The role of Gadd45β in apoptosis and autophagy

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1. Introduction

For every multicellular organism, programmed cell death (PCD) is a constant part of daily processes, at every developmental step and all ages. It is an essential mechanism to maintain cellular homeostasis by balancing biosynthetic and catabolic processes. It rids the organism of unwanted or damaged cells without causing an immune response. There are three major forms of PCD, type I cell death, also called apoptosis (Kerr et al. 1972) and type II cell death, referred to as autophagic cell death (Levine and Klionsky 2004). Type III cell death also known as necrosis, differs from autophagy and apoptosis. In contrast to type I and II PCD, this form of cell death is not immunological neutral: after swelling of the cell, the contents of cells subjected to necrosis are spilled into the surrounding tissue, often resulting in inflammation (Golstein and Kroemer 2007). Although necrosis was believed to be an unguided form of cell death, recent investigations in the field demonstrated a connection between the receptor-interacting serine/threonine-protein kinase (RIP) and necrosis, suggesting that necrosis is a partially regulated process (Holler et al. 2000; Vercammen et al. 1998). This led to the idea of necroptosis, a form of programmed necrotic cell death. Apoptotic cell death is characterised, but not exclusively defined by chromatin condensation, nuclear fragmentation, the presence of proteolytically active caspases and plasma membrane blebbing (Kroemer et al. 2009). Primarily, autophagy is a cell survival process during starvation. However, ongoing autophagy and thus resulting cell digestion was reported to be a cell death mechanism as well (Denton et al. 2011; Levine and Yuan 2005; Galluzzi et al. 2008). Nevertheless, the characteristics of autophagic cell death are beyond dispute: The sequestration of cytoplasmic content in a *de novo* formed double- or multi-membrane-bound structure, the so-called autophagosome (Stromhaug and Klionsky 2001), a process that can get out of control and result in digestion of the whole cell. All three forms of PCD have one thing in common: they are evolutionary conserved mechanisms that shape physiologically relevant processes, such as cell development, homeostasis and

differentiation. Moreover, apoptosis and autophagy are highly relevant in a multitude of different diseases, be it cancer, infection or inflammatory diseases (Mizushima et al. 2008; Ewald et al. 2011; Prescott et al. 2007). Apoptosis and autophagy intersect at multiple points, partly share certain upstream signalling molecules (Matsuzawa et al. 2012; Ivanov and Ronai 2000) and can act in a synchronised manner or mutually exclude each other (Maiuri et al. 2007).

1.1 Autophagy

1.1.1 General considerations about autophagy

The term autophagy is a combination of the greek words “auto” -self and “phagein” –to eat, thus meaning “to digest oneself”. True to its name, autophagy is a catabolic process, which involves the sequestration of cytoplasmic contents into membrane vesicles and their subsequent degradation. Originally, autophagy was found in 1962 by Ashford and Porter (Ashford and Porter 1962). They called their discovery “microbody”, which they observed in rat livers. They described it as portions of the cytoplasm set aside for hydrolysis with the general purpose of providing the protoplast with breakdown products for use in physiological reorientation. The term autophagy was coined one year later in 1963 (De Duve 1963). However, the molecular mechanisms of this engulfment process were largely discovered years later in yeast by the group of Ohsumi (Matsuura et al. 1997; Wada et al. 1992). The use of genetic analysis was mostly responsible for these findings. Progress in experiments with *Saccharomyces Cerevisiae*, *Hansenula polymorpha* and *Pichia pastoris* led to the identification of a whole gene family, which is conserved in all species from yeast to men (Reggiori and Klionsky 2002; Wang and Klionsky 2003). This fact emphasises the importance of this mechanism. The function of these so called *Au**T**opha**G*y-related, or *ATG*, genes were then dissected in numerous studies (Mizushima et al. 1998; Shintani et al. 1999; Tanida et al. 2001; Stromhaug and Klionsky 2001; Nemoto et al. 2003; Levine and Klionsky 2004).

The best-known function of autophagy is the digestion of unneeded/unwanted cellular content in order to maintain cell homeostasis and subsequent recycling of cellular content. Autophagy is one of two cellular mechanisms that performs this function, the other being ubiquitinylation and subsequent proteasomal degradation (Hershko et al. 1981). Autophagy is also needed for the defence of the cell against intracellular pathogens (Tattoli et al. 2012; Knodler and Celli 2011). In addition, the deregulation of the autophagic machinery plays a major role in many diseases, especially neurodegenerative ones (Rubinsztein et al. 2005).

1.1.2 The three different forms of autophagy and their role

There are three main forms of autophagy: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. Microautophagy is a non-selective lysosomal degradation process, which involves the direct engulfment of cytoplasmic cargo by autophagic tubes at the boundary membrane of a lysosome (Sahu et al. 2011; Li et al. 2012). These autophagic tubes mediate both the invagination and vesicle scission into the lumen. The contents of these vesicles are subsequently degraded by lysosomal acidification. Additionally, microautophagy is described as a selective process, but whether this is true in yeast only (Li et al. 2012) or also in mammals (Sahu et al. 2011) remains unclear. In contrast to this, CMA is reported to be an exclusive degradation process of individual soluble proteins by lysosomes. A unique feature of CMA is that proteins reach the lysosomal lumen by directly crossing the lysosomal membrane (Koga and Cuervo 2011). This is accomplished by the chaperone protein Hsc70 (heat shock cognate 70) and cochaperones. They recognize cytosolic proteins, which contain a KFERQ-like pentapeptide and bind to the lysososomal protein LAMP-2a, which acts as a receptor and delivers the unfolded proteins into the lysosomal lumen (Orenstein and Cuervo 2010; Mizushima and Komatsu 2011). However, best investigated is the process of macroautophagy, which is the focus of this work and is hereafter referred to simply as autophagy.

During autophagy, a damaged or otherwise obsolete organelle or portions of the cytoplasm are sequestered in the so-called phagophore, which becomes enlarged by elongation of the

membrane (Chen and Klionsky 2011; Tooze and Yoshimori 2010). When the phagophore is sealed and forms an enclosed structure, it is called an autophagosome (Fig. 1). During later steps of autophagosomal maturation, it fuses with a lysosome to become the autolysosome. In this way, the content becomes acidified and is thus able to break the constituents down into amino acids (Levine and Deretic 2007).

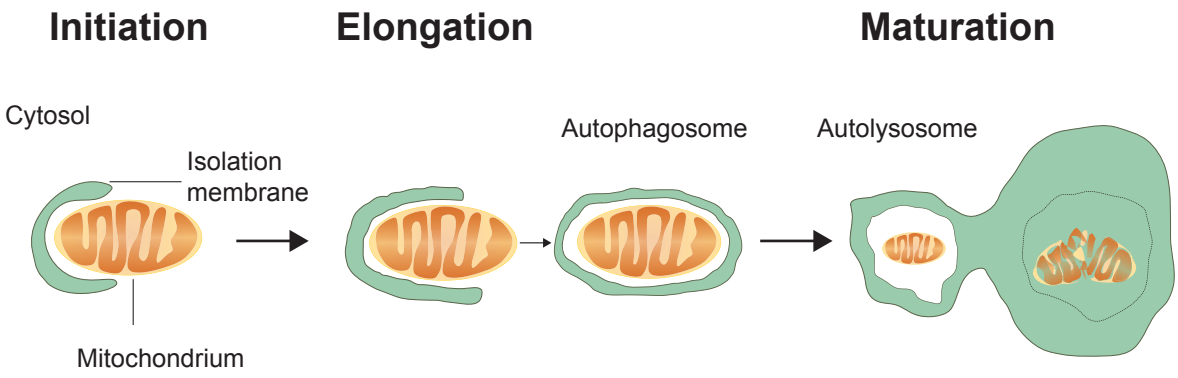


Figure 1: Schematics of the three main stages of the autophagic machinery: Initiation, elongation and maturation.
During initiation, the phagophore engulfs cytosolic contents and organelles by an isolation membrane. The phagophore is elongated and closes eventually, subsequently followed by fusion with a lysosome. Modified according to (Levine and Deretic 2007).

