes in the cell.

![Figure 1.1: Ubiquitin, isopeptide linkage and chain conformation variations. (A) Ribbon diagram of the main chain of the ubiquitin molecule with the seven lysine residues in stick representation. Ubiquitin is a small, single domain protein of 76 residues consisting of a single anti-parallel \( \beta \)-sheet curved over an adjacent \( \alpha \)-helix forming a highly stable, compact structure. The protruding C-terminus with the glycine 76 residue is used for isopeptide linkage of ubiquitin to a target lysine side chain. (B) Schematic representation of monoubiquitination, K48-linked and K63 linked polyubiquitination of target lysine residues. (Adapted from [14])](image)

The attachment of ubiquitin to protein substrates occurs in a three-step process [2] depicted in Fig. 1.2. The first step consists in the energy-dependent activation of ubiquitin by an ubiquitin activating enzyme (E1). The E1 first uses ATP to produce an ubiquitin-adenylate intermediate. This intermediate is then transferred to the E1 active site cysteine residue with release of AMP, resulting in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1
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cysteine sulfhydryl group. In the second step, the ubiquitin is transferred from E1 to the active site cysteine of a ubiquitin-conjugating enzyme (E2) via a trans(thio)esterification reaction. The final step of the ubiquitination cascade is carried out by one of the hundreds of ubiquitin-protein ligases (E3). E3 enzymes usually contain one of two domains: a RING (Really interesting new gene) domain or a HECT (Homologous to the E6-AP Carboxyl Terminus) domain. RING E3 ligases mediate the transfer from E2 to substrate directly. In the case of HECT E3 ligases, a covalent E3-ubiquitin intermediate is formed before transfer of ubiquitin to the substrate protein [15].

While only two E1 enzymes were discovered so far in eukaryotes [16], the multiplicity of E2 and E3 enzymes enables target-specific ubiquitination. However some E3 ligases can modify more than one substrate and even perform different types of ubiquitination: the cbl E3 ligase for example is known to polyubiquitinate the Abl tyrosine kinase and monoubiquitinate EGFR. Thus, still other molecular factors are obviously needed to explain the correct timing, localization, type and specificity of ubiquitination.

1.1.2 Functions of ubiquitination

Tag for degradation through the proteasome

The ubiquitin-proteasome proteolytic pathway was the first function attributed to ubiquitination (reviewed in [15]). The presence of K48-linked ubiquitin chains of at least four residues on a protein targets this substrate for degradation in the 26S proteasome. The 26S proteasome is a 2,5MDa complex composed of the barrel-shaped 20S catalytic core particle (CP) and one or two 19S regulatory particles (RP). The RP is made up of two parts: the lid and the base. The polyubiquitin chains are recognized by the Rpn10, a subunit of the RP base. The proteasome
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Figure 1.2: Ubiquitin is attached to a substrate by a three-step process. (1) Ubiquitin activation (1a) Formation of a ubiquitin-adenylate. (1b) Transfer of the ubiquitin-adenylate to the active site cysteine of the E1. (2) Transfer of the activated ubiquitin to the E2 enzyme. (3) E3-mediated transfer of ubiquitin to the substrate (adapted from [17]).

Lid is a highly conserved complex related to the COP9 signalosome and the eukaryotic initiation factor 3 (eIF3)[18]. The most conserved lid component is the metalloprotease Rpn11, which removes the polyubiquitin chains from the substrates, enabling their further processing. The base contains the six proteasomic ATPases and attaches to the α-ring of the CP. These ATPases yield the energy necessary for unfolding of the substrate and opening of the proteasome channel, prerequisites for translocation of the substrate in the inner chamber of the CP. The CP is made of four heptameric rings: two outer identical α-rings and two inner identical β-rings. The β-particles harbor the protease active sites. At the inner site of the proteasome, the polypeptide is hydrolyzed by a nucleophilic mechanism, producing small peptide fragments. They are short-lived and do not accumulate in the cell, most likely due to rapid hydrolization by downstream proteases and aminopeptidases. Some of the peptides generated are transported through the endoplasmatic reticulum (ER) and are presented to the immune system by MHC (Major histocompatibility complex) class I molecules, making the proteasome a central component of the antigen presentation machinery.
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Sorting signal in the biosynthetic and endocytic pathways

Rather than in the 26S proteasome, membrane-bound proteins are disposed through the endosomal/lysosomal pathway. This involves several ubiquitin-mediated trafficking steps that ultimately result in delivery of the proteins to the lysosome for degradation (Reviewed in [19], Fig. 1.3). First, ubiquitination mediates the internalization or endocytosis of proteins located in the plasma membrane and their targeting to early endosomes. At present it is thought, that the ubiquitin binding CLASPs (clathrin-coat-associated sorting proteins) epsin/eps15/eps15R recognize the ubiquitin endocytic sorting signal via their ubiquitin interacting motifs (UIM). Assembly of these CLASPs with phospholipids, AP-2, clathrin and each other then links the ubiquitinated cargo to the clathrin-dependent endocytic pathway [14]. Second, probably as a quality control mechanism, ubiquitination may also direct newly synthesized proteins from the trans-Golgi network to the early endosome.

At the endosome, ubiquitin mediated sorting into the lysosome is mediated by a vast network of protein-protein and protein-lipid interactions, whose central players are four highly conserved protein complexes, ESCRT-0, -I, -II and -III (Endosomal sorting complex required for transport). The ESCRT complexes are responsible for cargo selection and the production of lumenal vesicles, resulting from inward budding of the endosomal membrane. This leads to the formation of the multi-vesicular body (MVB) or late endosome. Fusion of the MVB with the lysosome finally leads to proteolysis of vesicle lipids and proteins [19].

All of the ESCRTs except ESCRT-III recognize and bind the ubiquitinated cargo through diverse ubiquitin-interacting motifs. ESCRT-III lacks such modules, instead it recruits deubiquitinating enzymes and the ESCRT disassembly machinery. ESCRT-0 is composed of Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducing adaptor molecule). The two molecules interact constitutively with each other through coiled-coil domains.
and expression of STAM is contingent to the presence of Hrs. Both ESCRT-0 components bind to clathrin leading to formation of Hrs-clathrin coats on endosomes, probably enabling thereby the encounter of ubiquitinated cargo with ESCRT-I [20]. Whereas depletion of Hrs leads to enlarged vacuolar structures with EGFR trapped at the membrane, depletion of the ESCRT-I component TSG101 promotes the accumulation of EGFR and EGF on tubular clusters and induces the formation of multicisternal structures with an internal matrix (Review of the ESCRT machinery in [21]).

**Figure 1.3:** Proteins derived from the cell membrane or newly synthesized from the Golgi apparatus are tagged by ubiquitin (red) to direct them to early endosomes. During maturation towards late endosome, tagged membrane proteins destined for degradation are sorted to lumenal vesicles, forming the so-called multivesicular body (MVB). The MVB/late endosome finally fuses with the lysosome, where the vesicles are degraded (Adapted from [19]).

The ubiquitin modifications involved in the lysosomal pathway are different to the K48 polyu-
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Ubiquitin chains targeting proteins to proteasomal degradation and exhibit great diversity ranging from single and multiple monoubiquitination to K63-linked diubiquitin or polyubiquitin chains. The current consensus view is that multiple ubiquitin residues increase the efficiency of sorting to the lysosomes, relatively independent of a particular conformation [19].

A major group of proteins regulated via the endosomal/lysosomal pathway are signal-transducing receptors, including G protein coupled receptors (i.e. CXCR4), receptor tyrosine kinases (i.e. EGFR, PDGFR, HGFR/c-met), immune receptors (i.e. IL2R, T cell receptor, IgG receptor) and costimulatory molecules (i.e. B7-2). They are usually constantly internalized and recycled back to the cell membrane at a low rate. Upon stimulation receptor internalization and/or lysosomal degradation are accelerated leading to a downregulation of the plasma membrane signaling molecules. This mechanism regulates the signal length, receptor activity and mediates the termination of ligand-induced signaling.

Another group of proteins that undergo ubiquitin-mediated endocytosis are ion channels (i.e. CFTR) [20].

1.1.3 The deubiquitinating enzymes

Classes of deubiquitinating enzymes

The deubiquitinating enzymes (DUB) belong to the superfamily of proteases, which is classified according to the catalytic mechanism in aspartic, serine, threonine, metallo and cysteine proteases (reviewed in [5, 22]). DUB are found in the groups of metallo and cysteine proteases, with the majority of the DUB being cysteine proteases, whose enzymatic activity, by definition, relies on the thiol group of a cysteine residue in their active site.
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This cysteine gets activated by deprotonation through an adjacent histidine residue. The now positively charged histidine is stabilized by a negatively charged residue, aspartate. These three amino acids constitute the catalytic triad of the cysteine proteases. During deubiquitination, the activated cysteine performs a nucleophilic attack on the carbonyl of the peptide bond between ubiquitin and its substrate. This generates an intermediate thiolester between the cysteine and the C-terminus of ubiquitin. Subsequently the histidine is deprotonated and the substrate released. The intermediate contains an oxyanion, that is stabilized by the so-called oxyanion hole, usually constituted by a aspartate, asparagine or glutamate residue together with the main chain of the catalytic cysteine. Finally, hydrolization of the thiolester intermediate regenerates free enzyme and ubiquitin.

In metalloproteases, in contrast, the active site residue is a zinc ion, that is stabilized by an aspartate and two histidines and binds and polarizes a water molecule for the nucleophilic attack.

According to sequence homologies and the structure of the catalytic domain, the DUB are divided in five subclasses.

1. USP (Ubiquitin-specific processing proteases)

This is the largest subfamily of DUB with more than 50 members. The catalytic domain of the USP contains two short and conserved motifs called histidine and cysteine box with all the residues necessary for proteolysis. Beside the conserved motifs the catalytic domain may also contain large unrelated sequences probably with regulatory function. There is a great variability in length of the catalytic domain, from 300 to 800 amino acids. The USP evolutionary co-evolved with the E3 ligases, the enzymes responsible for target specificity of the ubiquitin conjugation, suggesting a close relationship between the two groups of enzymes [23].
2. The UCH (Ubiquitin carboxy-terminal hydrolases)

This subclass has only four members identified so far. Structural and biochemical studies indicated that the active site of the UCH is partially covered by a loop, making it therefore difficult to access for substrates of more than 30 amino acids. Accordingly, UCH are thought to mainly play a role in ubiquitin salvage (the removal of small cellular nucleophiles) and in processing of newly synthesized ubiquitin [5].

3. The OTU(ovarian tumor)-related proteases

This family of cysteine proteases has been predicted based on bioinformatical analyses [24]. Today 14 members of this subfamily are known. Their common feature is the ovarian tumor (OTU) domain. Structural analysis revealed that the OTU proteases do not have the complete catalytic triad of other cysteine proteases and instead use another mechanism, involving a hydrogen bonding network [25].

The physiological function of only a few members is known. A20 is a specific feedback inhibitor of NFκB activation in the tumor necrosis factor (TNF), interleukin-1, and Toll-like receptor (TLR) pathways. The enzyme is exceptional in its dual enzymatic activity: while its N-terminal domain deubiquitinates K63-linked polyubiquitinated signaling mediators such as TRAF6 and RIP, its C-terminal domain acts as a ubiquitin ligase (E3) for K48-linked degradative polyubiquitination of the same substrates [26]. Also another member of the family, DUBA (Deubiquitinating enzyme A), is involved in innate immune responses. The enzyme negatively regulates the production of type I Interferon (IFN) by removing K63-linked polyubiquitin chains from the E3 ligase TRAF3 [27].

4. The Machado-Joseph disease protein domain proteases (MJD)

This family was discovered by a bioinformatics search for other families of ubiquitin deconjugating enzymes [28]. So far the only known members are Ataxin-3 and some Ataxin-3
like proteins. The Machado-Joseph disease is a neurological disease, that is caused by a misfolded form of the Ataxin-3 protein. There are no homologues for MJD in yeast. Other biological functions of the MJD are not determined so far.

5. The JAMM/MPN+ proteases

This family has been predicted on the basis of a shared JAB1/MPN/Mov34 metalloenzyme (JAMM) motif [29], with the consensus sequence Glu-XₙHis-X-His-X₁₀-Asp. This motif contains two absolutely conserved histidine residues and one aspartic acid residue that coordinate together a zinc ion essential for the proteolytic activity [30]. The POH1 metalloprotease (Rpn11 in yeast) is part of the proteasome 19S regulatory subunit. Another member of this family is AMSH (Associated molecule with the SH3 domain of STAM). AMSH is found in a complex with STAM and Hrs at the endosomes. The enzyme has a strong specificity for K63-linked ubiquitin chains and is supposed to be involved in endosomal sorting [31].

![Diagram of catalytic domains](image)

**Figure 1.4:** Structures of the catalytic domains of the five subclasses of deubiquitinating enzymes (Yellow) with ubiquitin (blue). Catalytic centers are shown as Van der Waals spheres: gray-carbon, blue-nitrogen, red-oxygen, orange-sulfur, purple-zinc (from [22].)

Despite the obvious sequence differences between the USP and UCH families of DUB, crystallographic analyses revealed that the three-dimensional structure of the catalytic core of both groups
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of enzymes are nearly similar to each other and close to that of classical cysteine proteases. Interestingly, without ubiquitin-binding, the active sites of these enzymes are in catalytically inactive conformations. Ubiquitin-binding then induces a conformational change which either removes steric obstructions in the active site or brings the catalytic residues in the proper positions. This ubiquitin-dependent activation explains the specificity of the deubiquitinating enzymes for removal of ubiquitin and ubiquitin-like molecules from protein substrates [5].

Functions of deubiquitinating enzymes

The common feature of all deubiquitinating enzymes is their capacity to cleave ubiquitin-linked molecules after the C-terminal glycine residue of ubiquitin [32]. This property is necessary for a variety of cellular mechanisms, reviewed in [5].

1. Processing of ubiquitin precursors

Ubiquitin is always synthesized in an inactive precursor form with a C-terminal extension beyond the terminal glycine [33]. This extension can result from coupling of the ubiquitin to ribosomal proteins or from the formation of ubiquitin multimers (in a head to tail manner, not to confuse with the ubiquitin chains that are linked through internal lysine residues). In either case the bond to be cleaved is a normal peptide bond. Activated ubiquitin is susceptible for nucleophilic attack by small molecules such as glutathione and polyamine. Because some of these components are present in quite high numbers in the cell, DUB are necessary to prevent ubiquitin to be titrated by these molecules. So far it is not yet exactly defined which DUB are responsible for cleavage of which kind of ubiquitin conjugates.
2. Rescue of ubiquitin substrates

DUB can have a role in editing and rescue of ubiquitin conjugates, in order to ensure the specificity of ubiquitination. In that case, proteins have a general susceptibility for deubiquitination by the editing DUB and their level of ubiquitination can be enhanced by inhibiting the activity of DUB, allowing a second level of regulation. Plus, this creates a time window for ubiquitination to take its effect, before the protein returns to a deubiquitinated state. The degradation by the proteasome for example requires ubiquitin chains of at least four residues to be attached to the substrate [34]. A constant deubiquitination rate ensures that proteins with longer ubiquitin chains have a higher probability to be degraded than inappropriately tagged substrates with just little more than four ubiquitins. One ex-
ample of such an editing deubiquitinating enzyme is UCH37, a intrinsic subunit of the 19S proteasome subunit [35].