Of note, the origin of this particular, double-layered membrane remains unknown. However, the mitochondrial membrane, the endoplasmatic reticulum (ER), the Golgi and the plasma membrane were reported to play a role in the formation of the autophagosomal membrane (Hailey et al. 2010; Tooze and Yoshimori 2010; Ravikumar et al. 2010). Although the exact mechanisms are not yet fully understood, it is commonly agreed that some unknown machinery must dock at, or be delivered to an assembly point or platform that has been named PAS (pre-autophagosomal structure or phagophore assembly site) (Tooze and Yoshimori 2010).

The maintenance of cell homeostasis during times of starvation or aging is probably one of the best-examined autophagy-inducers (Cuervo et al. 2005). Starvation initiates autophagy, so that supernumerous cellular contents are recycled and the amino acids can be utilised for important cellular processes necessary for cell survival (Jia and Levine 2007). Additionally, autophagy is able to rid the cells of unwanted and potentially harmful cellular content, here the clearance of mitochondria is probably the best known example (Mizushima and Klionsky

2007). Along these lines, mitochondria are removed by autophagy during the terminal differentiation step of erythrocytes and T cells (Novak 2012).

Autophagy is also used for the defence against intracellular pathogens, a process called xenophagy. How pathogens are specifically targeted for degradation and their individual escape mechanisms are highly diverse between different pathogens. For a variety of intracellular pathogens e.g. *Listeria monocytogenes, Mesorhizobium huakuii* and *Rickettsia conorii*, these mechanisms could already be studied, (Kirkegaard et al. 2004). *In vitro* studies also proved autophagy to be relevant in the clearance of viruses, such as herpes simplex virus type I (HSV-1) and the Sindbis virus (Liang et al. 1998; Orvedahl et al. 2007). Interestingly, remnants of autophagic breakdown seem to be later cross presented via MHC Class II molecules (Fig. 2), implying an important role of autophagy in an immunological context as well (Menéndez-Benito and Neefjes 2007; Schmid et al. 2007; Schmid and Münz 2007).

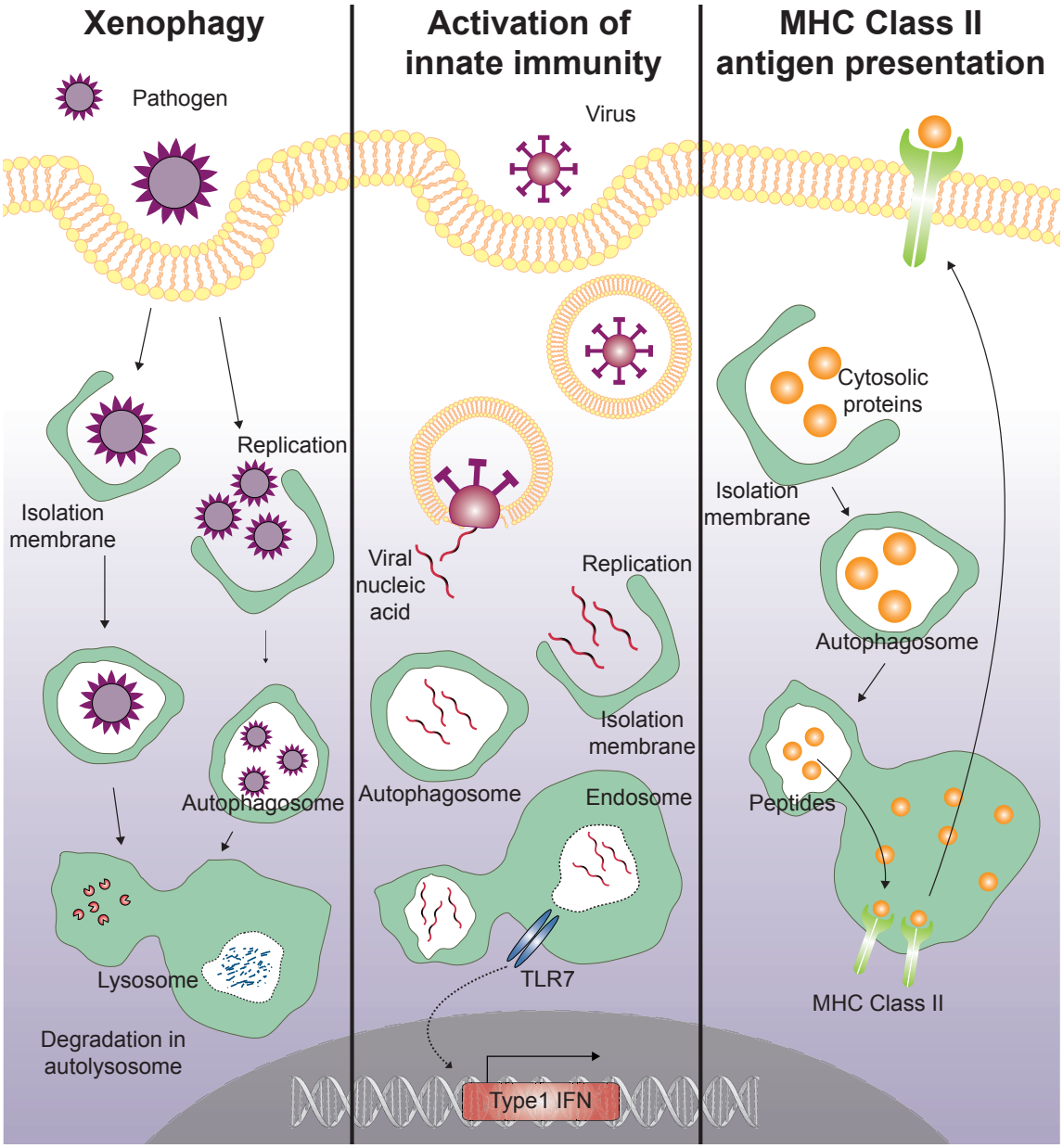


Figure 2: Schematic representation of three important aspects of macroautophagy in innate and adaptive immunity.
Left: In xenophagy, autophagy is directly involved in degradation of an intracellular pathogen. **Middle:** Autophagy may be involved in delivering viral nucleic acid to TLR7 in order to help the cell mount a type1 IFN immune response. **Right:** Autophagy might also be involved in adaptive immunity by mounting endogenously synthesised antigens onto major histocompatibility complex (MHC) class II molecules for antigen presentation to $CD4^+$ T cells. Modified according to (Levine and Kroemer 2008).

Apart from other implications, autophagy, or more specific its dysregulation, is also heavily involved in a number of diseases. Most strikingly are the neurodegenerative diseases, such as Alzheimer's, transmissible spongiform encephalopathy, Parkinson's and Huntington's (Steffan 2010; Rubinsztein et al. 2007). However, recent reports indicate that autophagy also

seems to be involved in cancer, for example by serving as a barrier to limit tumor initiation (Kimmelman 2011; Wojtkowiak and Gillies 2012; Hu et al. 2012).

1.1.3 Regulation and molecular events in autophagy

As described in 1.1.2, autophagy can be separated into three major steps: initiation, elongation and maturation. To simplify the description of the events regulating autophagy and to describe them in detail, the mammalian nomenclature is used. Additionally, only the most prominent isoform of each Atg protein will be used, although multiple ones exist in mammals, but not in yeast. The difference in function between diverse isoforms is a topic of ongoing research.

In the initial step, the main sensor of the overall state of the cell in respect to starvation or growth factor abundance is the mammalian target of rapamycin (mTOR). mTOR is a conserved Ser/Thr protein kinase that regulates cell growth, cell cycle progression, nutrient import, protein synthesis and autophagy (Sarbassov et al. 2005). mTOR is about 280 kDa in size and is a member of the phosphatidylinositol-related kinases (PIKK) group (Bhaskar and Hay 2007). It is evolutionary conserved and all eukaryotic genomes contain a single *TOR* gene (Pattingre et al. 2008). Functionally, mTOR acts in two different complexes: the mTORC1 and the mTORC2 complex.

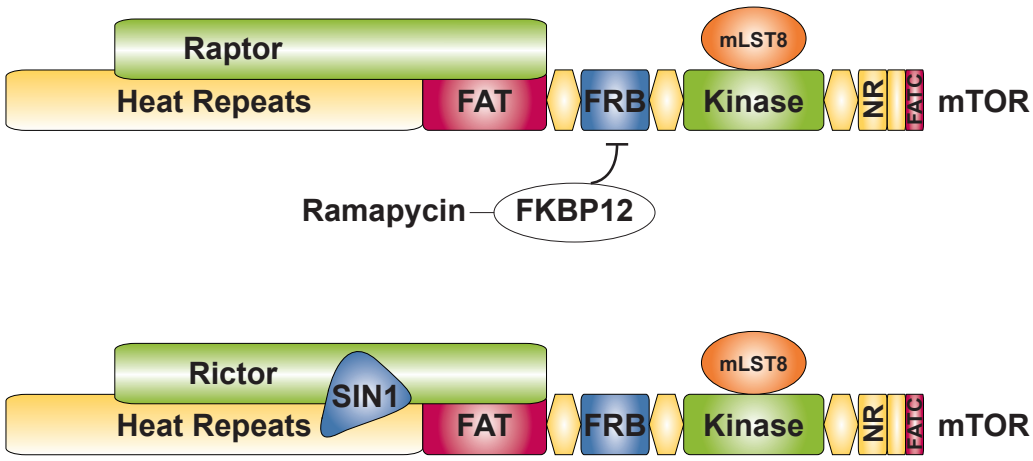


Figure 3: Depiction of the two distinct TOR complexes, mTORC1 and mTORC2.
mTORC1 is in complex with raptor and mLST8 and is rapamycin-sensitive. mTOR2 is rapamycin insensitive and forms a complex with rictor, mLST8 and SIN1. Modified according to (Bhaskar and Hay 2007).

mTORC1 is comprised of mLST8 and Raptor and is sensitive to rapamycin (Menéndez-Benito and Neefjes 2007; Hara et al. 2002; Schmid et al. 2007; Schmid and Münz 2007). mTORC2 is composed of mTOR, mLST8, rictor, SIN1 and protor and is, in contrast to mTORC1, insensitive to rapamycin treatment (Levine and Kroemer 2008; Cybulski and Hall 2009). Although mTORC2 is insensitive to rapamycin, long-term rapamycin treatment can inhibit the assembly of new mTORC2, thus indirectly inhibiting mTORC2 (Steffan 2010; Sarbassov 2005; Rubinsztein et al. 2007). The role of mTORC2 is much less defined than that of the mTORC1 complex. Publications show its involvement in the activation of Akt/PKB regulation by phosphorylation of a serine (Kimmelman 2011; Sarbassov 2005; Wojtkowiak and Gillies 2012; Hu et al. 2012). Interestingly, upstream events of mTORC1 did not seem to be altered when mTORC2 kinase activity was abolished by a knockdown of the rictor complex (Sarbassovet al. 2005; Hresko and Mueckler 2005). Therefore, mTORC2 does not seem to be located upstream of mTORC1. In summary, it can be concluded that the interplay between mTORC1 and mTORC2 is not yet fully understood and further experimental work has to be done (Bhaskar and Hay 2007; Lee et al. 2007).

The first step leading to the initiation of autophagy after mTOR senses starvation signals of the cell is the binding of the ULK1 complex. This complex consists of 4 proteins, the ULK1 serine/threonine protein kinase (mammalian ortholog of yeast Atg1), Atg13, FIP200 (mammalian homolog of Atg17) and the Atg13 binding protein Atg101 (Pattingre et al. 2008; Ganley et al. 2009; Hosokawa et al. 2009; Hosokawa et al. 2009). mTORC1 is inccoperated into this complex, phosphorylating ULK1 and Atg13 (Mehrpour et al. 2010). Under conditions of nutrient starvation or upon rapamycin treatment, mTORC1 disassociates from this complex and thereby enables ULK1 to autophosphorylate and also to phosphorylate Atg13 and FIP200 (Mehrpour et al. 2010).

Another complex responsible for the initiation of phagophore membrane is the Beclin 1:class III phosphatidylinositol 3-kinase (PI3K) complex. It consists of the class III PI3K hVps34, its regulatory protein kinase p150, Beclin 1 and the mammalian homolog of Atg14 (Bhaskar and Hay 2007; Itakura et al. 2008; Sun et al. 2008; Mehrpour et al. 2010). Beclin 1 is a major

interaction partner of multiple proteins, be it cellular ones such as MyD88, AMBRA1 and UVRAG, (Hara et al. 2002; Liang et al. 2006; Fimia et al. 2007; Pattingre et al. 2008) or antiapoptotic ones like the Bcl-2 family members, e.g. Bcl-X_L (Cybulski and Hall 2009; Levine et al. 2008). The interaction with Bcl-2 family members blocks the induction of autophagy by inhibiting the formation of phosphatidylinositol 3-phosphate (PtdIns3P), also known as omegasome (Sarbassov 2005; Liang et al. 1998; Furuya et al. 2005). In conclusion, Bcl-2 is not only an apoptotic, but also an autophagic inhibitor protein.