3. Ubiquitin recycling

After targeting proteins to the proteasome the ubiquitin chains are cleaved off to prevent ubiquitin itself to be degraded and to recycle it in the cellular pool of free ubiquitin. Two DUB are supposed to be involved in this process. UBP6(USP14) is associated with the regulatory subunit of the 26S proteasome and its activity is strongly enhanced by proteasome binding. The metalloprotease Rpn11 (POH1 in mammalian cells) is the only DUB essential for viability in yeast [4]. Its activity in the context of the 26S proteasome is ATP dependent, probably it is coupled to the activity of ATPases that unfold and translocate protein substrates in the proteasome. Wilkinson et al. suggested that in mammalian cells, the enzyme responsible for ubiquitin chain disassembly is isopeptidase T[36]. Naturally an ubiquitin recycling mechanism is also necessary for the second protein degradation machinery, the endosomal/lysosomal pathway. In yeast, the DUB involved in this process is supposed to be Doa4, which is localized to the endosomes and recycles ubiquitin from substrates already committed to inclusion to lumenal vesicles. Lack of Doa4 leads to exhaustion of the cellular pool of free ubiquitin and can be rescued by provision of additional ubiquitin [37].
1.2 UBPY

1.2.1 General facts

UBPY is a 1118 amino acids (human) or 1080 amino acids (mouse) protein. The first detailed description of the human UBPy was done by Naviglio et al. in 1998[8], three years later the mouse homologue was described by Gnesutta et al.[38]. The human UBPy is a 130kDa protein, while the mouse UBPy is of about 120kDa. The mouse and human sequences are highly homologous (Fig. 1.6). Notably in the C-terminal region with the cysteine and histidine boxes characteristic for USP the homology is very high (97% identity). The genomic locus of the gene consists of at least 19 exons, with the second bearing the start codon and is located on chromosome 2 in mouse and chromosome 15 in humans. The mouse UBPy protein was found in a variety of tissues at low levels, with strongest expression in brain and testis [38]. The deubiquitinating activity of UBPy was tested in rabbit reticulocyte lysate [8] and mouse testis extracts pretreated with N-methylmaleimide to inhibit endogenous DUB activity [38], which confirmed that UBPy is able to deubiquitinate high molecular weight substrates. The enzyme has also been shown to cleave linear ubiquitin multimers [8]. UBPy is able to breakdown K48 and K63 linked ubiquitin chains [39, 40]. The mouse UBPy protein contains several functional regions depicted in Fig. 1.6:

- Two coiled-coil domains(93-142; 465-547).
- A rhodanese domain (185-310). Rhodanese domains are structural modules conserved from bacteria to human. Some may contain sulfurtransferase activity, but the UBPy rhodanese domain is catalytically inactive. The domain mediates interaction of UBPy with the E3 ligase Nrdp1 (neuregulin receptor degradation protein 1)[41].
- Two SH3 (Src homology 3) binding motifs with the consensus sequence PX(V/I)(D/N)RXXKP. These motif were identified by binding studies with the SH3 domain of STAM. It also binds
1.2. **UBPY**

to the C-terminal SH3 domain of Grb2 (Growth factor receptor-bound protein 2), but, for example, not the N-terminal SH3 domain of Grb2 [42].

- The carboxy-terminal catalytic domain spanning amino acids 736-1068 with the cysteine box(740-758) and histidine box(1013-1032) characteristic for ubiquitin specific proteases.

- A 14-3-3 binding motif with consensus sequence RSYS^{680}SP at amino acid positions 677-682.

- A predicted MIT (microtubule interacting and transport) domain at amino acid positions 32-112.

**1.2.2 Interaction partners of UBPY**

Naviglio *et al.* showed that altering the amount of UBPY in U2OS cells led to dramatic changes in overall protein ubiquitination [8]. This implicates that UBPY is responsible for a key step in ubiquitin processing and/or deubiquitinates a broad spectrum of substrates.

A number of UBPY interaction partners has already been identified. The first reported binding partner of UBPY is the brain specific ras guanine exchange factor rasGRF-1 (Ras protein-specific guanine nucleotide-releasing factor 1)[38]. Guanine exchange factors are responsible of the exchange of Guanine nucleotides (GDP/GTP) on Ras proteins and important factors in ras/raf signal transduction.

UBPY was further found in a trimolecular complex with GRAIL and otubain-1 [13]. GRAIL (gene related to anergy in lymphocytes) is an E3 ligase undergoing autoubiquitination. The interaction with UBPY stabilizes the protein.

UBPY also interacts with Nrdp1 (*neuregulin receptor degradation protein 1*) [41], an E3 ligase responsible for maintenance of steady state levels of the neuregulin receptor tyrosine kinases.
Figure 1.6: Alignment of the mouse and human UBPY amino acid sequence with the main functional domains (adapted from [38]).
1.2. UBPY

ErbB3 and ErbB4. Mutational analysis revealed that the rhodanese and catalytic domain of UBPY are necessary for Nrdp1 binding. The protein undergoes autoubiquitination mediating its degradation unless stabilized by UBPY.

UBPY cDNA was also isolated in a screen for proteins binding to the SH3 domain of STAM [42]. STAM is tightly associated with Hrs through a coiled-coil motif. Deletion of either the coiled-coil motif or the SH3 domain of Hrs leads to defective degradation of PDGF and its receptor. Usually SH3 domains bind to proline-rich peptides containing the core sequence PXXP. Although UBPY contains six of such proline-rich sequences, the interaction with the STAM SH3 domain is mediated through two other, unusual SH3 binding domains.

In the same study in vitro binding assays showed that murine UBPY binds to the C-terminal SH3 domain of Grb2 (growth factor receptor bound protein 2). Grb2 is an adaptor protein that binds the epidermal growth factor receptor and contains one SH2 and two SH3 domains.

UBPY was also detected in a search for proteins binding to GADS [12]. Both GADS and Grb2 bind to phosphorylated LAT (Linker for activation of T cells) after TCR stimulation and are crucial elements in TCR signaling [43].

Three independent studies identified UBPY as an interaction partner with 14-3-3 proteins [44, 45, 46] through the 14-3-3 binding motif RSYS^{680}SP at amino acid positions 677-682. 14-3-3 proteins function at several key points in G(1)/S- and G(2)/M-transition by binding to regulatory proteins and modulating their function. The association with 14-3-3 proteins usually requires a specific phosphorylation of the protein ligand and mediates cell cycle arrest [47]. In the case of UBPY, binding is regulated by phosphorylation of the serine residue at position 680 [48]. Binding of 14-3-3 proteins to UBPY reduced its enzymatic activity. This could either be explained by a binding induced conformational change or, with the cysteine box only 60 amino acids downstream of the binding motif (Fig. 1.6), a masking of the catalytic domain.

A recent study identified UBPY as an interaction partner with several CHMP (charged MVB
proteins) proteins, which are part of the endosomal ESCRT machinery [39]. Binding to these proteins is conferred by the MIT domain of UBPY.

**1.2.3 The role of UBPY in cell proliferation**

The first reported function of UBPY was described by Naviglio et al. They observed that UBPY levels drop after growth inhibition through cell to cell contact or serum starvation and increase after restimulation, suggesting a role of UBPY in cell proliferation. This was further confirmed by knockout and knockdown models which consistently found that cells depleted of UBPY stop to proliferate and undergo growth arrest [8, 9]. More recent findings showed that UBPY is enzymatically inactivated through the binding of 14-3-3 proteins in a cell cycle dependent manner [48]. In a proteomic search, the binding of 14-3-3ζ to UBPY was detected only in interphasic, but not in mitotic cells [45]. Accordingly, Mizuno et al. showed that in cells treated with nocodazole, which arrests them in prometaphase, the 14-3-3 binding motif gets dephosphorylated, UBPY dissociates from 14-3-3 and is enzymatically activated [48]. For now it is not clear why UBPY gets specifically activated during early mitosis, especially in regard of its role in endosomal trafficking.

**1.2.4 UBPY as a regulator of endosomal trafficking**

A number of studies involving UBPY-depleted cells revealed an important role of UBPY in endosomal trafficking. All related work groups observed that UBPY knockdown or knockout leads to marked differences in endosomal morphology [49, 10, 11, 9]. Endosomes in all stages from early recycling endosome to lysosome are drastically enlarged and form unusual aggregates. Another clear effect of UBPY depletion is the accumulation of ubiquitinated substrates and more precisely of polyubiquitinated substrates. These ubiquitinated proteins colocalize with the en-
1.3. ROLE OF UBIQUITINATION AND DEUBIQUITINATION IN T CELLS

larged endosomes [10, 11, 9].

Two studies detected a (at least partial) loss of Hrs and complete loss of STAM [10, 9] in UBPY depleted cells. Hrs and STAM together form the ESCRT-0 complex located on early endosomes and responsible for endosomal trafficking. In cells depleted of Hrs or of both STAM isoforms ligand-induced EGFR degradation is impaired. According to that three studies also found impaired EGFR degradation in UBPY knockdown cells [10, 49, 50]. In these studies, EGFR accumulates in the cell and colocalizes with the enlarged early endosomes after EGF stimulation. In contrast, a previous study reported accelerated EGFR degradation in UBPY depleted cells [40] and immunohistochemical staining of EGFR in liver sections from UBPY knockout mice revealed that in vivo the EGFR is absent in most cells and displays intracellular localization in the few EGFR-positive cells. These apparently conflicting results led to two contradictory hypotheses concerning the role of UBPY in EGFR degradation. One attributes a stabilizing role to UBPY, where loss of UBPY leads to increased ubiquitination and thereby increased targeting of the receptor for lysosomal degradation. The other hypothesis establishes a functional homology of UBPY to the the yeast Doa4 deubiquitinating enzyme responsible for ubiquitin recycling at the vacuole. However, a defect in ubiquitin recycling would be accompanied by a depletion of the cellular pool of free ubiquitin, which is not the case in UBPY knockout cells [9].

1.3 Role of ubiquitination and deubiquitination in T cells

Covalent modification of proteins by Ubiquitin represents an essential regulatory mechanism that controls protein stability and a wide variety of molecular processes ranging from localization and transport of proteins to the modulation of signaling cascades and inter- and intramolecular protein interactions [51]. Thus it is not surprising that ubiquitination is of fundamental importance
1.3. ROLE OF UBIQUITINATION AND DEUBIQUITINATION IN T CELLS

in the regulation of the adaptive and innate immune system (for a recent review see [52]). In the last years, several components of the ubiquitin modification machinery have been shown to have substantial roles in the control of T cell development and differentiation, maintenance of immune tolerance and TCR signal transduction.

1.3.1 Thymocyte development and central tolerance

During thymocyte development, T cells are selected according to the interaction of their TCR with antigen/MHC molecules leading to activation of intracellular signal cascades. The strength and kinetics of these signaling responses determine whether T cells survive or are actively deleted, by positive and negative selection respectively. Thymocytes that are successfully selected pass beyond the double positive cd4+cd8 stage and mature into single positive cd4+ or cd8+ cells. Thymocytes from mice deficient for the E3 ligase c-cbl show enhanced and prolonged signaling through the TCR, leading to enhanced positive selection of CD4+thymocytes in MHC-classII restricted TCR transgenic mice [53, 54]. In contrast, thymocytes from mice deficient for the deubiquitinating enzyme CYLD show a reduced signaling through the TCR, leading to a developmental block at the double positive stage of thymocyte development. CYLD positively regulates a proximal step of TCR signaling, namely the recruitment of active Lck to its substrate, Zap70 [55]. CYLD deficient mice have hyperresponsive peripheral T cells mediating the spontaneous development of intestinal inflammation [56].

1.3.2 Peripheral tolerance

Thymocyte selection is one pathway through which the immune system establishes tolerance towards self-antigen. However, many self-antigens are first encountered by T cells only out-
1.3. ROLE OF UBIQUITINATION AND DEUBIQUITINATION IN T CELLS

side the thymus. In that case peripheral tolerance towards these self-antigens is established by several mechanisms including activation-induced cell death, suppression by regulatory T cells and anergy induction. T cells are rendered anergic by TCR stimulation without appropriate co-stimulation, leading to a sustained calcium signaling. Instead of being activated, the anergized T-cells fall into a state of unresponsiveness [57], characterized by the inability to produce IL-2 after restimulation with the antigen. The first E3 ligase that was linked to the induction of a clonal anergy state was c-CBL [58]. However c-cbl deficient mice fail to develop spontaneous autoimmune disease which is most likely explained by a compensatory function of the homologue CBL-B. CBL-B negatively regulates T-cell signaling by mediating the ubiquitination of the p85 subunit of Phosphoinositol-3-kinase (PI3K) which prevents its recruitment to the CD28 costimulatory molecule [59]. Consequently cells lacking CBL-B do not require costimulation by CD28 to mount a maximum IL-2 response and mice lacking CBL-B develop autoimmune disease [60, 61]. Several other E3 ligases are upregulated as a consequence of the sustained calcium signaling triggered by anergizing TCR signals, such as GRAIL, ITCH and Nedd4 [62]. These E3 ligases downmodulate the function of signaling molecules essential for T cell activation such as phospholipase Cγ1 (PLCγ1) and protein kinase Cθ (PKCθ). This leads to a block in downstream signaling pathways that are essential for IL-2 production and T-cell proliferation. Consistent with an essential role in anergy induction mice deficient for ITCH or CBL-b develop severe autoimmune disease [63, 61].

1.3.3 T cell receptor downmodulation

The TCR is a large multisubunit complex composed of at least eight subunits: TCRαβ, cd3εδ, cd3ϵγ and cd3ζζ. In resting T cells, the T cell receptor (TCR) is constitutively internalized through endocytosis. Constant surface levels of the receptor are maintained by its rapid recy-
1.3. ROLE OF UBIQUITINATION AND DEUBIQUITINATION IN T CELLS

clinging back to the plasma membrane [64]. Following TCR-mediated stimulation, the entire TCR complex is internalized and all of its components are rapidly degraded in lysosomes [65] leading to a state of unresponsiveness for new antigenic stimuli of the T cell for 72 hours or longer [66]. In case of the cd3-ζ chain, lysosomal degradation is mediated by ubiquitination through the E3 ligase cbl [67]. The interaction of c-cbl with the TCR is mediated by Zap-70 [67] and SLAP [68] The importance of this process was shown by a double knockout of c-cbl and cbl-b leading to development of hyperresponsive T cells, whose inability to downregulate TCR resulted in a lethal, probably autoimmune disease [69]. TCR levels are not only modulated in mature cells after antigenic stimulation, they are also developmentally regulated. At the CD4+CD8+ double-positive stage of development, thymocytes have a strict requirement for signals through the TCR for both positive and negative selection. The TCR expression at that stage is only 10% of the expression on single positive thymocytes and peripheral T cells [70]. The active maintenance of low surface TCR expression probably decreases their sensitivity for interactions with self peptide - MHC molecules so that quantitative differences in signaling can be distinguished more accurately. In immature thymocytes the rate of TCR assembly is kept low due to high degradation rates of newly synthesized TCR α chains. C-cbl deficient thymocytes exhibit higher levels of TCR, independently of TCR triggering [53]. It is conceivable that the developmental regulation of degradation rates might be mediated by regulation of the ubiquitination machinery.

1.3.4 NFκB signaling

One of the first immune responses that was shown to depend on ubiquitination is NFκB (Nuclear factor kappa B) activation (reviewed in [52]). Several immune receptors can trigger NFκB activation: TCR, TLR, TNFR, IL1R. Ligand binding causes phosphorylation of the inhibitory molecule IκB, complexed with the NFκB dimers. Phosphorylated IκB is recognized by a SCF
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complex which mediates K48-polyubiquitination of IκB. Ubiquitinated IκB is degraded in the proteasome, the liberated NFκB dimer enters the nucleus where it initiates transcription of NFκB target genes. Phosphorylation of IκB is equally dependent of an ubiquitination process. Triggering of the receptors activates the E3 ligase TRAF6 which mediates K63 ubiquitination of NEMO, a subunit of the IκB kinase complex (IKK). This activates the IKK complex enabling it to phosphorylate IκB (Fig.1.7).

Figure 1.7: Ubiquitin mediated NFκB activation(from [52]).
Chapter 2

Aim of the project

The importance of ubiquitin deconjugation for T-cell function was demonstrated only recently by the analysis of mice deficient for the ubiquitin carboxy-terminal hydrolase CYLD. Two independent studies showed that T cells derived from CYLD knockout mice display a hyperresponsive phenotype and that CYLD deficient mice spontaneously developed intestinal inflammation [56] or were more susceptible to induced colonic inflammation [71]. The role of CYLD as negative regulator of NFκB activation was already well established in vitro [72, 73, 74], however only the study of the knockout mouse models allowed to discern cell-type specific targets of the enzyme within the NFκB pathway such as Lck in thymocytes [55] and Tak1 in mature T cells [56], emphasizing the importance of mouse models for the understanding of protein function especially in the context of the immune system.

The deubiquitinating enzyme UBPY has an essential role in endocytic trafficking and ligand-induced downmodulation of receptor tyrosine kinases [10, 11, 9]. The correct regulation of TCR downmodulation after ligand stimulation is essential for maintenance of immune homeostasis [69] and intracellular compartmentalization of TCR signaling components is proposed to determine selection outcome in thymocytes [75]. UBPY was also reported to interact with a major
player in TCR signaling, the adaptor molecule GADS [12], and with a central regulator of T cell anergy, the E3 ligase GRAIL [13]. These results suggested a potential role of UBPY in T cell function. As the conventional knockout of UBPY is embryonically lethal and induced deletion in mature animals causes rapid death of the animals [9], cre/LoxP mediated targeting was used to generate mice lacking UBPY specifically in T cells.

In these mice the UBPY gene is deleted during the CD4-CD8- double negative stage 3 of thymocyte development, yielding UBPY deleted T cells of all later stages, from CD4+CD8+ double positive thymocytes to mature CD4+ and CD8+ T lymphocytes. Using this mouse model, the present work aimed to clarify the following questions: What is the consequence of T-cell specific deletion of UBPY in the context of the whole organism? Is UBPY in T cells required for a functional immune system and do T cells normally develop in the absence of UBPY? Are UBPY-deficient T cells functional in response to antigen stimulation and anergy induction? Is UBPY involved in intracellular trafficking of the TCR or in the signal transduction events triggered by TCR stimulation?
Chapter 3

Materials

3.1 Reagents and chemicals

- Acrylamide (30%) Bisacrylamide (0.8%) Roth, Karlsruhe, Germany
- Agarose NEEO Roth, Karlsruhe, Germany
- Ammonium persulfate (APS) Sigma-Aldrich, Steinheim, Germany
- Bromphenol Blue Sigma-Aldrich, Steinheim, Germany
- Bovine serum albumine (BSA) AppliChem GmbH, Darmstadt, Germany
- Brefeldin A Sigma-Aldrich
- Chlorophorm Merck, Darmstadt, Germany
- Complete protease inhibitor cocktail tablets (PIT) Roche, Mannheim, Germany
- Cycloheximide Sigma-Aldrich, Steinheim, Germany
- DNA from salmon sperm Sigma-Aldrich, Steinheim, Germany
- dNTPs (10mM) MBI Fermentas, St.Leon Rot, Germany
- $\alpha^{32}$P dCTP (3000 Ci/mmol) Amersham Biosciences, Munich, Germany
- Ethanol, 99% Merck, Darmstadt, Germany
- Ethidium bromide Sigma-Aldrich, Steinheim, Germany
- L-glutamine (200mM, sterile) Gibco, Invitrogen, Karlsruhe, Germany
### 3.2 Buffers and solutions

When not otherwise stated, deionised (Millipore) water was used as solvent.

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<th>Buffer/Chemical</th>
<th>Manufacturer/Source</th>
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<td>Annexin V staining buffer (10x)</td>
<td>0.1M Hepes (pH 7.4), 1.4M NaCl, 25mM CaCl₂</td>
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<td>APS solution</td>
<td>10% APS in water, prepared fresh</td>
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<tr>
<td>BD PharmLyse™ erythrocyte lysis buffer 10x</td>
<td>BD Biosciences, Pharmingen, Karlsruhe, Germany</td>
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<tr>
<td>Blocking solution (Western blotting)</td>
<td>5% low-fat milk in TBS, 0.1% Tween 20 or 3% BSA in TBS-T, 0.1% NaN₃</td>
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### 3.2. BUFFERS AND SOLUTIONS

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<td>Denhardt’s solution</td>
<td>2% (w/v) BSA; 2% Ficoll; 2% Polyvinylpyrrolidon</td>
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<td>DNA loading buffer</td>
<td>10x 0.4% Bromphenol Blue; 0.4% xylencyanol; 50% glycerol; 0.1M EDTA</td>
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<td>50mM Tris pH 8.0; 100mM EDTA; 100mM NaCl; 1% SDS; 400µg/ml Proteinase K;</td>
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<td>Electrophoresis buffer</td>
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<td>2% FCS, 2mM EDTA; 0.1% NaN₃ in PBS</td>
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<td></td>
</tr>
<tr>
<td>Ponceau S stain</td>
<td>0.1% Ponceau S in 5% acetic acid</td>
<td></td>
</tr>
<tr>
<td>Pre-hybridization solution (Southern)</td>
<td>5xSSC; 0.5% SDS; 100µg salmon sperm DNA, denatured; 5x Denhardt's solution</td>
<td></td>
</tr>
<tr>
<td>Protease inhibitor cocktail (50x)</td>
<td>1 Complete protease inhibitor tablet (Roche) dissolved in 1ml water</td>
<td></td>
</tr>
<tr>
<td>Restriction enzyme buffers (10x)</td>
<td>MBI Fermentas, St. Leon-Rot, Germany</td>
<td></td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>50mM TrisHcl pH 7.4; 150mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS; 5mM EDTA; 1x Protease inhibitor cocktail</td>
<td></td>
</tr>
<tr>
<td>SDS PAGE sample buffer (3x)</td>
<td>0.15M Tris/HCl, pH 6.8; 6% SDS; 30% (w/v) glycerol; 0.003% Bromphenol Blue; 75mM DTT</td>
<td></td>
</tr>
<tr>
<td>Separating buffer (Western) (4x)</td>
<td>1.5M Tris/HCl pH 8.8; 0.4% SDS</td>
<td></td>
</tr>
<tr>
<td>SSC (20x)</td>
<td>3M NaCl; 0.3M sodium citrate, pH 7.0</td>
<td></td>
</tr>
<tr>
<td>Stacking buffer (Western) (4x)</td>
<td>0.5M Tris/HCl pH 6.8; 0.4% SDS</td>
<td></td>
</tr>
<tr>
<td>Stripping solution (Southern)</td>
<td>0.5% SDS</td>
<td></td>
</tr>
<tr>
<td>Stripping solution (Western)</td>
<td>50mM Tris, 2% SDS, 100mM 2-mercaptoethanol, pH 6.8</td>
<td></td>
</tr>
<tr>
<td>TAE-buffer (50x)</td>
<td>2M Tris; 57% acetic acid; 100mM EDTA; pH 8.0</td>
<td></td>
</tr>
<tr>
<td>Taq-Polymerase Puffer (10x)</td>
<td>MBI Fermentas</td>
<td></td>
</tr>
<tr>
<td>TBS (Tris buffered saline) (10x)</td>
<td>0.5M Tris base, 9% NaCl, pH 7.6</td>
<td></td>
</tr>
</tbody>
</table>
3.3 Filters and membranes

3.3.1 Antibodies used for Fluorescence Activated Cell Sorting (FACS)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorochrome</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V</td>
<td>APC, FITC</td>
<td>BD Biosciences PharMingen, San Diego, USA</td>
</tr>
<tr>
<td>B220 (RA3-6B2)</td>
<td>APC</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD3ε (145-2C11)</td>
<td>biotin, APC</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD3ζ (H146-968)</td>
<td>PE</td>
<td>EuroBioSciences</td>
</tr>
</tbody>
</table>
3.4. **ANTIBODIES**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 (L3T4)</td>
<td>FITC, PE, APC</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD5 (53-7.3)</td>
<td>biotin</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD8a (53-6.7)</td>
<td>FITC, PE</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD24 (HSA, M1/69)</td>
<td>PE</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD25 (PC61)</td>
<td>APC</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD25</td>
<td>FITC</td>
<td>Southern Biotechnology Associates, USA</td>
</tr>
<tr>
<td>CD44</td>
<td>biotin</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD45RB</td>
<td>FITC</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD62L (MEL-14)</td>
<td>PE</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>CD69 (H1.2F3)</td>
<td>PE</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>TCRβ (H57-597)</td>
<td>biotin, PE</td>
<td>BD Biosciences PharMingen</td>
</tr>
<tr>
<td>TCRγδ (GL3)</td>
<td>PE</td>
<td>BD Biosciences PharMingen</td>
</tr>
</tbody>
</table>

For detection of biotinylated antibodies by FACS, streptavidin coupled with FITC, PE or APC was used (BD Biosciences Pharmingen).

### 3.4.2 Antibodies used for thymocyte stimulation

Purified NA/LE Hamster Anti-mouse CD3e (145-2C11); BD Biosciences PharMingen

Purified NA/LE Hamster Anti-mouse CD28 (37.51); BD Biosciences PharMingen

Purified anti-Armenian and Syria Hamster IgG (G192-1); BD Biosciences PharMingen

### 3.4.3 Antibodies used for Western Blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin (I-19)</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>c-Cbl (C-15)</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Cbl-b</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>c-Fos (H125)</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>ERK1 (MK1)</td>
<td>Mouse</td>
<td>BD Biosciences Pharmingen</td>
</tr>
</tbody>
</table>
3.5. **RECOMBINANT CYTOKINES AND PROTEINS**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fyn</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Gads</td>
<td>Rabbit</td>
<td>Upstate, USA</td>
</tr>
<tr>
<td>GRAIL (H11-744)</td>
<td>Rat</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Grb2 (C-7)</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Hrs</td>
<td>Rabbit</td>
<td>Raiborg et al., EMBO J 20, 5800, 2001</td>
</tr>
<tr>
<td>Itch</td>
<td>Rabbit</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>LAT</td>
<td>Rabbit</td>
<td>Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>Lck</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>Phospho-ERK1/2</td>
<td>Rabbit</td>
<td>Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>(Thr202/Tyr204,)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-LAT (Tyr191)</td>
<td>Rabbit</td>
<td>Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>Phospho-Tyr (4G10)</td>
<td>Mouse</td>
<td>AG Schraven</td>
</tr>
<tr>
<td>Phospho-Zap70</td>
<td>Rabbit</td>
<td>Cell Signaling Technology, Inc.</td>
</tr>
<tr>
<td>(Tyr319/Syk(Tyr352))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLP-76</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>STAM2</td>
<td>Rabbit</td>
<td>Bache et al., J Biol Chem 278, 12513, 2003</td>
</tr>
<tr>
<td>Ubiquitin (FL-76)</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology, Inc.</td>
</tr>
<tr>
<td>UBPY (31aa)</td>
<td>Rabbit</td>
<td>University of Oxford, Institute of Molecular Medicine</td>
</tr>
<tr>
<td>Zap70 (99F2)</td>
<td>Rabbit</td>
<td>Cell Signaling Technology, Inc.</td>
</tr>
</tbody>
</table>

Secondary antibodies from donkey against goat, mouse, rabbit and rat coupled to horseradish peroxidase (HRP) were from Santa Cruz Biotechnology, Inc.

### 3.5 Recombinant cytokines and proteins

Interleukin-2 from mouse, recombinant expressed in E.coli; Sigma-Aldrich, USA
3.6 Enzymes

NcoI; MBI Fermentas
Proteinase K(10mg/ml); PAN Biotech GmbH
Taq DNA Polymerase; MBI Fermentas

3.7 Kits

murine IL-2 ELISA Ready-SET-Go! eBioscience
NucleoSpin™ExtractII kit Macherey-Nagel, Düren, Germany
QIAexII™Gel extraction kit Qiagen, Hilden, Germany
Rediprime™II Amersham Biosciences, Munich, Germany
APC BrdU Flow Kit BD Pharmingen

3.8 Primers used for Southern Blot probe

sense GGT AGC ACG GAG AGT AAT TT
antisense CCG GGT TGC CAG AAT TAG GT

3.9 Cell culture media

3.9.1 Basic components and supplements

RPMI 1640 PAA Laboratories, Pasching, Germany
Fetal calf serum (FCS, heat inactivated 30 min on 56°C) Biochrom, Berlin, Germany
### 3.10. Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamine</td>
<td>Cambrex, Verviers, Belgium</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Cambrex, Verviers, Belgium</td>
</tr>
<tr>
<td><strong>3.9.2 Media</strong></td>
<td></td>
</tr>
<tr>
<td>Complete T cell medium</td>
<td>RPMI, 10%FCS, 0.2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin</td>
</tr>
<tr>
<td>Starving medium</td>
<td>RPMI, 100U/ml penicillin, 100µg/ml streptomycin</td>
</tr>
<tr>
<td>Stimulation medium</td>
<td>RPMI, 100U/ml penicillin, 100µg/ml streptomycin, 10µg/ml anti-CD3e, 2µg anti-CD28, 10µg anti-hamster antibodies</td>
</tr>
<tr>
<td><strong>3.10 Equipment</strong></td>
<td></td>
</tr>
<tr>
<td>Automated cell counter</td>
<td>CASY Scharfe System, Reutlingen, Germany</td>
</tr>
<tr>
<td>ELISA reader</td>
<td>Tecan, Kreisheim, Germany</td>
</tr>
<tr>
<td>Flow cytometer FACSCalibur, with CellQuest software</td>
<td>BD Biosciences, Heidelberg, Germany</td>
</tr>
<tr>
<td>GenAmp®PCR System 9700</td>
<td>Perkin Elmer, Wellesley, USA</td>
</tr>
<tr>
<td>Mastercycler®Gradient</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Mini-gel system for vertical electrophoresis (PAGE)</td>
<td>Bio Rad, USA</td>
</tr>
<tr>
<td>Chambers for horizontal electrophoresis</td>
<td>Bio Rad, USA</td>
</tr>
<tr>
<td>TransBlot SD Semidry Transfer cell</td>
<td>Bio Rad, USA</td>
</tr>
<tr>
<td>IMAGE Reader</td>
<td>Fujifilm, Düsseldorf, Germany</td>
</tr>
<tr>
<td>Phosphoimager</td>
<td>Amersham Bioscience, Buckinghamshire, UK</td>
</tr>
<tr>
<td>UV Stratalinker® 2400</td>
<td>Stratagene, CA, USA</td>
</tr>
<tr>
<td>Photometer</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Gased incubator</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td>Laminar flow benches</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td>Scintillation counter</td>
<td>Wallac, Turcu, Finland</td>
</tr>
</tbody>
</table>
3.11. MICE

Centrifuges: Beckman Coulter, Krefeld, Germany; Eppendorf, Hamburg, Germany; Hettich, Tuttlingen, Germany.

3.11 Mice

UBPY<sub>flox/flox</sub>-deficient mice (on C57/BL6 - 129/Ola mixed background) were generated as described [9].

CD4<sub>cre</sub> mice (on C57/BL6 - 129/Ola mixed background) were provided by Chris Wilson, University of Washington.

All experiments described in this work were performed with 4-12 weeks old mice housed under specific pathogen-free conditions in accordance with the German animal protection law.
Chapter 4

Methods

4.1 DNA and RNA Methods

4.1.1 Phenol Chloroform extraction

The phenol/chloroform extraction is used to separate proteins and nucleic acids and to remove salts by the subsequent precipitation by isopropanol. A DNA solution was transferred to Phase-Lock-tubes (Eppendorf). 500µl of a Phenol/Chloroform/Isoamylalcohol solution (25:24:1) were added to the tube and mixed vigourously. Phases were separated by centrifugation for 5 minutes at 20,000xg. Then 500µl of a Chloroform/Isoamylalcohol solution (24:1) were added and again mixed vigourously. The tubes were again centrifuged for 5 minutes at 20,000xg. The aqueous phase containing the extracted DNA could then be further processed.

4.1.2 DNA precipitation

For DNA precipitation, a DNA solution was carefully mixed with an equal volume of isopropanol. The DNA was then pelleted by centrifugation for 15 minutes at 20,000xg. The su-
4.1. DNA AND RNA METHODS

permatant was discarded, the DNA pellet washed with 70% Ethanol and air-dried. The DNA was then resuspended in water or TE buffer for further processing.

4.1.3 Isolation of genomic DNA from cells and organs

Pelleted cells, tissues (≈20mg) or mouse tails (≈10mm) were resuspended in 700µl DNA lysis buffer (680µl TENS buffer supplemented with 60µg/ml proteinase K) and incubated over night at 56°C until complete dissolution of the tissue. Subsequently the DNA was isolated using the Phase-lock-tubes (Eppendorf). The DNA was precipitated, resuspended in 150µl TE buffer and dissolved overnight at 56°C.

4.1.4 Determination of DNA concentration by photospectrometry

This method can be applied to concentrations above 50µg/ml of nucleid acids. In a photometer the absorption at 260 and 280nm of the diluted sample (1/20 to 1/200) is measured using the solvent as reference. In case of doublestranded DNA, OD$_{260}$ = 1 corresponds to a concentration of 50µg/ml. In case of single stranded RNA or DNA OD$_{260}$ = 1 corresponds to a concentration of 40µg/ml. Purity of the probe is given by the quotient OD$_{260}$/OD$_{280}$, with optimal values being 1.8 for DNA probes and 2.0 for RNA probes.

4.1.5 Digestion of DNA with restriction enzymes

50µg of genomic DNA were incubated overnight with 1 unit/µg of restriction enzyme at the conditions indicated by the manufacturer.
4.1. DNA AND RNA METHODS

4.1.6 Electrophoretic separation of DNA fragments

For the electrophoretic separation of DNA fragments, 1% agarose gels were used. The gels were prepared by boiling the agarose in 1xTAE-buffer in the microwave oven, and then cooling to approximately 60°C. For the visualization of DNA fragments, ethidium bromide is added in final concentration of 10ng/ml. The mixture is poured in horizontal casting chambers and allowed to polymerize. The samples are prepared for loading by mixing with 0.1 volume 10x loading buffer. The fragment separation is performed in TAE buffer, under 70-100V.

4.1.7 Extraction of DNA fragments from agarose gels

The DNA bands were visualized by exposure to UV light and the desired fragment excised. The gel fragment was transferred to an Eppendorf tube. Extraction of the DNA was performed using the QIAexII Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions.