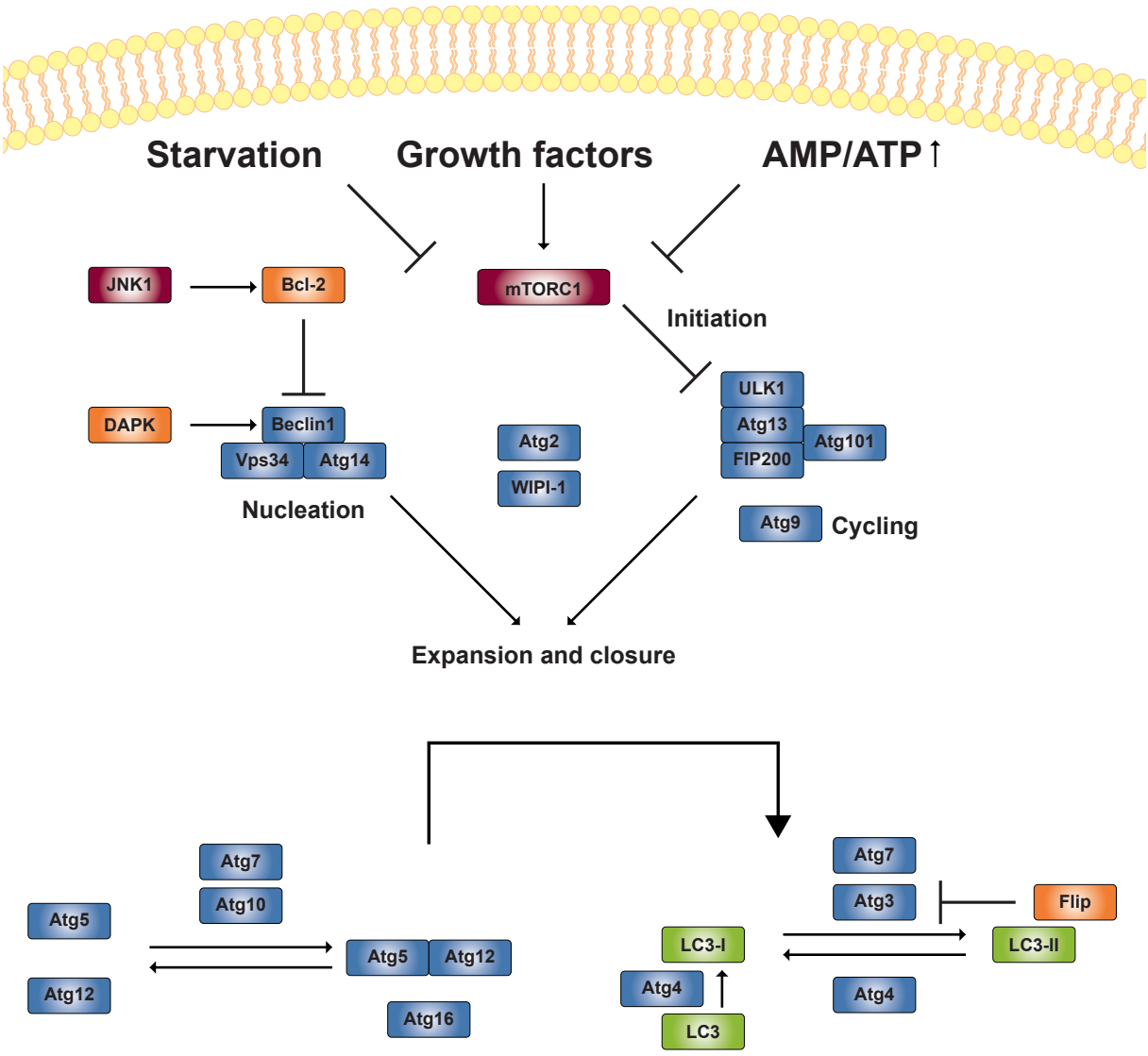


Figure 4: Regulation of autophagy and its relationship with apoptotic mediators.
Depicted are the main complexes responsible for the initiation, the expansion and the maturation of the autophagosomal membrane. Depicted in red are the mediators of apoptosis involved in autophagy. Purple are protein kinases with substrates in the autophagic machinery. Green is LC3. Modified according to (Mehrpour et al. 2010).

Subsequently, the Beclin 1/hVps34 complex interacts with WIPI-1 via its PtdIns3P-binding site (Hresko and Mueckler 2005; Proikas-Cezanne et al. 2004). WIPI-1 and Atg2 are both linked to the biogenesis of the autophagosome, but the exact mechanism remains unresolved (Lee et al. 2007; Mehrpour et al. 2010). Also not known is how the Beclin 1/hVps34 complex acts in unison with the ULK1 complex (Fig. 4). In summary, both complexes together initiate the formation of the autophagosomal membrane and provide the interface between sensing the general state of the cell and the activation of the autophagic machinery.

After initiation, the elongation phase is coined by two different ubiquitin-like conjugation systems, the LC3 (Atg8 in yeast) and the Atg12 system (Ganley et al. 2009; Ohsumi 2001; Hosokawa, Sasaki, et al. 2009; Yorimitsu and Klionsky 2005; Hosokawa et al. 2009). Atg12 is a 186-amino-acid hydrophilic protein, which is homologous to ubiquitin (Itakura et al. 2008; Mizushima et al. 1998; Sun et al. 2008; Mehrpour et al. 2010). Here, it was reported that Atg12 covalently binds to Lys149 of the Atg5 (Liang et al. 2006; Mizushima et al. 1998; Fimia et al. 2007; Pattingre et al. 2008). This complex formation is dependent on Atg7 and Atg10 (Levine et al. 2008; Mizushima et al. 1998). Atg16 stabilises the Atg12-Atg5 complex and anchors it to the membrane (Liang et al. 1998; Matsushita et al. 2007; Furuya et al. 2005).

It should be noted, that Atg5 is a key protein in autophagosome formation and in the autophagic process in general. Although there are reports of an Atg5-independent autophagosomal pathway (Mehrpour et al. 2010; Nishida et al. 2009), the majority of autophagy seems to be Atg5-dependent. A fact which is emphasised by a study from Kuma and colleagues: $Atg5^{-/-}$ mice do not survive the early neonatal starvation period (Kuma et al. 2004). The fatality of this defect clearly shows the unique role Atg5 plays in the autophagosomal pathway. Of note, Atg5 expression also seems to be directly connected to certain autoimmune diseases, for example multiple sclerosis and autoimmune demyelination (Mehrpour et al. 2010; Alirezaei et al. 2009).

Subsequently, the Atg12-Atg5 conjugation system initiates the second conjugation system. This one is important for the elongation process, namely the LC3 (Atg8) conjugation system

(Fig.5). In this system the COOH terminus of LC3 is conjugated to the polar head of phosphatidylethanolamine (PE) (Ohsumi 2001; Scarlatti et al. 2009; Yorimitsu and Klionsky 2005). LC3-PE formation (also known as LC3-II) depends on Atg7 and Atg3, which have a function similar to the E1 and E2 enzymes in the ubiquitin pathway, respectively (Mizushima et al. 1998; Ichimura et al. 2000; Tanida et al. 1999; Meijer and Codogno 2004). Atg4 primes LC3 for conjugation by exposing a glycine residue at the COOH terminus (the form of LC3 with this glycine exposed is also known as LC3-I) (Scarlatti et al. 2009). Interestingly, LC3 is recruited to both the internal and the external surface of the expanding autophagosomal membrane, a fact which predestines the protein as a marker for autophagosomes (Noda et al. 2009; Mehrpour et al. 2010). After the closure of the autophagosomal membrane, LC3-PE is released in an Atg4-dependent way, along with all other Atg proteins assembled on the phagophore (Kirisako et al. 2000). In contrast to this, the LC3 on the inner surface of the autophagosomal membrane remains and is later subjected to acidification and lysis along with the rest of the autophagosomal cargo (Ichimura et al. 2000; Kabeya et al. 2000).