4.1.8 Polymerase chain reaction (PCR)

The standard PCR reaction mix was composed as follows:
0.5μM sense primer
0.5μM antisense primer
1x Taq polymerase buffer
1.5mM MgCl₂
200μM dNTP-Mix
2.5U Taq polymerase
5-100ng DNA template
The volume was adjusted to 25μl with water.
For amplification of the Southern probe, the PCR was carried out in a thermocycler with the following program:
### 4.1. DNA AND RNA METHODS

<table>
<thead>
<tr>
<th>REACTION STEP</th>
<th>CYCLES</th>
<th>TEMPERATURE</th>
<th>DURATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>95°C</td>
<td>1min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95°C</td>
<td>50sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>15</td>
<td>60°C</td>
<td>50sec</td>
</tr>
<tr>
<td>Elongation</td>
<td></td>
<td>72°C</td>
<td>3min</td>
</tr>
<tr>
<td>Final elongation</td>
<td>1</td>
<td>72°C</td>
<td>7min</td>
</tr>
</tbody>
</table>

#### 4.1.9 Southern Blot

**Blotting of the DNA**

The digested genomic DNA was separated on a 0.7% agarose gel at 70V. The DNA was visualized by exposure to UV light and photographed together with a ruler, allowing size assignment of detected fragments. The Southern Blot was assembled as follows (from bottom to top): three layers of Whatman paper with their ends soaking in a buffer reservoir filled with transfer solution (0.4N Sodium hydroxide), the gel, the nylon membrane (adjusted to gel size), three layers of whatman paper, a stack of paper towels, a glass plate and a weight on the glass plate. The capillary transfer was performed at room temperature for 15 to 24 hours. The membrane was rinsed with 2xSSC to remove the sodium hydroxide and used for hybridization with the Southern probe.

**Preparation of the radioactively labeled probe**

The specific probes were made by PCR amplification (as described in Section 4.1.8) with the primers listed in Section 3.8 using genomic DNA from wild type mouse embryonic fibroblasts as template. The amplified fragments were purified by NucleoSpinR ExtractII kit (Macherey-Nagel, Duren, Germany) according to the manufacturers instruction. For the radioactive labeling of the probe, the kit Rediprime®II (Amersham Biosciences) was used according to the manufacturers instruction. Shortly, 2-20ng of the probe were diluted with TE buffer up to 45µl.
4.1. DNA AND RNA METHODS

denatured for 5 minutes on 95°C and cooled on ice for 5 minutes. The denatured probe was added to the Rediprime-mix together with 5µl of [\(\alpha\)-32P]dCTP and incubated 30 minutes on 37°C. During this step, the enzyme Klenow-polymerase synthesizes the second cDNA strand using the random priming principle and builds in the radioactive dCTPs in the nucleotide strands. The unbound radioactive dCTPs are removed by another purification step with the NucleoSpinR ExtractII kit. The purified radioactive probe was denatured again on 95°C, cooled on ice for 5 minutes and used immediately for hybridization.

**Hybridization, washing and exposure**

The pre-hybridization (blocking) of the membrane was performed in 5ml of pre-hybridization solution (5x SSC; 0,5% SDS; 100µg/ml denatured salmon sperm DNA; 5x Denhardt’s solution) on 63°C for 6 hours. After the pre-hybridization, the radioactively labeled, purified and denatured probe was added to the solution and incubation was continued on 63°C overnight. In order to remove the unspecifically bound radioactive probe, the membrane was washed first with the Wash solution I for 20 minutes on 55°C (low stringency wash buffer) and then two more times with the Wash solution II (higher stringency wash buffer), each time 20 minutes on 55°C. Washed membrane was placed on the phosphoimager screen and exposed overnight. Finally, the screen was scanned with the Phosphoimager.

**Stripping and reprobing**

In order to prepare the membrane for the new hybridization, the membrane was 'stripped' with boiling 0.5% SDS solution. The hybridization procedure was repeated with the new probe in the same way as described above, starting with the pre-hybridization step.
4.2 Histology

4.2.1 Preparation of tissue sections

For fixation, tissues were removed, washed if necessary in ice-cold PBS and incubated overnight in acid formalin and stored in 10% buffered formalin. Water from the tissues was removed by serial incubation steps with ascending concentrations of ethanol and the tissues embedded in paraffin. From the paraffin blocks, 4μm sections were cut, stretched at 37°C and attached to coated microscope slides. Before staining, the tissue sections were deparaffinized by incubating two times for five minutes in xylol, after which the sections were rehydrated to 30% ethanol. Finally the slides were washed in water and incubated in PBS for five minutes.

4.2.2 Eosin/Hematoxylin staining

Eosin/hematoxylin staining was performed according to standard protocols.

4.2.3 Immunohistochemistry

For immunostaining, 4μm thick sections of formalin-fixed, paraffin-embedded tissue was cut, deparaffinized, and subjected to a heat induced epitope retrieval step. The slides were rinsed in cool running water and washed in Tris-buffered saline (pH 7.4) before incubation with primary antibodies against CD3 (#N1580, Dako, Glostrup, Denmark, dilution 1:10) and Foxp3 (FJK-16s, eBioscience, 1:100) for 30 minutes. For detection biotinylated donkey anti-rat or donkey anti-rabbit (Dianova, Hamburg, Germany) secondary antibodies were used followed by the streptavidin-AP kit (K5005, Dako) or the EnVision peroxidase kit (K 4010, Dako). Alkaline phosphatase was revealed by Fast Red as chromogen and Peroxidase was developed with a highly sensitive diaminobenzidine (DAB) chromogenic substrate for approximately 10 minutes.
4.3. PROTEIN METHODS

Negative controls were performed by omitting the primary antibodies. (Histology was performed by Prof. Dr. Christoph Loddenkemper, Charité Berlin)

4.3 Protein methods

4.3.1 Preparation of RIPA protein extracts

The total protein extracts from cells were obtained by lysis in the RIPA buffer supplemented with a protease inhibitor cocktail (Roche). The amount of the lysis buffer was adjusted to contain $5 \times 10^5$ cells/µl. Cells were pelleted by centrifugation for 5 minutes at 560xg and resuspended in a small drop of PBS (approximately the size of the cell pellet). The cells were incubated in the lysis buffer on ice for 30 minutes and then centrifuged for 30 minutes on 20,000xg on 4°C. Clarified lysates were transferred into fresh tubes and the protein concentration was determined by Bradford method (Section 4.3.2). The proteins were denatured by adding 1/2 volume of the 3xSDS PAGE sample buffer and boiled for 5 minutes on 95°C. After denaturing, the samples were cooled on ice, briefly centrifuged and used immediately for electrophoresis or frozen on -20°C.

4.3.2 Protein concentration determination (Bradford assay)

For determination of protein concentrations in protein extracts, the 5x Roti®-Nanoquant solution (Roth) was used. BSA standard dilutions in a range from 1 to 50µg/ml were prepared with a total volume of 200µl. For the zero value of the standard curve, 200µl of water were used. The protein samples were appropriately diluted (usually 1:10 to 1:50 with the RIPA extracts prepared as described in section 4.3.1) in water in a total volume of 200µl. The protein standards and the diluted samples were pipetted in polystyrene cuvettes. The 5x Nanoquant solution was diluted 1:5 with
4.3. PROTEIN METHODS

water, 800μl of this working solution added to each cuvette and carefully mixed by inverting the cuvettes several times. The OD$_{595}$ of each sample was measured in a spectrophotometer using pure water as reference. The OD$_{595}$ of the standards were used to establish a standard curve which was then used to determine the protein concentrations of the samples.

4.3.3 SDS polyacrylamide gel electrophoresis (SDS-Page)

The proteins were separated by size on vertical polyacrylamide gels in an electrophoresis chamber (Bio-Rad) filled with electrophoresis buffer. The gels consisted of a low-percentage stacking gel (pH 6.8) on top of a separation gel (pH 8.8). The composition of the gels are indicated in the tables below. Proteins loaded onto the gel were denatured by prior heat-treatment in SDS-PAGE sample buffer. β-Mercaptoethanol in the sample buffer reduces disulphide bonds in proteins and negatively charged SDS associates with hydrophobic residues of the proteins. The stacking gel ensures simultaneous entry of proteins of all sizes into the separation gel. The negatively charged proteins are then separated in the electric field proportional to the logarithm of their mass.

<table>
<thead>
<tr>
<th>SEPARATING GEL</th>
<th>Final acrylamide concentration in separating gel (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solutions</td>
<td>5 7,5 8 9 10 12 15 20</td>
</tr>
<tr>
<td>30%acrylamide/ 0,8% bisacrylamide (ml)</td>
<td>2,5 3,75 4 4,5 5 6 7,5 10</td>
</tr>
<tr>
<td>4xTris.Cl/SDS pH 8.8 (ml)</td>
<td>3,75 3,75 3,75 3,75 3,75 3,75 3,75 3,75</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}$ (ml)</td>
<td>8,75 7,5 7,25 6,75 6,25 5,25 3,75 1,25</td>
</tr>
<tr>
<td>10% APS (μl)</td>
<td>50 50 50 50 50 50 50 50</td>
</tr>
<tr>
<td>TEMED (μl)</td>
<td>10 10 10 10 10 10 10 10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>STACKING GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solutions</td>
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<tr>
<td>30%acrylamide/ 0,8% bisacrylamide (ml)</td>
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<tr>
<td>4xTris.Cl/SDS pH 6.8 (ml)</td>
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<tr>
<td>$\text{H}_2\text{O}$ (ml)</td>
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<tr>
<td>10% APS (μl)</td>
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<td>TEMED (μl)</td>
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</table>
4.3.4 Detection of Proteins immobilised on membranes with antibodies

(Western Blot)

The polyacrylamide gel was removed from the electrophoresis chamber and cut on the glass plate to an appropriate size. Proteins were transferred from the gel onto a nylon membrane (PVDF membranes were used for very small proteins <30kDa) by semi-dry blotting (TransBlot SD Semi-Dry Transfer Cell, Bio Rad). Six layers of Whatman 3MM chromatography paper were soaked in transfer buffer and placed onto the anode. The nylon membrane (prewetted in transfer buffer) or the PVDF membrane (prewetted in 100% methanol) and the gel slice were positioned on top of the filter paper. Finally six further layers of Whatman paper soaked in transfer buffer were applied. Air bubbles were removed after each step. A constant voltage of 20 V for 1 hour transferred the proteins. Before blocking, successful and homogeneous transfer of the proteins to the membrane was tested by Ponceau S staining. The membrane was incubated for 5 minutes at room temperature in Ponceau S solution. After that the membrane was washed with TBS-T until distinct bands were visible. Optionally a picture of the Ponceau S stained membrane was taken. The membrane was then incubated in blocking buffer appropriate for the individual antibody and remained in the solution for at least 1 hour at room temperature to saturate non-specific binding sites on the membrane. The membrane was incubated with the primary detection antibody overnight at 4 °C on a nutator. After washing three times for 10 min in TBST, the secondary HRP-coupled antibody in blocking solution (1:3000) was added and incubated for 1 h. After a second washing step with three times TBST for 10 min, the freshly mixed ECL detection solutions (Immobilon™Western) were applied for at least 1 min. Excess liquid from the membrane was drained with filter papers. Light produced by catalytic oxidation of luminol in the detection solution through the horse radish peroxidase was detected with the Image Reader (Fujifilm).
4.3.5 **Stripping**

Blots were incubated 2x 20 min at 50 °C in pre-warmed blot stripping buffer, with gentle shaking. Afterwards, the membrane was rinsed 3x 5 min in TBS, then reblocked.

4.3.6 **Preparation of protein extracts from stimulated thymocytes**

Thymocyte single cell suspensions and lysis of erythrocytes was carried out as described in 4.4.1. The cell suspensions were then adjusted to a cell concentration of $4 \times 10^7$ cells/ml in starving T cell medium. For each timepoint, $4 \times 10^6$ cells were used. For starvation, the cells were incubated for 15 minutes at 37°C. After that, the cells were pelleted and incubated in stimulating medium (complete T cell medium supplemented with 10µg/ml CD3ε antibody, 10µg/ml Hamster IgG antibody, 2µg/ml CD28 antibody) at 37°C with gentle rocking (200rpm). At each timepoint a 100µl sample of the stimulated cell suspension was taken and transferred in an eppendorf tube with 50µl of boiling 3xSDS sample buffer and incubated for 15 minutes at 95°C with vigorous shaking (1000rpm). The resulting SDS buffer protein samples were stored at -20°C until used for Western Blotting.

4.4 **Cell biological methods**

4.4.1 **Preparation of single-cell suspensions of primary cells**

**Isolation of primary cells**

After sacrificing the mice by CO$_2$ inhalation, organs (spleen, lymph nodes, thymus) were removed, shortly washed with PBS and placed in PBS on ice. Single-cell suspension from organs were obtained by pressing small pieces of the isolated organs with the tampon of a syringe.
4.4. CELL BIOLOGICAL METHODS

through a 40µm nylon cell strainer in a petri-dish with cold PBS. The cell strainer was then transferred onto a 50ml Falcon tube and the PBS with the cells pipetted through the cell strainer in the tube. Remaining cells on the petridish were collected by rinsing once with PBS and also pipetted in the tube through the cell strainer. Cells were further resuspended by careful pipeting several times. Single cell suspensions were stored on ice until further processing.

**Lysis of erythrocytes**

For lysis of erythrocytes, cells were pelleted by centrifugation for 15min at 600xg. The cell pellet was then resuspended in 1x PharmLyse™ buffer (BD Biosciences) and incubated for 7 minutes at room temperature. 4ml of buffer were used for one spleen and 2ml for one thymus. Then the reaction was stopped by addition of 12ml of PBS and the cells pelleted again. The cell pellet was then resuspended in PBS or medium and stored on ice.

**4.4.2 Fluorescence Activated Cell Sorting (FACS)**

Cell samples of 0.5-1x10^6 cells were resuspended in 100µl FACS buffer with 0.5µl of desired fluorochrome-coupled or biotinylated antibodies (maximum of 3 different fluorochromes was used) and incubated for 20 minutes on room temperature, protected from light or, when indicated, for one hour at 4°C. After incubation, excess antibody was removed by washing the cells two times with FACS buffer. In case biotinylated antibodies were used, streptavidin coupled with fluorochrome was used in the second staining step (0.5µl of fluorochrome coupled streptavidine in 100µl FACS buffer) and incubated 20 minutes on room temperature, protected from light, or, when indicated, for one hour at 4°C. After labeling, cells were washed once, resuspended in 150µl FACS buffer and analyzed.
4.4. **CELL BIOLOGICAL METHODS**

4.4.3  *In vivo* BrdU labeling

Mice 4 weeks of age were killed 2 h after intraperitoneal injection with 100 ml of a 10mg/ml solution of BrdU. BrdU incorporation in thymocyte subsets was analyzed with the BD Pharmingen BrdU Flow Kit according to the manufacturer’s instructions.

4.4.4  Survival assay

Thymocyte single cell suspension were prepared and erythrocytes lysed as described in 4.4.1. The cells were resuspended in starving medium at a concentration of 4.10^6 cells/ml. 100µl of this cell suspension was added per well of a 96-well plate and the plate incubated at 37°C. After 0, 24 and 48 hours the cells were transferred to FACS tubes, pelleted and resuspended in 1x Annexin staining buffer with 2.5µl AnnexinV-APC and 2.5µl Propidium iodide (PI). Staining was carried out for 20 minutes at room temperature. After that further 150µl of 1x AnnexinV staining buffer were added and the cells acquired by Flow Cytometry. Viable cells were determined as AnnexinV negative cells within the Propidium iodide-negative gate and expressed as percentage of the viable cells at \( t = 0h \).

4.4.5  Proliferation assay

A 96well round bottom plate was coated with 30µl per well of CD3ε antibody dilutions in PBS (at the following concentrations: 0.1; 1; 10; 50 µg/ml) for 2 hours at 37°C. After this incubation the plates were washed three times with 200µl PBS per well. Thymocyte single-cell suspensions were prepared and erythrocytes lysed as described in 4.4.1. The concentration of the cell suspensions was adjusted to 10^6 cells/ml in complete T cell medium. 200µl of the cell suspensions were added per well and incubated at 37°C for 96 hours. For additional interleukin-2 stimulation, recombinant mouse Interleukin-2 (Sigma-Aldrich) was added at a final concentration of 100u/ml
4.4. **CELL BIOLOGICAL METHODS**

to the medium. During the last twenty hours, cells were pulsed with 1µCi $^3$H-thymidine (GE Healthcare) per well. After the incubation period, cells were harvested on filtermats (Wallac) using a semi-automated cell harvesting apparatus (Inotech). The filtermats were dried, coated with Melt-on scintillation sheets (Wallac) and counted in a scintillation counter (Wallac).

### 4.4.6 Interleukin-2 ELISA

For detection of Interleukin-2 in cell culture supernatants of stimulated thymocytes we used the mouse Interleukin-2 ELISA-Ready-SET-Go! Kit (eBioscience).

**Coating**

First the ELISA plates are coated with 100µl per well of a dilution of the capture antibody in coating buffer (48µl capture antibody in 12 ml coating buffer), sealed and incubated overnight at 4°C. The coated plates can be stored for maximum one week at 4°C.

**Blocking**

The next day the coated plates are washed five times: the wells were aspirated, 250µl wash buffer per well were added and let 1 minute to soak, then the wash buffer was aspirated and the plates were blotted on absorbent paper to remove residual wash buffer. This was repeated five times. Then the plates were blocked by incubating the well with 200µl Assay diluent for one hour at room temperature. Plates were covered during each incubation step. After blocking, the plates were washed five times as previously described.
4.4. CELL BIOLOGICAL METHODS

Preparation of standards

Using assay diluent, serial dilutions of an Interleukin-2 standard were prepared. Dilution of 4µl standard in 20ml assay diluent gave the top standard dilution (200pg/ml). Starting with the top standard dilution, 2-fold serial dilutions were performed to make the standard curve (range 2-200pg/ml).

Capture

100µl of the standards and 100µl of the samples to be tested were added in triplicates to the appropriate wells and incubated at room temperature for 2h (or overnight at 4°C in a sealed plate for maximal sensitivity) Then the plates were washed five times as previously described.

Detection

100µl of the detection antibody were added per well and incubated for one hour at room temperature. The plates were washed again five times. 100µl of the enzyme solution were added to each well and incubated for 30 minutes at room temperature. The plates were washed seven times. 100µl of substrate solution were added to each well and incubated for 15 minutes at room temperature. The reaction was stopped by adding 50µl of the stop solution to each well and the plates were read at 450nm in an ELISA plate reader.

4.4.7 In vitro induction of apoptosis

A 96-well round bottom tissue culture plate was coated overnight with antibodies against CD3ε and CD28 (50µg/ml each) at 4°C. The next day, the plate was washed three times with PBS and 100µl of a thymocyte single cell suspension (4.10^6cells/ml) in complete T cell medium prepared as described in section 4.4.1 added per well. The plates were incubated overnight at
4.4. CELL BIOLOGICAL METHODS

37°C. The next day, the cells were resuspended in 100µl 1xAnnexin staining buffer with 2.5µl AnnexinV-APC and 2.5µl Propidium iodide (PI) and staining was performed for 20 minutes at room temperature. Then 150µl 1x Annexin staining buffer were added to the samples and the samples acquired by Flow Cytometry. Viable cells were determined by negative staining for both the Apoptosis marker Annexin V and the vital dye Propidium iodide. Cells in early apoptosis are positive for AnnexinV, but still negative for PI staining. Cells in late apoptosis, as well as necrotic cells are positive for both stains.

4.4.8 Detection of ligand induced surface TCR downmodulation

Thymi were removed and single-cell suspensions prepared as described in section 4.4.1. After lysis of the erythrocytes, the cells were counted using the Casy® cell counter and the cell concentration adjusted to 4.10^6 cells/ml in ice-cold complete T cell medium. For each timepoint 1.10^6 cells were used and transferred to FACS tubes. First, the cells were stained for 30 minutes at 4°C with 3µl biotinylated TCRβ antibody. To start the stimulation, cells were pelleted by centrifugation for 5 minutes at 560xg at 4°C and resuspended in 250µl complete T cell medium supplemented with 1.5µl anti-hamster IgG prewarmed to 37°C. The resuspended cells were then incubated at 37°C for the desired times. After the desired incubation time, 250µl of ice-cold PBS were added to the tubes, to stop the reaction and the tubes stored on ice. When all samples were collected, the cells were pelleted again by centrifugation for 5 minutes at 560xg at 4°C and stained in 100µl FACS buffer with CD4-APC, CD8α-FITC and streptavidin-PE (0.5µl each) for 30 minutes at 4°C. Since the cells were not permeabilized previous to the staining, streptavidin-PE binds only to the TCR-biotinylated antibody complexes remaining at the surface. The levels of surface TCR at t were expressed as percentage of the surface TCR present at t = 0 (%SF₀), using the median fluorescence intensity (MFI) of PE in the CD4SP or DP gates with the follow-
4.4. CELL BIOLOGICAL METHODS

Ligand independent regulation of TCR surface levels

Thymi were removed and single-cell suspensions prepared as described in section 4.4.1. After lysis of the erythrocytes, the cells were counted using the Casy® cell counter and the cell concentration adjusted to $4 \times 10^6$ cells/ml in ice-cold complete T cell medium. For each time-point $1 \times 10^6$ cells were used and transferred to FACS tubes. To start the measurement, cells were pelleted by centrifugation for 5 minutes at 560xg at $4^\circ$C and resuspended in 250µl complete T cell medium alone or supplemented with 10µg/ml Brefeldin A or supplemented with 50µg/ml cycloheximide prewarmed to $37^\circ$C. The resuspended cells were then incubated at $37^\circ$C for the desired times. After the desired incubation time, 250µl of ice-cold PBS were added to the tubes, to stop the reaction and the tubes stored on ice. When all samples were collected, the cells were pelleted again by centrifugation for 5 minutes at 560xg at $4^\circ$C and stained in 100µl FACS buffer with CD4-APC, CD8α-FITC and TCRβ-PE (0.5µl each) for 30 minutes at $4^\circ$C. Since the cells were not permeabilized previous to the staining, TCRβ-PE binds only to the TCR remaining at the surface. The levels of surface TCR at $t$ were expressed as percentage of the surface TCR present at $t$ ($\%SF_0$) in untreated cells, using the median fluorescence intensity (MFI) of PE in the CD4SP or DP gates with the following formula: $\%SF_0 = (100 \times \text{MFI}_{t}\text{tr}(t))/\text{MFI}_{\text{untr}}(t)$ with $\text{MFI}_{t}\text{tr}(t)$ being the MFI of the treated cells at a timepoint and $\text{MFI}_{\text{untr}}(t)$ being the MFI of untreated cells at the same timepoint.
Chapter 5

Results

5.1 Generation of mice with a T cell specific UBPY knockout

\( \text{(UBPY} \Delta ^T \text{)} \)

The conventional knockout of UBPY causes embryonic lethality and the induced global knockout in mature animals leads to the death of the animals within seven days [9], thus making these mouse models unsuitable for the study of T cell specific functions of UBPY. Therefore a T cell-specific UBPY gene knockout mouse was generated using the cre/LoxP system. This is a naturally occurring site specific recombination system derived from the bacteriophage P1. The cre recombinase mediates site-specific recombination between two sequences of 34bp, called LoxP sites. A loxP site (locus of X-ing over) consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region conferring directionality to the site. Two LoxP sites in direct orientation on a DNA molecule dictate excision of the intervening DNA between the sites leaving one LoxP site behind. Previous work in our lab used gene targeting to create mice with exon 4 of the UBPY gene flanked by two LoxP sequence motifs (UBPY \text{flox/flox}) [9]. Cre-mediated
5.1. GENERATION OF MICE WITH A T CELL SPECIFIC UBPY KNOCKOUT (UBPY$^\Delta^T$)

deletion of exon 4 causes a shift in the reading frame and introduces several stop codons in the RNA. This RNA may produce a severely truncated protein of only 88 amino acids, lacking all functional domains, including the catalytic region and the diverse protein binding domains (see Fig.1.6) However, it is likely that this truncated protein is instable and rapidly degraded.

The UBPY $^{flox/flox}$ were bred with mice expressing the cre transgene under the control of the cd4 promoter. In the thymus, maturation of thymocytes proceeds through three stages classified according to expression of the surface markers CD4 and CD8. The earliest stage is the double-negative (DN) stage, in which neither of both coreceptors is expressed. The DN stage is further subdivided in four stages according to expression of the surface markers CD44 and CD25 (DN1-DN4). Thymocytes having successfully rearranged the T cell receptor beta chain (TCR$\beta$) during DN3 are selected, expand and proceed to the double-positive stage. DP thymocytes rearrange the TCR $\alpha$ chain and undergo a second round of selection based on the specificity of the mature TCR heterodimer for self-antigen associated to molecules of the major histocompatibility complex. As a result of this process, a relatively small fraction of DP thymocytes mature to either CD4+ or CD8+ single positive (SP) cells and finally emigrate to peripheral lymphoid organs as naive T cells. Since CD4 expression is initiated after successful rearrangement of the TCR$\beta$ during DN3, the expression of the cre recombinase also starts at that time. Therefore in UBPY $^{flox/flox}/$CD4 cre$^+$ mice, UBPY deletion begins at the DN4 stage and should be completed in the double-positive thymocytes, single positive CD4+ and CD8+ single positive thymocytes and the peripheral CD4+ and CD8+ T cells.

The UBPY deletion was tested with a Southern probe recognizing a 5kb NcoI fragment in wild-type DNA. After cre mediated recombination the fragment recognized by the probe is 6kb long, due to the excision of a NcoI restriction site (Fig.5.1A). The majority of double negative thymocytes still carry the floxed UBPY allele. This is in accordance with the expectations, since cre expression under the cd4 promoter only starts at double negative stage 3. The highest deletion
5.1. GENERATION OF MICE WITH A T CELL SPECIFIC UBPY KNOCKOUT (UBPY$^{\Delta T}$)

rate is achieved in double positive thymocytes, with nearly 80% of the cells bearing the UBPY$^{\Delta}$ allele. Higher levels of the nonrecombined UBPY allele were detected in FACS-sorted peripheral T cells from spleen and mesenterial lymph nodes, indicating the presence of T cells derived from thymocytes with an incomplete deletion of UBPY. In accordance to the results of the Southern Blot, immunoblot analysis of protein extracts from total thymocytes revealed the presence of a fraction of UBPY expressing cells, with the majority probably being double-negative thymocytes. Indeed, in sorted DP thymocytes the fraction of cells still expressing UBPY is so small, that UBPY expression is below detection level. These results show that UBPY $^{\text{floxed/floxed}}$CD4 cre$^{+}$ mice yield at least in part UBPY depleted DP and SP thymocytes, as well as peripheral CD4+ and CD8+ T lymphocytes, representing therefore a convenient tool for the study of the role of UBPY in T cells. Subsequently UBPY $^{\text{floxed/floxed}}$CD4 cre$^{+}$ mice will be referred to as UBPY$^{\Delta T}$
5.1. GENERATION OF MICE WITH A T CELL SPECIFIC UBPY KNOCKOUT (UBPY$^{\Delta T}$)

Figure 5.1: Generation of UBPY$^{\Delta T}$ mice. (A) Diagram showing the wildtype Ubpym genomic locus, the loxP-flanked (UBPYflox), and the deleted (ΔUBPY) alleles. Filled boxes indicate exons (E2-E5). Arrowheads indicate loxP sites. Restriction enzyme sites and the location of the probe used for Southern blot analysis are depicted. NcoI fragments are in kilobases. N, NcoI. (B) Southern Blot of NcoI digested genomic DNA using a $^{32}$P-labeled probe in sorted thymocytes (upper panel) and in sorted peripheral T cells (lower panel) from wildtype (WT) and UBPY$^{flox/flox}$/CD4 cre$^+$ mice (KO). MLN, mesenterial lymph nodes. Data are representative of n = 3 pooled mice. (C) Graphic representation of UBPY-deletion rates in thymocyte subsets and peripheral T cell populations in UBPY$^{flox/flox}$/CD4 cre$^+$ mice. For quantification of the UBPY deletion, the relative density of the 6kb band was measured using the ImageQuant software (Molecular Dynamics) and expressed as percentage of the density of all bands in each lane. (D) Immunoblot of UBPY deletion in total thymocytes and sorted CD4+CD8+ double positive thymocytes.
5.2  SPONTANEOUS DEVELOPMENT OF INFLAMMATORY BOWEL DISEASE IN UBPY$^{\Delta T}$ MICE

5.2  Spontaneous development of inflammatory bowel disease in UBPY$^{\Delta T}$ mice

UBPY$^{\Delta T}$ mice were born at the expected mendelian frequency and were kept under standard specific pathogen-free conditions. As early as six weeks of age, they developed signs of colitis, including diarrhea and rectal prolapse. The animals became rapidly wasted and moribund and all mice died within 20 weeks (Fig.5.2A). Gross examination of the organs of UBPY$^{\Delta T}$ mice, which already developed signs of disease, revealed a thickening of the bowel wall (Fig.5.2B). This was usually accompanied by splenomegaly, lymphadenopathy and sometimes, probably depending on progression of the disease, thymic loss. Histologically, the most prominent phenotypical changes were found in the colon. Hematoxylin/eosin staining of sections of the colon revealed a massive hyperplasia of the mucosa in UBPY$^{\Delta T}$ animals. Crypt abscesses could also be detected as well as a massive cellular infiltration (Fig.5.2C) in the lamina propria of UBPY$^{\Delta T}$ mice. In order to determine the nature of these infiltrating cells, colonic sections of wildtype and UBPY$^{\Delta T}$ mice were stained by immune histology with antibodies directed against CD3 as a T lymphocyte specific marker and FoxP3 as a marker for regulatory T cells. These stainings clearly showed that the infiltrating cells were essentially CD3 positive T cells (Fig.5.2D), with some interspersed regulatory T cells (Fig.5.2D, inset, arrow). No chronic inflammatory changes were detected in any other organ (data not shown). These mice were of mixed genetic backgrounds, which may have contributed to variations in disease progression in individual mice. There was no colitis in heterozygous or control (UBPY$^{wt/wt}$cd4cre$^+$, UBPY$^{wt/flox}$cd4cre$^+$, UBPY$^{flox/flox}$CD4cre$^-$) UBPY mice.

The colon pathology in the absence of chronic inflammatory changes in other organs in UBPY$^{\Delta T}$ mice is characteristic for inflammatory bowel disease (IBD), a chronic inflammation
5.2. SPONTANEOUS DEVELOPMENT OF INFLAMMATORY BOWEL DISEASE IN UBPYΔT MICE

of the mucosa. As a general rule, IBD is the result of an inappropriate response of the immune system towards the normal microflora of the gut. Several factors influence the development of the disease and similar clinical features appear in many mouse strains with diverse genetic defects (reviewed in [76]). A genetic predisposition of the mouse strain used determines whether a given genetic defect leads to the development of colitis or not. Second, the gut microflora is required for triggering or maintenance of the inflammation. Mice kept under germ-free conditions usually do not develop IBD. Genetic abnormalities in epithelial cells, for example, leading to the loss of their barrier function towards lumenal microorganisms can lead to colitis. Mucosal inflammation can also be due to genetic defects in cells of the adaptive immune system, if affecting the function of antigen-presenting cells, macrophages or natural killer cells. However in UBPYΔT animals, the defect must reside within the T cell population. In that case IBD results either of excessive effector function or defective regulatory T cell function. An excessive effector function is caused for example by an intrinsic overproduction of proinflammatory cytokines such as TNFα (i.e. TNFARE mice, [77]), or IFNγ (i.e. Stat4 transgenic mice, [78]). A defective regulatory function might be due to a failure to produce regulatory cytokines (i.e. IL-10 [79]) or to a defect in the development of regulatory T cells (i.e. IL-2 knockout mice, [80]). Immune histological stainings of colonic sections from UBPYΔT mice against FoxP3 revealed the presence of at least some regulatory T cells. Therefore the colitis in these mice is not due to the absence of regulatory T cells. In order to confirm that the pathological changes in UBPYΔT mice are indeed caused by a disturbance of immune homeostasis, we proceeded with the analysis of the peripheral T cells.
5.2. SPONTANEOUS DEVELOPMENT OF INFLAMMATORY BOWEL DISEASE IN UBPY$^T$ MICE

![Figure 5.2: UBPY$^T$ mice develop an inflammatory bowel disease and die prematurely. (A) Survival curve of wildtype and UBPY$^T$ showing the lethality of the UBPY$^T$ phenotype. (B) Anatomy of WT and UBPY$^T$ animals showing the thickening of the colon and the rectal prolapse in UBPY$^T$ mice. (C) Histological analysis of colonic mucosa. The figure shows hematoxylin and eosin staining of colons from wildtype (WT, left) and UBPY$^T$ (KO, right) mice. Left, normal crypt architecture in wildtype (WT) animals. Right, in UBPY$^T$ (KO) mice, marked mucosal thickening and epithelial hyperplasia and elongated crypts. Some crypt abscesses (arrow). Cellular infiltrates. (D) Immune histological staining of colonic section from wildtype (WT) and UBPY$^T$ (KO) animals with antibodies directed against CD3 (red) and FoxP3 (brown). Visible are the infiltrates of CD3 positive cells in the lamina propria. The inset in the right box highlights the presence of FoxP3+ regulatory T cells in UBPY$^T$ animals (arrow).]
5.3 Frequency and activation state of UBPY$^{ΔT}$ peripheral T cells

To detect disturbances in immune homeostasis, first frequencies and total cell numbers of the peripheral T cells in young (4-6 weeks of age) wildtype (WT) and UBPY$^{ΔT}$ (KO) mice were determined. At this age, UBPY$^{ΔT}$ mice still had a healthy appearance, without the typical symptoms of colitis (rectal prolapse, diarrhoe and weight loss). Cells from lymph nodes and spleens were stained with fluorescent labeled antibodies against B220, a B lymphocyte specific marker, CD3, specific for T lymphocytes, CD4 and CD8 and the stained cells were analyzed by Flow Cytometry. The total numbers of the different lymphocytes subsets (B cells, T cells, CD4+ and CD8+ lymphocytes) in the spleen were calculated using the frequencies determined by Flow Cytometry. Fig.5.3A shows the results obtained by Flow Cytometry with the frequencies obtained. Fig.5.3B allows the comparison of total lymphocyte numbers in WT and KO spleens. While the frequency and numbers of B lymphocytes, characterized by the expression of B220, were unchanged, staining against CD3 revealed a significant reduction of the mature CD3+ T lymphocyte compartment. This reduction was detectable in both spleen and lymph nodes. Staining with anti-CD4 and anti-CD8 antibodies showed that both CD4+ and CD8+ T lymphocyte compartments were affected in spleen and lymph nodes. However the reduction was more severe in the CD4+ compartment, leading to an altered CD4:CD8 ratio of 0.9 in UBPY$^{ΔT}$ mice compared to 1.5 in wildtype animals (Fig.5.3B).