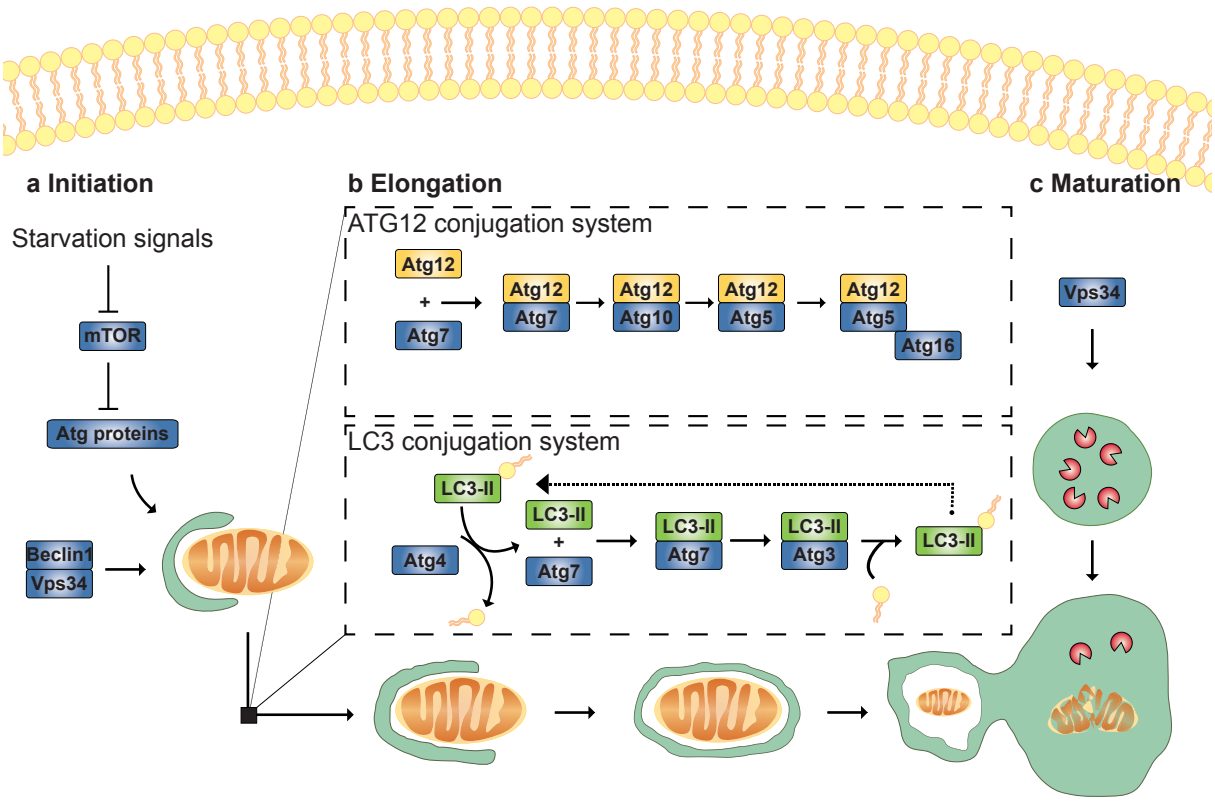


Figure 5: Schematic overview of the molecular events in autophagosomal maturation.
Special emphasize lies on the elongation system and its two ubiquitin-like conjugation systems: Atg12 and LC3. Modified according to (Levine and Deretic 2007).

Additionally, LC3 performs another important role in selective autophagy: LC3-II is able to bind to p62/SQSTM1 and thus targets ubiquitinylated proteins for autophagosomal degradation (Bjørkøy et al. 2005; Bjørkøy et al. 2006). In detail, p62 can bind ubiquitinylated proteins through its ubiquitin-associated (UBA) domain and LC3-II through its LC3-interacting region (LIR) (Bjørkøy et al. 2005; Bjørkøy et al. 2006). p62 thereby seems to be the missing link between ubiquitinylation and autophagy. Interestingly, although both forms of LC3 can bind p62, only the lipidated form of LC3, bound to the autophagosomal membrane, is involved in p62 recruitment to autophagosomes (Fig. 6) (Shvets et al. 2011).

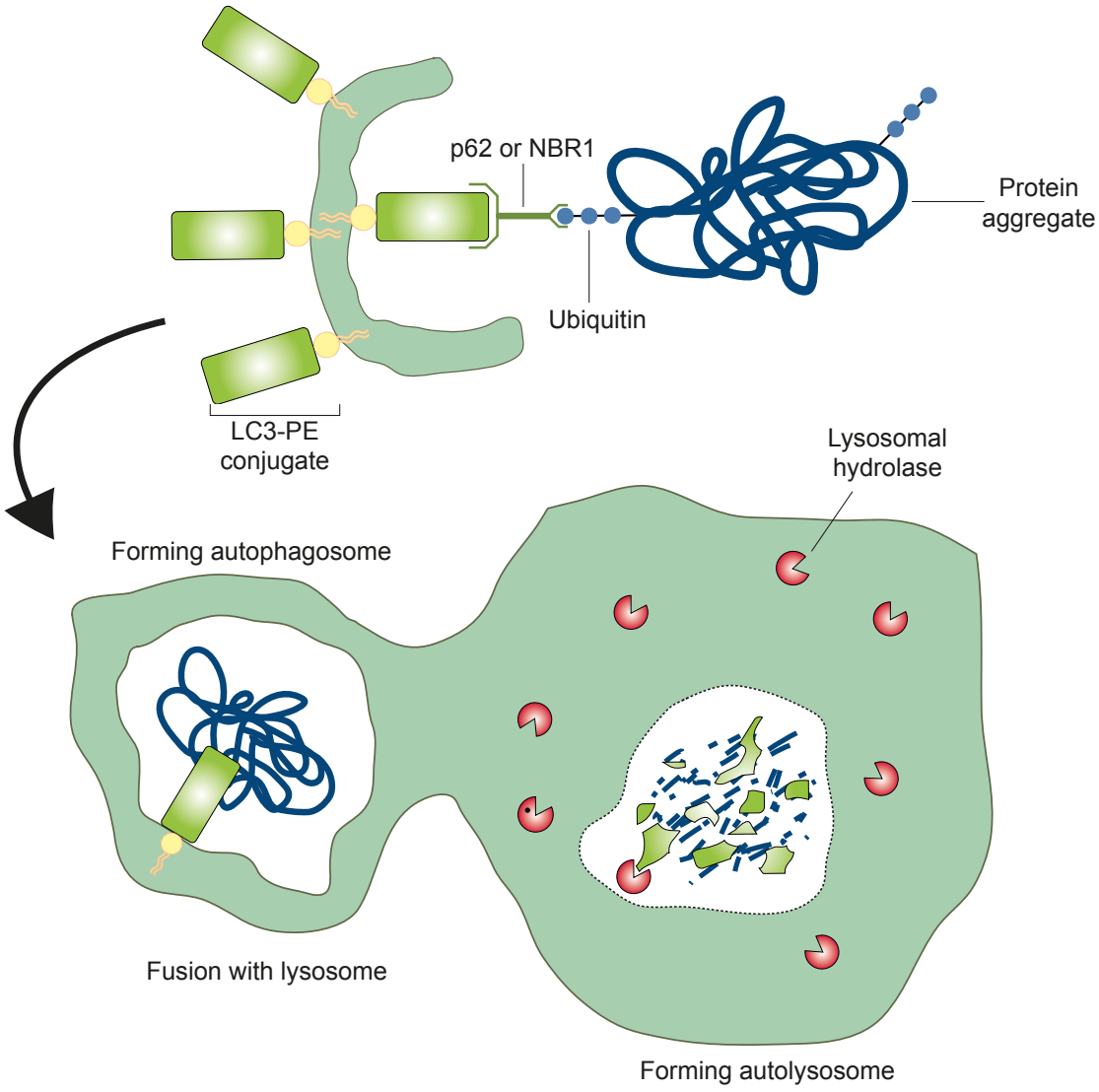


Figure 6: A model for the selective autophagic degradation of protein aggregates.
p62 binds to ubiquitylated proteins and LC3-PE (LC3 II) and thus specifically targets proteins for autophagosomal degradation. Modified according to (Tyedmers et al. 2010).

The last step in the autophagosomal process is maturation, which is largely defined by the fusion of the autophagosome with a lysosome (Wang et al. 2002; Wang et al. 2003). This results in the formation of an autophagolysosome and subsequent degradation of internalised proteins (Teter et al. 2001; Nakamura et al. 1997). However, a closer look at this subject shows that a direct fusion of autophagosomes with lysosomes seems to be the exception, not the norm. Before the autophagosome fuses with a lysosome, it usually merges with an endosome or a multivesicular body (Razi et al. 2009; Stromhaug and Seglen 1993; Fass et al. 2006).

The maturation of autophagosomes, here explicitly the fusion of autophagosomes with lysosomes, is controlled by proteins which are involved in controlling the membrane fusion during intracellular transport (Rab GTPase, SNARE and ESCRT proteins, and the function of acidic degradative compartments (v-ATPase, LAMP proteins, lysosomal carriers, and lysosomal hydrolases)) (Mehrpour et al. 2010; Eskelinen 2005; Fader and Colombo 2009; Rusten and Stenmark 2009).