Peripheral T cells had lower surface levels of TCR, as determined by staining with antibodies against TCR$^{β}$ and quantification by Flow Cytometry (Fig.5.3C). In the spleen there even seemed to be a distinct CD4$^{+}$TCR$^{β}$− population.

Next, the activation state of the peripheral T cells was analyzed. Splenocytes and lymph node cells were stained with antibodies against the positive activation markers CD69 and CD25 and
the negative activation marker CD45RB and analysed by Flow Cytometry. Fig.5.3C shows the overlays of the signals obtained with these markers in CD4+ cells from spleen and lymph nodes of wildtype and UBPYΔT mice. Surface expression of CD25 and CD69 was increased and surface expression of CD45RB was reduced in knockout animals, indicating a hyperactivated state of the peripheral T cells. Using the surface markers CD44 and CD62L it is possible to differentiate between naïve T cells (CD44\textsuperscript{low}CD62L\textsuperscript{high}), memory T cells (CD44\textsuperscript{high}CD62L\textsuperscript{high}) and effector T cells (CD44\textsuperscript{high}CD62L\textsuperscript{low}). The frequencies obtained were used to calculate the total numbers of these T cell subsets. Fig.5.3D shows that in CD4+ compartments of UBPYΔT lymph nodes, the frequency of naïve and memory T cells was drastically reduced, with the large majority of the T lymphocytes bearing an effector phenotype.

The analysis of the peripheral T lymphocyte populations shows that UBPY knockout in T cells leads to a severe lymphopenia in UBPYΔT animals. Clearly, immune homeostasis is disturbed, with the few remaining T lymphocytes being aberrantly high activated and mostly displaying an effector phenotype, while naïve T cells are almost absent. Hyperactivation is already detectable at 4 weeks of age, and therefore precedes onset of the colitis. The T cells in UBPYΔT mice have reduced surface levels of T cell receptor. This might reflect the hyperactivated state of the T cells, resulting in enhanced ligand induced downmodulation or alternatively might be caused by a maturation defect during thymocyte development. Disturbed T cell development could also be causative for the overall reduction of the T lymphocyte populations. Therefore the next step consisted in the detailed analysis of thymocyte development in UBPYΔT mice.
5.3. FREQUENCY AND ACTIVATION STATE OF UBPY ΔT PERIPHERAL T CELLS

Figure 5.3: Lymphopenia and hyperactivation of peripheral T cells in UBPY ΔT mice. (A) Flow cytometry of lymphocyte subsets in spleen and lymph nodes (LN) from 6-week-old wildtype (WT) and UBPY ΔT (KO) mice after staining with antibodies against CD3, B220, CD4 and CD8 as indicated. Numbers in quadrants indicate the frequency of subpopulations (mean ± sem of n ≥ 3). (B) Total numbers of lymphocyte subsets (horizontal axes) in spleens from 6-week-old WT mice (gray bars) and UBPY ΔT mice (open bars), mean ± sem of n ≥ 3, asterisks (*) indicate statistically significant differences (p ≤ 0.02). (C) Flow cytometry of activation markers CD25, CD69 and CD45RB and TCR β chain in CD4+ T cells from spleen and lymph nodes (LN) of wildtype (filled histograms) and UBPY ΔT (open histograms) mice. Results are representative of at least three independent experiments. (D) Flow Cytometry of functional subpopulations in splenic CD4+ T cells of wildtype (WT) and UBPY ΔT (KO) animals. Numbers in quadrants indicate frequencies of naïve (CD44lowCD62Lhigh), effector (CD44high, CD62Llow) and memory (CD44highCD62Lhigh) T cell subsets (mean ± s.e.m.; n ≥ 4, 4-8-week old mice). Total numbers of the functional subpopulations in splenic CD4+ T cells were calculated using the frequencies obtained by Flow Cytometry and represented in the stacked columns diagram on the right. Dark gray, memory T cells; light gray, naive T cells; white, effector T cells.
5.4 Thymocyte development in UBPY\(\Delta^T\) mice

In order to analyse the development of the thymocytes, the thymi of 4-6 weeks old UBPY\(\Delta^T\) animals and wildtype controls were removed and single-cell suspensions prepared. The different thymocyte subsets were stained with antibodies coupled to fluorescence markers and analysed by Flow Cytometry: CD4 and CD8 antibodies were used for the four main thymocyte subsets. The DN stages were analysed using antibodies against the surface markers CD4, CD8, CD44 and CD25. The TCR\(\gamma\delta\) thymocytes were determined as the CD3+TCR\(\gamma\delta\)+ subset by staining with CD3 and TCR\(\gamma\delta\) antibodies. The regulatory T cell subset was defined as the CD8-CD4+CD25+ subset by staining with antibodies against these three surface markers. Total thymocyte numbers were counted and total numbers of the thymocyte subsets calculated using the frequencies obtained by Flow Cytometry (Fig.5.4C). The analysis of the CD4 and CD8 expression showed significantly reduced frequencies of CD4+ and CD8+ single positive (SP) compartments, by similar frequencies of the double negative compartment and increased frequency of the double positive compartment (Fig.5.4A, upper panel). The CD25 and CD44 expression in the DN compartment revealed that the DN stages are not affected in UBPY\(\Delta^T\) animals (Fig.5.4A, lower panel). Also the frequency of the TCR\(\gamma\delta\) subset of thymocytes was not affected by the UBPY knockout (Fig.5.4B, upper panel). Frequency and numbers of CD8-CD4+CD25+ cells within the whole thymocyte compartment were reduced (Fig.5.4C). This was not surprising, given the overall reduction in CD8-CD4+ cells in UBPY\(\Delta\) thymocytes. The percentages of CD4+CD25+ cells within the CD8- compartment however were similar in wildtype and UBPY\(\Delta\) mice (Fig.5.4B, lower panel). Total thymocyte numbers in young, preclinical UBPY\(\Delta\) mice were not altered in comparison to wildtype animals. This confirms the presumption that thymic loss observed in several UBPY\(\Delta\) mice in later stages of the colitis is a disease associated symptom. Numbers of thymocyte subsets therefore reflected the differences in frequencies: there was a significant
5.4. THYMOCYTE DEVELOPMENT IN UBPYΔ^T MICE

reduction of CD4 single positive thymocytes, including the regulatory T cells, and CD8 single positive cells, while total numbers of double negative, double positive and TCRγδ thymocytes were unchanged in UBPYΔ mice (Fig.5.4C). The reduction in numbers in UBPYΔ SP thymocytes was more drastic in the CD4SP compartment than in the CD8SP compartment, resulting in a ratio of CD4SP to CD8SP of 0.9 compared to 1.8 in wildtype animals. These results indicate a maturation defect in UBPYΔ thymocytes, leading to a block in thymocyte development at the double positive stage.

To further investigate that thymic defect, the expression of the developmental stage specific surface markers CD5, CD3ε, CD69 and CD24(heat shock antigen, HSA) was analysed by Flow Cytometry (Fig.5.5A). CD5 is expressed at very low levels on early DN thymocytes, however signals transduced by the pre-TCR upregulate CD5 and the levels of CD5 expression reflect the extent or intensity of pre TCR signaling [81]. At the DP stage most thymocytes express intermediate levels of CD5 and low TCR signals are sufficient to maintain this expression. Only those DP thymocytes that express TCRs that can interact with MHC-ligand with sufficiently high affinity to promote either positive or negative selection upregulate CD5 surface expression to high levels. DP thymocytes express low levels of TCR and TCR levels are upregulated after receipt of a selecting TCR signal, bearing SP thymocytes with ten-fold increased surface levels. CD69 is used as a marker to identify the thymocytes that are auditioning in positive selection. CD69 upregulation is initiated after TCR and CD5 upregulation and is crucially dependent upon TCR–MHC–peptide interactions[82]. HSA is used as a marker for the late stage of SP thymocyte maturation [83] and downregulation of HSA correlates with increasing maturation. The SP thymocytes with no expression of HSA at their surface are the most mature thymocytes and ready for emigration to the periphery. At the DN developmental stage, UBPYΔ^T thymocytes expressed normal quantities of CD3ε, CD69 and CD24. CD5 expression however was lower,
5.4. THYMOCYTE DEVELOPMENT IN UBPYΔT MICE

Figure 5.4: Defective thymocyte development in UBPYΔT mice. (A-B) Thymocyte subsets from 6-week-old wildtype (WT) and UBPYΔT mice (KO) were analyzed by Flow Cytometry. Numbers in quadrants and in boxed areas indicate the frequency of subpopulations (mean±sem). (A) Upper panel, frequencies of the four main thymocyte subsets classified by expression of CD4 and CD8, n = 9. Lower panel, frequencies of double negative stages classified by expression of CD44 and CD25 in the CD4−CD8− gate (n = 4). (B) Upper panel, frequencies of the TCRγδ thymocytes, (n = 3). Lower panel, frequencies of regulatory T cells, determined as CD4+CD25+ cells within the CD8− gate, (n = 3). (C) Total numbers of thymocyte subsets (horizontal axes) in 6-week-old WT mice (gray bars) and UBPYΔ mice (open bars) are represented as mean±sem, asterisks (*) indicate statistically significant differences (p ≤ 0.02)
5.4. THYMOCYTE DEVELOPMENT IN UBPY$\Delta^T$ MICE

suggesting a defective pre-TCR signaling. This is not in contradiction to the normal DN-DP transition in UBPY$\Delta^T$, since CD5 expression is not required for thymocyte maturation [84]. No significant differences in the expression of any of the the four markers could be detected between DP thymocytes of wildtype and UBPY$\Delta^T$ mice, probably due to the fact that positively selected thymocytes constitute only a minor fraction of the DP subset. By contrast, in the CD8SP compartment, CD5, CD3$\epsilon$ and CD69 expression were drastically reduced, while a mild reduction was observed in CD4SP thymocytes. The most mature CD4SP and CD8SP thymocyte subsets, characterised by low expression of HSA are almost lacking in UBPY$\Delta^T$ thymocytes.

Several studies showed that UBPY has an essential role in cell proliferation with lack of UBPY inducing a growth arrest in several cell lines [8, 9]. The defective development of thymocytes in UBPY$\Delta^T$ animals was therefore likely to be caused by a defect in cell cycle progression. In order to test this, mice were injected intraperitoneally with BrdU, a thymidine analogue, that is incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle. Two hours after injection, the mice were sacrificed and the BrdU incorporation in the thymocyte subsets was assessed with a BrdU-specific antibody by Flow Cytometry (Fig.5.5B). The percentages of BrdU incorporating, thus replicating cells in the thymocyte subsets of UBPY$\Delta^T$ animals were not reduced compared to control animals. Within the CD8 single positive compartment this percentage was even highly increased, probably reflecting the highly immature phenotype of this subset in UBPY$\Delta^T$ animals. These results clearly show that, in thymocytes, lack of UBPY does not induce cell cycle arrest and that the defect in thymocyte development is not caused by impaired cell proliferation.

UBPY$\Delta^T$ animals are clearly impaired in thymocyte development, leading to a severe reduction of frequencies and absolute cell numbers in both single positive departments. The lower CD4:CD8 ratio indicates that the CD8SP compartment is less severely affected, in terms of
5.4. THYMOCYTE DEVELOPMENT IN UBPYΔΤ MICE

Figure 5.5: Impaired thymocyte maturation in UBPYΔΤ mice. (A) Flow Cytometry of differentiation markers CD5, CD69, CD24 and CD3ε in thymocyte subsets of WT (filled histograms) and UBPYΔΤ animals (open histograms). The histogram overlays are representative of at least three independent experiments. (B) In vivo BrdU incorporation in thymocyte subsets (horizontal axis) of 4-week-old mice wildtype mice (WT, gray bars) and UBPYΔΤ mice (KO, open bars), presented as mean ± s.e.m. (n = 6 mice per group). The asterisk (*) indicates statistically significant difference (p ≤ 0.0005)

frequencies and numbers. However the analysis of the maturation markers shows that this compartment in UBPYΔΤ mice mainly consists of immature CD8SP cells. Analysis of different developmental markers revealed that additionally to the reduction in numbers of SP thymocytes, UBPYΔΤ mice have a drastic thymocyte maturation defect yielding SP thymocytes with low surface TCR expression and high expression of HSA. The lower expression of CD5 and CD69 in UBPYΔΤ SP thymocytes, markers whose expression is proportional to the signals received through the TCR, suggests a defective signaling through the TCR.

The selection processes, lineage commitment and maturation steps during thymocyte development, which are affected in UBPYΔΤ mice, all depend on tightly balanced responses to TCR stimulation. The next step therefore consisted in the analysis of the responsiveness of UBPYΔΤ thymocytes to stimulation through the TCR.

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5.5 **UBPYΔ^T thymocytes exhibit a defective responsiveness to TCR stimulation**

Developing thymocytes are destined to die by apoptosis, unless interaction of the TCR with MHC-self-antigen complexes induces a rescue signal leading to proliferation of the cell. To determine the responsiveness of UBPYΔ^T thymocytes to TCR stimulation three read-outs were therefore used: the proliferation rate, the rate of apoptosis induction and the amount of the growth factor interleukin-2 produced.

For assessment of the proliferation rate *in vitro*, thymocytes of 6 week old wildtype and UBPYΔ^T mice were seeded in triplicates on 96-wells plates coated with increasing concentrations of plate-bound anti-CD3 antibody and with or without addition of recombinant mouse Interleukin-2 for four days at 37°C. Twelve hours before acquisition the cells were loaded with ^3H-labeled thymidine. At the end of the incubation period, the cells were harvested on filter plates and incorporation of the radioactive thymidine measured in a scintillation counter. Since only proliferating cells are capable of incorporating the ^3H-thymidine, the scintillations count is directly proportional to the proliferation rate in a sample.

A depicted in Fig.5.6A, after this *in vitro* incubation period and independently of the concentration of plate-bound CD3 antibody used, no proliferation of UBPYΔ^T thymocytes could be detected in the absence of Interleukin-2, while the proliferation rate in wildtype thymocytes raised with increasing concentrations of anti-CD3. However the proliferation rate in UBPYΔ^T thymocytes was restored almost to wildtype level by addition of recombinant Interleukin-2 to the medium. IL-2 is a crucial regulator of T cell proliferation, survival and programmed cell death (apoptosis) (reviewed in [85]). However, at low concentrations, IL-2 prevents apoptotic death of thymocytes induced by CD3-stimulation *in vitro* [86]. The reduced proliferation rate *in vitro* of UBPYΔ^T thymocytes therefore might result from an increased susceptibility to apoptotic death.
5.5. **UBPYΔ^T thymocytes exhibit a defective responsiveness to TCR stimulation**

Viability of wildtype and UBPYΔ^T thymocytes with or without TCR stimulation was therefore compared. In a survival assay, thymocytes were incubated without stimulating agents for 0h, 24h and 48h. At each time point, cells were stained with the apoptosis marker Annexin V and a vital dye, Propidium iodide (PI). Vital cells were identified as AnnexinV^−PI^− cells and their frequencies determined (Fig.5.6C). This survival assay shows that there are no differences in the viability of wildtype and UBPYΔ^T thymocytes without TCR stimulation. Next, apoptosis induction was tested by incubating thymocytes with plate-bound CD3 antibodies and soluble CD28 antibodies for 24h. After this incubation, the thymocytes were stained with fluorescent coupled Annexin V and the vital dye Propidium iodide (PI) and analysed by Flow Cytometry. Annexin V has a high affinity towards phosphatidylserine, a phospholipid of the plasma membrane. The translocation of PS from the inner to the outer leaflet of the plasma membrane, making it accessible to Annexin V, is one of the very first indications of apoptosis. Propidium iodide binds to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis. Cells in early apoptosis are therefore stained by Annexin V, but not by Propidium iodide, in contrast to cells in late apoptosis or dead cells, which are stained by both. The results of this assay showed no differences in the susceptibility to apoptosis after *in vitro* stimulation between wildtype and UBPYΔ^T thymocytes (Fig.5.6D).

To test the ability of UBPYΔ^T thymocytes to produce interleukin-2, single-cell thymocyte suspensions were prepared and seeded in 96-well plates with plate-bound CD3 antibodies for 72hours. Concentrations of Interleukin-2 in the cell supernatants were then tested by ELISA. This assay revealed that UBPYΔ^T thymocytes do not produce detectable levels of Interleukin-2 in response to stimulation with anti-CD3 antibodies(Fig.5.6B).

These results show that UBPYΔ^T thymocytes have a disturbed responsiveness to TCR stimulation *in vitro*, as detected by the absence of proliferation and of interleukin-2 production. This seems not to be caused by a decreased viability or an increased susceptibility to apoptosis of the
5.5. \textit{UBPY}^\Delta T \textit{THYMOCYTES EXHIBIT A DEFECTIVE RESPONSIVENESS TO TCR STIMULATION}

UBPY$^\Delta T$ thymocytes \textit{in vitro}. Consequently, in the next steps, the regulation of TCR surface levels and the TCR signal transduction pathways were evaluated.
5.5. **UBPYΔ^T THYMOCYTES EXHIBIT A DEFECTIVE RESPONSIVENESS TO TCR STIMULATION**

**Figure 5.6:** Impaired responsiveness of UBPYΔ^T thymocytes to TCR stimulation. (A) Proliferation assay. In vitro ³H Thymidine incorporation in thymocytes of 6-week-old wildtype (WT, black circles) and UBPYΔ^T (KO, open circles) after stimulation with increasing concentrations of plate-bound CD3 antibody (in µg/ml) and with or without addition of recombinant mouse Interleukin-2 (100u/ml) for 96h. Representative of two independent experiments with n = 3 mice each. (B) IL-2 production in thymocytes from 6-week-old WT (gray bars) and UBPYΔ^T (open bars) mice (n = 3) with or without stimulation with plate-bound anti-CD3 antibody for 72h, determined by ELISA. (C) Survival assay. Thymocytes from wildtype (WT) and UBPYΔ^T (KO) mice were incubated for the indicated times in medium without stimulating agents, stained with fluorescent coupled apoptosis marker Annexin V and the vital dye propidium iodide (PI) and analysed by Flow Cytometry. Left, frequencies of living cells (AnnexinV⁻PI⁻) in WT (black circles) and KO (open circles) thymocytes over time (mean±sem of n = 4 mice). Right, Annexin V staining in the PI⁻ gate of WT and KO thymocytes after t=0h, 24h and 48h incubation. (D) Stimulation induced apoptosis. Thymocytes from wildtype (WT) and UBPYΔ^T (KO) mice were incubated for 24h with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody, stained with fluorescent coupled apoptosis marker Annexin V and the vital dye propidium iodide (PI) and analysed by Flow Cytometry (Left). Cells in early apoptosis were determined by Annexin V⁺PI⁻ staining, cells in late apoptosis and dead cells were detected by Annexin V⁺PI⁺ staining. Right, frequencies of early and late apoptotic cells in wildtype (WT) and UBPYΔ^T (KO) thymocytes (mean±sem, n = 5).
5.6 Normal intracellular trafficking of TCR in UBPyΔT thymocytes

Analysis of the thymocyte development in UBPyΔT mice indicated that UBPyΔT thymocytes fail to upregulate the T cell receptor (TCR) surface levels during their maturation to single positive (SP) thymocytes. The analysis of three TCR components, the TCRβ, CD3ε and CD3ζ chains, in the different thymocyte subsets by Flow Cytometry showed that all three are expressed at lower levels in UBPyΔT SP thymocytes compared to wildtype (Fig. 5.7A), with the exception of the CD3ζ chain, whose expression in CD4SP, but not in CD8SP, thymocytes equaled wildtype levels. Several publications showed that UBPy plays an important role in endosomal trafficking of receptor tyrosine kinases such as the EGFR [9, 11, 10], with a drastic reduction of EGFR levels in UBPy depleted cells [9]. Similar to receptor tyrosine kinases, TCR are constitutively internalized, but rapidly recycled back to the cell surface in order to maintain steady levels of surface TCR [64]. Upon ligand stimulation surface TCR levels are downmodulated, probably by increased targeting of the TCR for degradation in lysosomes instead of recycling [65]. Both EGFR and TCR modulation are mediated by ubiquitination through the activity of the E3 ligase cbl [69, 87]. Therefore low TCR surface levels in UBPyΔT SP thymocytes might be caused by a defective endosomal trafficking of the TCR.

In order to determine the TCR downmodulation rate after ligand stimulation, thymi from 6-week old wildtype and UBPyΔT mice were removed and single-cell suspensions prepared. After lysis of the erythrocytes, total thymocytes were stained with a biotinylated hamster anti-TCRβ antibody on ice. At \( t = 0 \) TCR were crosslinked by addition of an anti-hamster antibody and the cells were incubated at 37°C for different times. At \( t \) the reaction was stopped by addition of ice-cold PBS. The TCR remaining at the surface were stained with phycoerythrin(PE)- labeled streptavidin and PE-fluorescence measured by Flow Cytometry. Additional staining with fluorescent
5.6. NORMAL INTRACELLULAR TRAFFICKING OF TCR IN $UBPY^{\Delta T}$ THYMOCYTES

labeled CD4 and CD8 antibodies allowed the gating on thymocyte subsets. For quantification of TCR downmodulation, the median fluorescence intensity (MFI) associated with TCR-antibody complexes at $t$ was expressed as percentage of the MFI at $t = 0$ and plotted in function of time (Fig.5.7B). In both CD4SP and DP thymocyte subsets the MFI from the surface TCR decreased over time, indicating a successful downmodulation after ligand stimulation. However there was no difference between wildtype and $UBPY^{\Delta T}$ thymocytes. This result clearly shows that UBPY is not involved in the regulation of ligand induced TCR downmodulation.

For analysis of the ligand-independent dynamics, thymocytes from wildtype and $UBPY^{\Delta T}$ mice were incubated for various times in medium alone (unstimulated controls), in medium supplemented with Brefeldin A (10µg/µl) or in medium supplemented with cycloheximide (50µg/µl) at 37°C to allow trafficking of the TCR. At each time point the reaction was stopped by addition of ice-cold PBS (phosphate buffered saline) and the samples stored on ice until all samples were collected. Then the thymocytes were stained with fluorescent coupled antibodies against TCRβ, CD4 and CD8 at 4°C and the fluorescence associated with TCRβ in the DP and CD4SP gates quantified by Flow Cytometry. The percentages of TCR downmodulation under the different conditions in the DP or CD4SP subset (Fig.5.7D) were determined from the median fluorescence values using the unstimulated controls as reference, or the median fluorescence value at $t = 0$ for the untreated samples themselves. Cycloheximide interferes with translation elongation and thereby blocks protein synthesis. Brefeldin A (BFA) severely alters the cellular organelle organization, leading to an extensive tubulation of endosomes, the trans-Golgi network (TGN) and the lysosomes. During BFA treatment, the Golgi mixes with the endoplasmatic reticulum leading to a blockage of the transport of newly synthesized proteins from the endoplasmatic reticulum to the Golgi complex [88, 89]. Additionally BFA induces fusion of the trans-Golgi network with early endosomes, which, although initially reported to leave cycling between plasma membrane and endosomes of transferrin unaffected [88], has meanwhile been shown to impair the endocytic
transport of the TCR [64, 90]. Incubation in medium only revealed that the TCR surface levels in CD4SP remained stable over the observed period of six hours, with no significant difference between wildtype and UbPyΔT mice. In contrast, in DP thymocytes the surface levels constantly increased up to 160% of Sf0 after six hours, with no difference between wildtype and UbPyΔT animals, probably reflecting the ongoing differentiation with TCR upregulation towards SP thymocytes of the DP subset. This result shows that surface TCR levels in UbPyΔT SP thymocytes are not per se instable. Treatment with cycloheximide had no effect on TCR expression in wildtype or UbPyΔT SP thymocytes, but led to a drastic reduction of surface levels compared to untreated cells in DP thymocytes. Therefore, in contrast to surface expression in DP thymocytes, surface expression of the TCR in SP thymocytes is independent of newly synthesized complexes. In DP thymocytes, treatment with BFA had the same effect on TCR expression as treatment with cycloheximide, reflecting the importance of protein synthesis in this thymocyte subset, and probably masking the minor influence of endosomal trafficking. In SP thymocytes, the effect of BFA on TCR expression was different from that of cycloheximide and resulted in a partial reduction of TCR membrane levels of about 20% within the first hour, which was maintained for a couple of hours. However, after six hours, surface levels were restored again, probably due to a compensatory mechanism or incomplete action of Brefeldin A.

Neither treatment with Brefeldin A, nor treatment with cycloheximide revealed any differences in TCR surface modulation between wildtype and UbPyΔT thymocytes. From this it can be concluded that endosomal trafficking and synthesis rates of the TCR are not defective in UbPyΔT thymocytes. This was also supported by the observation that, in contrast to the aforementioned studies showing alterations in endosomal sorting in UbPy-depleted cells [9, 11, 10], the levels of Hrs and STAM were unaltered in UbPyΔT thymocytes (Fig5.7C). As altered responsiveness of UbPyΔT thymocytes might alternatively caused by defects in TCR signal transduction, the signal transduction pathways after TCR stimulation were analysed in the next chapter.
5.6. NORMAL INTRACELLULAR TRAFFICKING OF TCR IN UBPYΔT THYMOCYTES

Figure 5.7: Regulation of TCR surface levels in UBPYΔT mice. (A) TCR surface expression. Flow cytometry of TCRβ, CD3ε, and CD3ζ chains in thymocyte subsets of wildtype (WT, filled histograms) and UBPYΔT mice (KO, open histograms). DN, double negative, DP, double positive, SP single positive. (B) Ligand induced TCR downmodulation in wildtype (WT, filled circles) and UBPYΔT (KO, open circles) thymocytes. Thymocytes were stained on ice with biotinylated hamster anti-TCRβ, the TCR crosslinked with anti-hamster antibody and incubated at 37°C for the indicated times. At t remaining surface TCR were labeled with streptavidin-PE and measured by FACS in CD4SP(left) and DP(right) gates. The percentage of surface expression was determined from the mean fluorescence values of treated cells, using the untreated controls as reference. (mean ± s.e.m.; n = 5). (C) Western Blot of Hrs and STAM in total thymocytes from wildtype (WT) and UBPYΔT animals. (D) Ligand independent modulation of TCR surface levels in wildtype (WT, filled circles) and UBPYΔT (KO, open circles) thymocytes. Thymocytes were incubated for the indicated times in medium alone, in medium with 50μg/μl cycloheximide (CX) or in medium with 10μg/μl Brefeldin A (BFA). At t cells were stained with a fluorescent-coupled anti-TCRβ, anti-CD4 and -CD8 antibodies and TCR surface levels of gated CD4SP and DP thymocytes acquired by FACS. The percentage of surface expression was determined from the mean fluorescence values of treated cells, using the untreated controls as reference (mean ± s.e.m.; n ≥ 4).
5.7 TCR SIGNAL TRANSDUCTION IN UBPYΔT MICE

5.7 TCR signal transduction in UBPYΔT mice

The defective thymocyte development in UBPYΔT mice and the impaired responsiveness of UBPYΔT thymocytes suggested that UBPY might regulate TCR signaling.

To examine that possibility tyrosine phosphorylation induced by stimulation with CD3 and CD28 antibodies in wildtype and UBPYΔT thymocytes was analysed. For that, thymi from 6-week old mice were removed and single-cell suspensions prepared. The cells were resuspended in starving medium (RPMI 1640 medium with penicillin and streptomycin (P/S)) and incubated for 15 minutes at 37°C. After the starving incubation, cells were pelleted, resuspended in prewarmed stimulation medium (RPMI with P/S, 10µg/ml CD3 antibody, 2µg/ml CD28 antibody and 10µg/ml anti-hamster IgG) and incubated at 37°C with moderate shaking (200rpm). At each time point a sample of the cell suspension was taken, immediately transferred into boiling SDS buffer and incubated at 95°C for 15 minutes with vigorous shaking (1000rpm). The resulting SDS protein samples were separated by SDSPage and analysed by Western Blot with specific antibodies. First, overall tyrosine phosphorylation was tested using a Phospho-tyrosine antibody (4G10) provided by Prof. Burkhardt Schraven from the University of Magdeburg (Fig.5.8A). Overall stimulation induced tyrosine phosphorylation was weaker in UBPYΔT thymocytes, particularly visible for the bands at 70kDa and ≈110kDa. In contrast to that, a band at ≈45kDa was stronger after 2 and 5 minutes of stimulation in UBPYΔT thymocytes compared to the wildtype (arrow). The tyrosine kinase ERK has two isoforms of 42 and 44 kDa. Therefore the phosphorylation of ERK1/2 after TCR stimulation was assessed in WT and UBPYΔT thymocytes by Western Blot using an antibody directed against ERK1/2 phosphorylated at Thr202 and Tyr204 of ERK1 (Thr185 and Tyr187 of ERK2). As shown in Fig.5.8B, ERK1/2 was indeed stronger phosphorylated in UBPYΔT thymocytes compared to wildtype after 2 minutes of TCR stimulation. Similarly LAT phosphorylation after 10 minutes of TCR stimulation was stronger.
5.7. TCR SIGNAL TRANSDUCTION IN UBPYΔT MICE

in UBPYΔT thymocytes compared to wildtype, while phosphorylation of Zap70 seemed unaffected. This enhanced signal, however, was only transient, as shown in Fig.5.8C. After 30 minutes of TCR stimulation, ERK1/2 phosphorylation of UBPYΔT thymocytes was markedly lower compared to wildtype thymocytes. LAT phosphorylation in UBPYΔT thymocytes dropped to wildtype levels after 60 minutes of TCR stimulation. Analysis of the immediate early gene c-Fos showed that the aberrant ERK signal in UBPYΔT thymocytes was not able to induce wildtype expression levels of this factor.

It was suggested that deubiquitinating enzymes were functionally linked to E3 ligases due to the coevolution of the two groups of enzymes [23], with the deubiquitinating enzymes being responsible for stabilization of E3 ligases by counteracting their autoubiquitination. In an attempt to find a substrate of UBPY the constitutive expression levels of several E3 ligases, of components of the early TCR signaling complex and the overall pattern of ubiquitination RIPA protein extracts of wildtype and UBPYΔT thymocytes were tested (Fig.5.8D). In contrast to the published UBPY knockout models in different cell lines [9, 11], UBPY knockout in thymocytes did not lead to an accumulation of ubiquitinated substrates. However, for none of the E3 ligases tested, a destabilization in UBPYΔT thymocytes could be observed. Also, all the components of the early TCR signaling complex tested displayed unaltered levels in UBPYΔT thymocytes, implicating that the aberrant TCR signaling in UBPYΔT thymocytes is probably not caused by an altered composition of the early TCR signaling complex.

These results provide evidence for a specific role of UBPY in TCR signal transduction, with lack of UBPY leading to stronger, but more transient ERK phosphorylation. These altered kinetics of the TCR signal are probably not caused by a destabilization of the TCR signaling complex, suggesting a degradation-independent effect of deubiquitination by UBPY on TCR signaling.
5.7. TCR SIGNAL TRANSDUCTION IN UBPYΔΤ MICE

Figure 5.8: Aberrant TCR signal transduction in UBPYΔΤ mice. (A-C) Total thymocytes from 6-week old wildtype (WT) and UBPYΔΤ mice were left untreated (US) or were stimulated with anti-CD3 (10µg/ml), anti-CD28 (2µg/ml) and cross-linked with anti-hamster IgG (10µg/ml). At the times indicated (above lanes) total protein extracts were produced by transferring the cells in boiling SDS sample buffer and the samples subjected to immunoblot analysis. (A) Total protein phosphorylation was analysed by immunoblot with a phospho-tyrosine specific antibody (Phospho-Tyr). The arrow indicates a band with stronger, but more transient phosphorylation. (B) Phosphorylation of ERK1/2, LAT and Zap70 at early time points. (C) Phosphorylation of ERK1/2 and LAT and expression of c-Fos at later time points (detected by high exposure of the immunoblots). (D) Immunoblot analysis of E3 ligases, of components of the early TCR signaling complex and of the ubiquitination pattern in RIPA total protein extracts of untreated wildtype (WT) and UBPYΔΤ (KO) thymocytes. 100µg protein extract/lane, antibodies are indicated on the left side of the blots. Actin was used as loading control and deletion was confirmed with UBPY specific antibody.