Most interestingly, Beclin 1, previously mentioned to play a role in the initiation of autophagy, is also involved in the maturation of autophagosomes. During autophagosome maturation, it can interact with Rubicon or UVRAG (Liang et al. 2006; Matsunaga et al. 2009; Zhong et al. 2009). When forming a complex with hVps34, UVRAG and Rubicon, Beclin 1 represses the maturation of autophagosomes. In contrast the same Beclin 1/hVps34/UVRAG complex, but without Rubicon, enhances autophagosomal maturation (Matsunaga et al. 2009; Zhong et al. 2009). These results demonstrate that Beclin 1 acts at two distinct steps of the autophagosomal pathway and is thereby an important regulator of autophagy.

1.1.4 Autophagy in T cells

One of the first studies that could prove the essential role of autophagy in T cells was investigating the role of Atg5 and in T cell survival and proliferation (Pua et al. 2007). The authors were the first to show autophagosome formation in primary mouse T lymphocytes. Interestingly, Pua and colleagues identified fully mature lymphocytes in $Atg5^{-/-}$ bone marrow

chimeric mice. However, the $Atg5^{-/-}CD8^{+}$ lymphocytes were largely decreased in numbers and $Atg5^{-/-}CD4^{+}$ lymphocytes were unable to proliferate efficiently upon T cell receptor (TCR) stimulation (Pua et al. 2007). More studies followed that could demonstrate a role of autophagy in T cells. More recently, T cells with a conditional Atg7-deficiency were found to exhibit a proliferation defect similar to caspase $8^{-/-}$ T cells upon lymphocytic choriomeningitis virus (LCMV) infection (Ch'en et al. 2011). The group further showed that in resting T cells the autophagosomal content was mainly composed of mitochondria, whereas in activated T cells it was largely made of cytosolic contents (Ch'en et al. 2011). This would argue for an energy-providing function in activated T cells as opposed to a cell proteome- and organelle-renewal function in resting T cells. Also using $Atg7^{-/-}$ T cells, a recent study by the same group that demonstrated the importance of Atg5 in T cell proliferation, revealed that upon a severe defect of autophagy, influx of Ca^{2+} is impaired (Jia et al. 2011). They demonstrated that this defective Ca^{2+} influx is due to the inability of autophagy-defective T cells to deplete the intracellular Ca^{2+} stores (Jia et al. 2011). Moreover, mice with lymphocytes deficient in Vps34 also showed a severely reduced T lymphocyte compartment (Mcleod et al. 2011). The reason for this reduction was found to be a large decrease in expression of IL-7Rα on the cell surface, an essential surface receptor for naïve T lymphocytes (Mcleod et al. 2011). These experiments clearly show the important role of autophagy in maintaining cellular homeostasis in T cells, be it in providing energy or in signalling events.

However, autophagy in T cells has been found to have pro-apoptotic functions as well, a fact that will be discussed later in this work (chapter 1.3.4). Additionally, recent publications could link autophagy to autoimmune diseases, including systemic lupus erythematosus (SLE). T cells have been shown to play a cardinal role in SLE initiation, progression and execution (La Cava 2009). Recent publications could link mTOR activation to abnormal activation of lymphocytes in SLE (Fernandez and Perl 2009; Fernandez and Perl 2010). Here, mTOR is involved in SLE through its mitochondrial hyperpolarisation (MHP) function, which is triggered by glutathione depletion and increased nitric oxide levels, both of which are

commonly observed in SLE patients (Nagy et al. 2004). Additionally, single-nucleotide polymorphisms (SNPs) in Atg5 could be linked to increased SLE susceptibility in a genome-wide study of 1745 individuals (Zhou et al. 2011). Taken together, these studies demonstrate the importance of autophagy in T cell survival, homeostasis and immunity.

1.2 Apoptosis

1.2.1 General considerations about apoptosis

Apoptosis is one of the most-investigated cell biological topics, which also exerts a high impact on immunology worldwide and still is a subject of intense research. The term apoptosis is Greek and can be translated as apo- off ptosis- falling, thus, the falling of a dead leaf. As already mentioned, apoptosis is characterised by several different cellular events, such as e.g. membrane blebbing (Kroemer et al. 2009). Normally, it occurs during development and aging and is a homeostatic process that depletes unnecessary cell populations in tissues (Elmore 2007). Apoptosis can be induced by a multitude of different stimuli like e.g. exposure to ultraviolet light (Dunkern et al. 2001) or chemical substances (Zhang et al. 2000). In both cases, apoptosis is usually mediated in a p53-dependent way (Nayak and Cooper 2012). Apoptosis can also be triggered by external signalling molecules through death receptor stimulation (Yurchenko et al. 2012).

The process of apoptosis is well defined and can be separated into early and late events. Early morphological events in apoptotic cell death include cell shrinkage and pyknosis (Kerr et al. 1972), which are both visible under the light microscope. Pyknosis is greek and means “to condense”, thus meaning an irreversible condensation of chromatin in the nucleus. Membrane inversion and the resulting exposition of phosphatidylserine on the outer cell surface is also a hallmark of early apoptosis (Taylor et al. 2008). At the same time, these externalised phosphatidylserines also serve as an “eat me signal” that causes the cell to be recognised and subjected to phagocytosis by macrophages (Fadok et al. 1992). A late event

in apoptosis is the fragmentation of DNA into oligonucleosomal fragments (Wyllie et al. 1980; Wyllie et al. 1984).

In order to measure apoptotic cell death, these processes can be used to determine the amount and the dynamic of on-going apoptosis. The importance of tightly regulated apoptosis can be observed via its absence: If apoptosis is impaired, tumor growth increases (Ewald et al. 2011; Ivanov and Ronai 2000). Moreover, apoptosis is vital in keeping autoimmunity in check (Nagata et al. 2010). Interestingly, too much apoptosis or defects in engulfment of apoptotic bodies can result in autoimmunity as well (Nagata et al. 2010). Thus, a well-balanced apoptotic machinery is vital to an organism. In order to estimate the impact of apoptosis in the context of negative selection in the thymus, it was shown by the use of transgenic mice that two thirds of positively selected thymocytes in these mice were subjected to negative selection (van Meerwijk et al. 1997; Laufer et al. 1999). The majority of these positively selected T cells are usually deleted during maturation and are potentially autoreactive, which can lead to severe autoimmune diseases (Lamhamedi-Cherradi et al. 2003).

1.2.2 Caspases

Caspases are the central components of apoptosis (Shi 2002). They are named because of their function as cysteine proteases, since they cleave their substrates following an Aspartate residue (Alnemri et al. 1996). Caspases are a family of conserved proteases, which exert various different functions and can by these means be separated into 3 different subgroups, namely the inflammatory, the initiator and the executioner caspases (Denault and Salvesen 2002). All caspases do have in common that they are synthesised as a catalytically inactive pro-enzymatic form (zymogen) (Riedl and Shi 2004). Caspases have been reported to play a role in species ranging from yeast to men (Madeo et al. 2002; Kumar and Doumanis 2000; Ahmad et al. 1997). The inflammatory caspases 1, 4, 5, 11, 12 and 14 are mainly important for the proteolytic cleavage of inflammatory cytokines including IL-1β and IL-18 (Denault and Salvesen 2002).

Apoptotic caspases are mainly responsible for the regulation of apoptosis and are divided into two subgroups, namely initiator and effector caspases (Riedl and Shi 2004). The initiator caspases consist of caspase 2, 8, 9, and 10. They are characterized by two distinct subunits: the large (~p20) and the small (~p10) subunit (Riedl and Shi 2004). Initiator caspases have a large pro-domain, which is typically needed for protein-protein interactions (Taylor et al. 2008). The activation of these caspases takes place by dimerization of multi-protein complexes via the Death Effector Domain (DED) for caspase 8 and 10 or via the Caspase Activating Recruitment Domain (CARD) for caspase 2 and 9 (Boatright et al. 2003).