Data (A-D) are representative of at least three experiments.
6.1 Successful generation of a T cell specific knockout model for the *in vivo* study of UBPy function

The function of the isopeptidase UBPy has been studied in several cell culture models using different knock-down strategies, from antisense RNA[8] to siRNA[11, 10], overexpression of dominant negative mutants[50] or cre-induced deletion of the loxP-flanked gene[9], all in immortalized cell lines. These studies agreed on a role of UBPy in cell proliferation [8, 9], with UBPy deletion leading to a growth arrest. Also, the accumulation of ubiquitinated substrates at the endosomes associated with aberrant enlargement was congruently observed by several independent work groups[11, 10, 9, 50], establishing UBPy as an important factor in trafficking of ubiquitinated substrates to endosomes and lysosomes. Endosomal trafficking plays a central role in T cell development and function. Since UBPy was also reported to interact with a major player in TCR signaling, the adaptor molecule GADS [12], and with a central regulator of T cell anergy, the E3 ligase GRAIL [13], this lead us to investigate the role of UBPy in T cells. How-
ever, the conventional knockout turned out to be embryonically lethal and cre-mediated induction of overall UBPY deletion in adult mice lead to their rapid death due to a severe liver failure, thus limiting the use of primary material from these mouse models [9]. This work attempted to circumvent these difficulties by establishing a mouse line bearing the UBPY knockout only in T cells. The previously described [9] UBPY\textit{flox/flox} mouse line was crossed to a transgenic mouse strain expressing the cre recombinase under the control of the CD4 promoter. The resulting UBPY\textit{flox/flox} \textit{CD4cre}+ (UBPY\textit{Δ}$^T$) mice effectively deleted UBPY in thymocytes starting from double negative stage 4 with the highest deletion rate of around 80% in double positive thymocytes (Fig.5.4B). The mice also yielded mature UBPY deleted T cells, as could be detected in spleen and lymph nodes. However the deletion rates in these organs were lower than in the thymus, indicating a developmental advantage of the few thymocytes escaping UBPY deletion. The mice were viable and exhibited no obvious phenotypical changes until the age of around six weeks, after which they started to develop a lethal colitis, which will be discussed later. The UBPY\textit{Δ}$^T$ mice are the first T cell-specific knockout mouse model for a ubiquitin-specific protease. These mice represent a convenient tool for the study of UBPY function \textit{in vivo} and \textit{ex vivo}. They allowed to study the impact of UBPY deletion on the immune system and, since UBPY deletion already starts at the T cell precursor stage, on thymocyte development. These mice were also a suitable source of UBPY depleted primary cells (thymocytes) permitting the study of endosomal trafficking by tracing TCR levels. The UBPY\textit{Δ}$^T$ animals also enabled to determine a previously undescribed role of UBPY in TCR signal transduction.

### 6.2 The role of UBPY in T cell development

Thymocyte development proceeds through three main stages at which signal transduction through the pre-T-cell-receptor (pre-TCR) or through the mature $\alpha/\beta$ TCR is required (reviewed in [91]).
6.2. THE ROLE OF UBPY IN T CELL DEVELOPMENT

The first signaling checkpoint takes place at the DN3 stage of development. Thymocytes that have successfully rearranged the TCRβ chain express the pre-TCR composed of the TCRβ chain together with the invariant pre-Tα polypeptide. Effective pre-TCR signaling induces allelic exclusion of the TCRβ locus, thus inhibiting further recombination events, followed by a brief period of strong proliferation leading to expansion of the T cell bearing the functional TCRβ chain. Also, CD4 and CD8 coreceptor expression is initiated. Before entering the DP stage, cells stop to proliferate and enter a cell cycle arrest enabling rearrangement of the TCRα chain. Patterns of CD44 and CD25 surface expression in UBPYΔT DN thymocytes were unaltered and the frequencies and total numbers of DP thymocytes were similar in UBPYΔT and wildtype animals. Only the CD5 expression in DN thymocytes was slightly reduced in UBPYΔT mice (Fig.5.4). Southern Blot analysis showed that in DN thymocytes from UBPYΔT mice the floxed UBPY allele is already deleted in a fraction of the cells (Fig.5.1). The effect on CD5 expression could be due to a starting reduction of UBPY protein levels interfering with ongoing pre-TCR signaling. Even so, the levels of UBPY in DN thymocytes of UBPYΔT mice are obviously sufficient for their normal expansion and differentiation to DP thymocytes.

The second signaling checkpoint occurs at the DP stage of thymocyte development. The DP thymocytes rearrange the TCRα chain and express a mature αβ TCR at their surface. In the thymic cortex, the thymocytes move around until they find a self-peptide MHC complex on which they can be positively selected. Lack of binding leads to death by neglect. After positive selection the thymocytes differentiate into single positive thymocytes and migrate to the thymic medulla, where they again encounter self-peptide MHC complexes, this time presented by medullary thymic epithelial cells (mTEC), specialized for central tolerance. High affinity interaction with these complexes leads to clonal deletion or alternatively to positive selection of regulatory cells. While frequencies and numbers of DN and DP thymocytes in UBPYΔT were
unchanged, both CD4 and CD8 SP thymocyte populations were drastically reduced. The remaining CD4 and CD8 SP cells displayed immature surface phenotypes, with lower expression of CD3ε, CD5 and CD69, markers typically upregulated after positive selection. The most mature thymocytes, with low to absent expression of HSA, were almost absent in UBPYΔ^T mice.

After positive selection, the thymocytes undergo activation and cellular proliferation. Since several publications reported a growth arrest in cells depleted of UBPY [8, 9], it was likely that positive selection in UBPYΔ^T mice was impaired due to a proliferation defect. However, BrdU incorporation experiments in vivo showed that the percentages of proliferating cells within the different UBPYΔ^T thymocyte subsets were not reduced, thus demonstrating that UBPY is not required for cell cycle progression in thymocytes. In vitro, though, proliferation of thymocytes in response to TCR stimulation was impaired, unless exogenous interleukin-2 was added to the medium. An increased susceptibility to apoptosis after TCR stimulation in vitro could not be detected. However, since even in wildtype cells less than 10% of the cells remained viable after 48h of culture, this test might not be suitable to detect differences in apoptosis induction after longer incubation periods. UBPYΔ^T thymocytes also failed to produce interleukin-2 in response to TCR stimulation. Thus, these results showed a defective responsiveness of UBPYΔ^T thymocytes to TCR stimulation, which could be caused by a decreased viability or susceptibility of the thymocytes after longer periods of incubation. These results do not allow a conclusion on defects in positive and/or negative selection in UBPYΔ^T mice. Further studies using mice transgenic for the H-Y TCR will be needed to answer this.

Molecular analysis of the signal transduction events in UBPYΔ^T thymocytes showed that signals through the TCR are transmitted, but display altered kinetics. ERK phosphorylation was stronger at early timepoints but more transient. Recent evidence indicates that beside the strength of the signal transmitted through the TCR also the duration of the signal are crucial factors both for the decision between positive versus negative selection as for lineage commitment (reviewed in
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While negative selecting ligands induce a stronger, but more transient signal, as measured by ERK phosphorylation, positive selecting ligands induce a weaker but more sustained ERK phosphorylation. Similarly, lineage commitment seems to be dependent on the kinetics of TCR signaling, with development of CD4 SP thymocytes requiring stronger and/or longer signals. In UBPYΔT thymocytes, the development of CD4SP thymocytes is more severely affected than the development of CD8SP thymocytes, in terms of frequencies and numbers. Rather than an abrogation of TCR signaling, a defect in mounting a sustained ERK signal in UBPYΔT thymocytes could underlie both the defective positive selection and the alterations in lineage commitment observed in UBPYΔT mice.

How could UBPY possibly regulate TCR signal kinetics? First, we tested if lack of deubiquitination by UBPY would lead to a destabilization of certain components of the TCR signaling machinery or of certain E3 ligases, but for none of the molecules tested an increased degradation could be detected. There is increasing evidence that ubiquitination can regulate TCR signaling in a degradation-independent way. Ubiquitination of the p85 subunit of Phosphatidyl-inositol 3 kinase (PI3K) by cbl-b for example was reported to prevent its recruitment to CD28 and TCRζ, without affecting its stability [59]. Also, negative regulation of TCR signaling through ubiquitination of PLC-γ and Lck was argued to be a proteolysis-independent mechanism [55, 93]. In c-cbl/cbl-b double-knockout mice, a defect in ligand-induced TCR downmodulation leads to prolonged TCR signaling and hyperresponsiveness in mature T cells [69]. It was therefore easily conceivable, that, conversely, increased TCR downmodulation could cause the shortened TCR signals in UBPY deficient thymocytes. However, we could not detect any aberrations in the regulation of TCRβ surface levels neither with nor without stimulation. This result clearly shows that UBPY is not involved in intracellular trafficking of the TCR. In addition to this result, we could not detect an accumulation of ubiquitinated substrates [10, 11, 9], nor could we detect a reduction in the levels of Hrs and STAM as described in previous studies of UBPY depletion. Thus,
endosomal trafficking of ubiquitinated substrates seems not to be globally affected in UBPyΔT thymocytes.

Other than the TCR, intracellular localization of several TCR signaling molecules may influence TCR signaling outcome. A recent study proposed that selection outcome in thymocytes is dependent on subcellular localization of several components of the Ras/ERK pathway [75]. Negative selecting ligands induced a strong and transient ERK phosphorylation, and phosphorylated ERK was preferentially targeted to the plasma membrane. Positive selecting ligands instead induced less ERK phosphorylation more slowly, and phosphorylated ERK accumulated throughout the cell. Similar localization patterns were observed for other members of the pathway such as Ras, Raf-1, Grb-2 and SOS. Differential ubiquitination has been shown to regulate compartmentalized signaling of Ras, with ubiquitinated HRas preferentially associating with endosomes [94]. Deregulation of Ras/MAPK compartmentalization could therefore represent a plausible explanation for the aberrations observed in UBPy-deficient thymocytes, embracing the previously described functions of UBPy in endosomal trafficking and cell growth. Future work will be needed to address this issue.

6.3 The role of UBPy in T cell homeostasis and function

UBPy-deficient mice have defects in T cell homeostasis and function that are manifested by decreased frequencies of naïve and increased frequencies of effector T cell subsets and development of spontaneous colitis.

Colitis is caused by an imbalance between effector and regulatory T cell responses, shifting the homeostasis of the mucosal immune system towards inflammation [76]. As depicted in Figure 6.1, this imbalance can result from either a particularly robust effector response or a weakened
regulatory response.

Figure 6.1: Pathways to mucosal inflammation. Normal mucosal homeostasis is maintained by a delicate balance of effector (green circles) and regulatory (red circles) T cells (upper panel). Disturbance of this balance by impaired regulatory T cell function (middle panel) or, alternatively, by excessive responses of effector T cells despite normal regulatory T cell function (lower panel) leads to mucosal inflammation.

Analysis of young UBPYΔT mice, before onset of disease showed that the peripheral T cells are aberrantly high activated and mostly display an effector phenotype, while naïve and memory T cells were nearly absent.

This is in sharp contrast to the defective responsiveness of thymocytes in UBPYΔT mice, but at a closer view not necessarily contradictory. Under normal conditions, TCR signals transmitted in a thymocyte reflect the affinity/avidity of its TCR to MHC-self antigen complexes and trigger the appropriate selection process. However, in UBPYΔT thymocyte development is impaired, with defects in thymocyte selection probably caused by aberrant TCR signaling. This might alter selection outcome and lead to positive selection of hyperreactive T cells. UBPYΔT thymocytes were not able to proliferate and to produce interleukin-2 in response to TCR stimulation. It is therefore puzzling at first, how any UBPY deleted T cells could appear in the periphery. Two results might contribute to the answer. First, the mature T cell compartment in UBPYΔT mice was only partly composed of UBPY-deficient cells and contained a large fraction cells with the non-recombined UBPY allele, probably due to leaky cre expression in
these cells. Second, proliferation in UBPY-depleted thymocytes could be restored by addition of recombinant Interleukin-2 in the medium. Proliferation and maturation of the UBPY-depleted T cells was therefore likely made possible by the interleukin-2 produced by the wildtype cells. Knockout of the deubiquitinating enzyme CYLD (cylindromatosis gene product), leads to a similar defect in thymic development [55] as in UBPY$^T$mice and to development of colitis due to hyperresponsive peripheral T cells [56]. The group explained this by divergent functions of CYLD in thymocytes and mature T cells. Similarly, UBPY could have a specific function in mature T cells. So, UBPY was proposed to stabilize GRAIL, an E3 ligase necessary for anergy induction [13]. Lack of UBPY in peripheral T cells could therefore interfere with anergy induction as a mechanism of peripheral tolerance.

GRAIL was also shown to be involved in induction of regulatory T cells [95]. We could not detect decreased levels of GRAIL in UBPY$^T$ total thymocyte extracts, showing that UBPY is not required for maintenance of constitutive levels of GRAIL in thymocytes. Stabilization of GRAIL by UBPY might only be needed for the upregulation of the E3 ligase in regulatory T cells and in mature T cells after anergy induction [96]. Regulatory T cells derive from the thymus and are positively selected through a high-affinity recognition of self-peptides presented by thymic stromal cells [97]. In UBPY$^T$ mice, total numbers of CD4+CD25+ regulatory T cells were also reduced. However, instead of the total number, the percentage of CD4+CD25$^{high}$ relative to total CD4+ cells should be taken into account for the evaluation of the regulatory T cell compartment and this frequency is unaltered in UBPY$^T$ mice. Again, the aberrations in TCR signaling might alter outcome of regulatory T cell selection.

UBPY$^T$ mice exhibited a hyperactivation of peripheral T cells prior to onset of the disease. In addition, they had defects in thymocyte selection and lymphopenia resulting from decreased thymic output. A combination of these abnormalities is likely to contribute to the development of colitis in UBPY mice. However, the present state of work does not allow a definitive conclusion.
on this point. Future work should involve \textit{ex vivo} testing of the responsiveness of naïve T cells from \textit{UBPY}$^{\Delta T}$ mice to TCR stimulation. Also, defects in regulatory T cell function should be assessed by transfer experiments.
Summary

Mono- and polyubiquitination controls a multitude of cellular processes beside proteasomal degradation, including the activity of signaling cascades, DNA replication and repair, subcellular localization of proteins and virus budding [20]. The reversibility of ubiquitination is ensured by a large group of deubiquitinating enzymes with important regulatory functions [5, 22].

The ubiquitin specific peptidase UBPY has a vital and non-redundant role, demonstrated by the fact that the conventional knockout of UBPY is embryonically lethal [9]. Further studies revealed a function of UBPY in endocytic trafficking and in ligand-induced downmodulation of receptor tyrosine kinases [10, 11, 9]. Both mechanisms play a central role in T cell development and function. UBPY was also reported to interact with a major player in TCR signaling, the adaptor molecule GADS [12], and with a central regulator of T cell anergy, the E3 ligase GRAIL [13].

To investigate the T cell specific functions of UBPY, cre/LoxP targeting was used to generate a knockout mouse model lacking UBPY exclusively in T cells (UBPY\(\Delta^T\)). UBPY\(\Delta^T\) mice have defects in immune homeostasis, causing hyperactivation of the peripheral T cells, the spontaneous development of inflammatory bowel disease and premature death of the animals. This phenotype probably underlies a defective thymocyte development in UBPY\(\Delta^T\) mice leading to reduced levels of single positive thymocytes and of mature T cells. UBPY, however, is not necessary for normal proliferation of thymocytes and not for endosomal trafficking of the TCR. Rather, UBPY depletion alters TCR signal transduction, leading to stronger but more transient ERK phosphorylation after TCR stimulation. This work could thus determine a previously undescribed function of a deubiquitinating enzyme in the regulation of TCR signal kinetics, with potential importance for other signal transduction pathways.
Zusammenfassung


Um daher die T-Zell-spezifischen Funktionen von UBPy zu analysieren, wurde mittels cre/LoxP targeting ein Mausmodell generiert, in dem UBPy ausschliesslich in T-Zellen ausgeschaltet ist (UBPy\(_{\Delta T}\)).

Die UBPy\(_{\Delta T}\) Mäuse haben ein defektes Immungleichgewicht, welches sich in der Überaktivierung der peripheren T-Zellen und der spontanen Entwicklung einer entzündlichen Darmerkrankung manifestiert und zu einem vorzeitigen Tod der Tiere führt. Diesem Phänotyp dürfte die gestörte Thymozytenentwicklung in UBPy\(_{\Delta T}\) Mäusen zugrundeliegen, die zu einer Verminderung der einfach positiven (single positive) Thymozyten und der reifen T Zellen führt. UBPy ist nicht notwendig für die Proliferation von T Zellen und steuert nicht den Transport des TZR im Endozy-
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toseweg. UBPY Depletion führt hingegen zu einer stärkeren aber verkürzten ERK-Phosphorylierung nach Stimulation durch den TZR. Diese Arbeit konnte daher eine bisher noch nicht beschriebene Funktion eines Deubiquitinierungsenzyms in der Regulation der Kinetik von TZR-vermittelten Signalen zeigen, die auch in anderen Signalwegen eine bedeutende Rolle spielen könnte.
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Curriculum vitae

(Darf aus Datenschutzgründen online nicht veröffentlicht werden.)
Posters, Talks and Publications

Posters


Talks

Regulation of T cell development and immune function by the deubiquitinating enzyme UBPY, FMP and MDC PhD Retreat, Joachimsthal 2007.

Publications


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