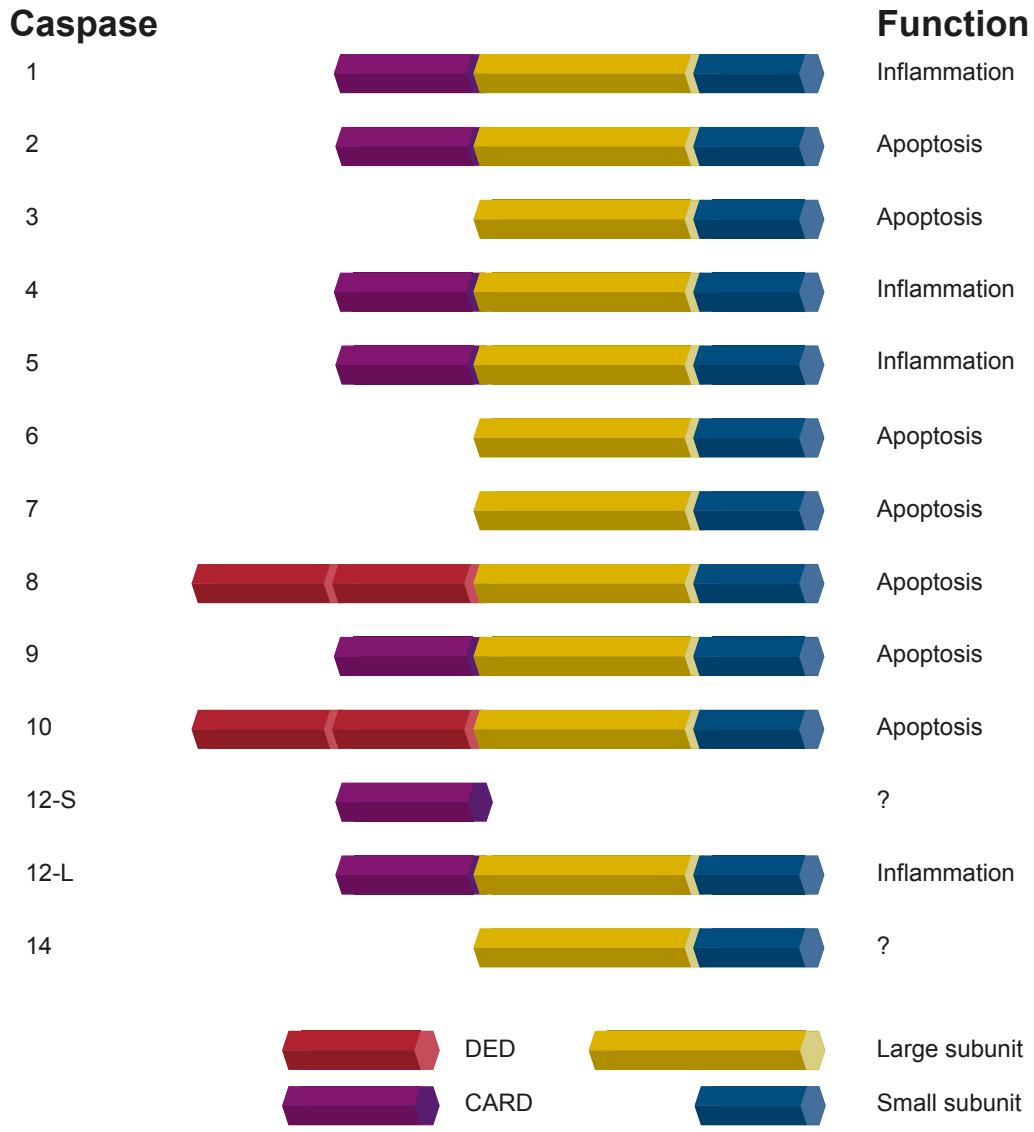


Figure 7: Schematic overview of the two different types of apoptotic caspases and the inflammatory ones, their function and structure.
Caspases are separated into three different subgroups according to their function: Effector caspases, initiator caspases and inflammatory caspases. Depicted are the different caspases, their classification and their composition. Modified according to (Taylor et al. 2008).

The effector caspases form the second group, which comprise caspase 3, 6 and 7. These caspases are located in the cytoplasm in an already dimerised form and only need to be cleaved by the initiator caspases (Riedl and Shi 2004). After being cleaved, the active catalytic centre is accessible and the caspases proteolytically cleave a broad spectrum of cellular targets, ultimately leading to cell death (Salvesen and Abrams 2004).

1.2.3 Regulation and molecular events in apoptosis

Two major pathways are known to act during apoptosis and are activated depending on the origin of the death stimulus, namely the extrinsic and the intrinsic pathway (Riedl and Shi 2004). The binding of an extracellular death ligand to its cell surface receptor activates the extrinsic, also called receptor-mediated apoptosis pathway (Wajant 2002). The probably best-studied example here is the binding of the CD95L/ FasL to its receptor, the CD95/Fas/Apo-1 (Nagata 1999). CD95 is a member of the tumor necrosis factor (TNF) superfamily of receptors and its main function is the induction of apoptosis, although other functional roles begin to emerge (Wajant 2002). CD95 receptors form pre-associated homodimers on the cell surface upon expression (Siegel 2000; Papoff et al. 1999). After activation of the CD95 receptor, the death-inducing signalling complex (DISC) is formed which comprise trimerized CD95, FADD and caspase 8 (Kischkel et al. 1995). Due to its death domain (DD), activated CD95 is able to bind the Fas-associated DD containing protein (FADD) (Chinnaiyan et al. 1995; Boldin et al. 1995). FADD itself also contains another protein-protein interaction domain, the death effector domain or DED. By means of this, it can recruit certain caspases e.g. caspase 8, which also contains a DED domain (Scaffidi et al. 1997). These two proteins together form the key components of the DISC, as caspase 8, in high concentrations, is believed to be able to autoproteolytically cleave and thereby activate itself (Medema et al. 1997; Salvesen and Dixit 1999). Subsequently, the initiator caspase 8 cleaves and activates the effector caspase 3, which ultimately leads to cell death (Fig.8) (Peter and Krammer 2003).

The intrinsic apoptotic pathway is generally triggered by death stimuli that are sensed from within the cell. Examples of these stimuli are oncogene activation, radiation, toxins, free

radicals and DNA damage and its inactivation is considered an indicator of cancer (Hanahan and Weinberg 2000).

The pathway starts with the so-called BH3-only proteins (Taylor et al. 2008). These normally inactive proteins become active under the various stresses described earlier. BH3-only proteins (BAD, BIK, BID, HRK, BIM, BMF, NOXA and PUMA) have a conserved BH3 domain that can bind and thereby inhibit the anti-apoptotic potential of the BCL-2 proteins (BCL-2, BCL-XL, BCL-W, A1A and MCL1) (Taylor et al. 2008; Youle and Strasser 2008). Thus, the pro-apoptotic BCL-2 family members BAX and BAK are no longer inhibited themselves and can induce the mitochondrial outer membrane permeabilisation (MOMP) (Wei et al. 2001; Tait and Green 2010). As a consequence of this disruption of the mitochondrial outer membrane integrity, the membrane potential gets lost and therefore pro-apoptotic proteins from the intermembrane space are released into the cytosol (Saelens et al. 2004; Elmore 2007). Of the pro-apoptotic molecules released after MOMP, cytochrome *c* is the best investigated one (Wang 2001). Upon release from the outer mitochondrial membrane (OOM), cytochrome *c* binds to APAF1 and thus leads to the assembly of the so-called apoptosome, a heptameric protein ring (Fig. 8). In turn, the APAF1- cytochrome *c* complex can bind and activate pro-caspase 9 and induce apoptotic cell death (Wang 2001; Shi 2006; Youle and Strasser 2008).

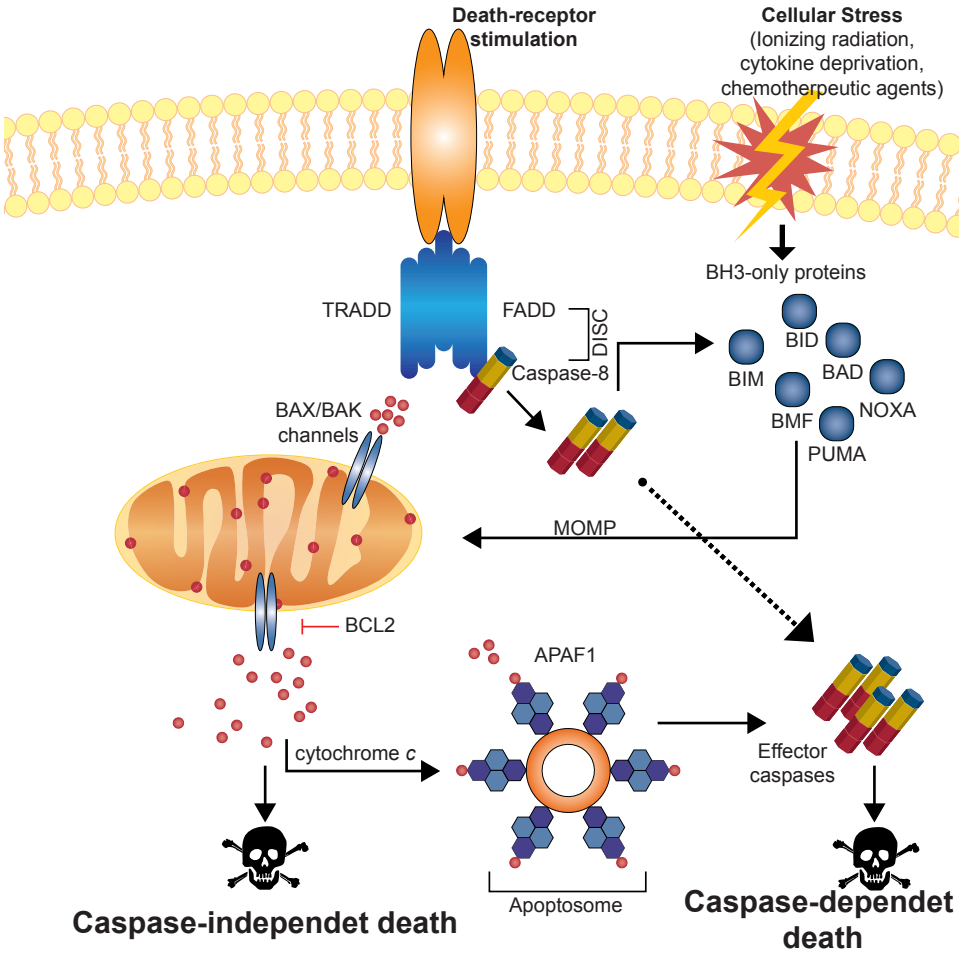


Figure 8: Schematic overview of extrinsic and intrinsic cell death:
The death receptor for the extrinsic pathway, as well as the most prominent BCL-2 family members are depicted. The relationship of the two pathways in respect to caspase activation and ultimately cell death are shown. Modified according to (Maiuri et al. 2007).

Interestingly, the two distinct apoptotic pathways are connected to each other via caspase 8 cleavage of the BH3-only protein BH3 interacting domain death agonist (BID). This results in truncated BID or tBID, which is thought to act together with BIM on BAX, thus giving rise to the MOMP (Youle 2007; Walensky et al. 2006). This model is called the direct-activation model. However, a direct binding of BIM or BID to BAX has not been shown. Additionally, a significant effect on apoptotic events in cells of the *Bim*$^{-/-}$/*Bid*$^{-/-}$ double deficient mice could not be shown, further strengthening the point that the effect of BID is an indirect one, through interaction with the anti-apoptotic BCL-2 family members (Willis et al. 2007).

The direct connection of the extrinsic and intrinsic apoptosis pathways through BID is especially important for so-called type II cells. In contrast to type I cells where the extrinsic apoptotic pathway is sufficient to induce cell death, type II cells cannot muster enough

initiator caspases to induce cell death (Samraj et al. 2006; Scaffidi et al. 1998). In order to activate caspase 3 properly, type II cells therefore need the intrinsic apoptosis pathway in addition to the extrinsic one.

1.2.4 Apoptosis in negative selection of T cells in the thymus

It is mandatory for an intact immune system to be able to distinguish between self and non-self. In this way, potentially harmful foreign organisms can be eliminated without posing a threat to the body. Along these lines, the immune system must tolerate self-antigens but has to be able to recognise degenerated tissue, thus cancer, and eliminate it before it spreads (Chen et al. 1993). Non-pathogenic foreign antigens, e.g. food antigens, also must be prevented from triggering an immune response. The cornerstone of an intact immune system is therefore the correct priming of immune cells. Lymphocytes are the key players in the body that mediate this balance between inflammatory response and tolerance (Hogquist et al. 2005). The thymus is the organ in which lymphocytes, thus the name T cell, mature and it is also the place where T cells are confronted with self-antigens to convey tolerance (Hogquist et al. 2005). Hematopoietic stem cells from the bone marrow are the progenitor cells of lymphocytes and these seed the thymus and undergo maturation there (Palmer 2003). The thymus is comprised of two anatomically different regions: The outer region or cortex, which contains immature thymocytes and the inner region or medulla, which holds mature thymocytes (Petrie 2002).

One of the earliest steps during thymocyte development is the expression of an antigen receptor or $\alpha\beta$ **T c**ell **r**eceptor (TCR) (Starr et al. 2003; Schatz and Ji 2011). T cells without such a receptor are so-called double-negative (DN) thymocytes, as they are DN for the co-receptors of the TCR, cluster of differentiation 4 and 8 (CD4 and CD8). DN thymocytes form a pre-T cell receptor, which is characterised by a β chain and a pre-T cell α receptor chain (Palmer 2003). This pre-α-TCR chain could be identified as a crucial component of thymocyte development (Fehling et al. 1995). During the maturation process, the T cells pass through several developmental DN steps, characterised by the expression of the cell surface

markers CD44 and CD25 (Lind et al. 2001). During these developmental steps, the antigen receptor evolves (Starr et al. 2003). This is characterised by the antigen receptor re-arrangement. This re-arrangement is a stochastic process, in which the genes that encode the receptor are re-combined as arrays of variable (V), diversity (D) and joining (J) gene segments. The RAG protein complexes (RAG1 and RAG2) introduce a double-strand brake in the DNA at specific recombination signal sequences (RSSs), flanking each V, D and J gene segment (Schatz and Ji 2011). As a result, a large variety of different, specific receptors are generated (Davis and Bjorkman 1988). From a mathematical point of view, up to $3x10^{11}$ different combinations are possible. After V(D)J recombination, the thymocytes are called double positive (DP), as they have become DP for the expression of the surface proteins CD4 and CD8. These double positive thymocytes can undergo three different cell fates: positive selection, negative selection and death by neglect (Starr et al. 2003). Around 90% of the generated TCRs are not able to receive a survival signal via MHC from an antigen presenting cell (APC) and therefore the thymocytes die. This process is called death by neglect (Zilberman et al. 1999). The remaining 10% of the thymocytes can interact with an MHC. For these, two different outcomes are possible: On the one hand the TCR interacts with an MHC molecule with a rather low affinity, which results in positive selection of these thymocytes and subsequent differentiation into either $CD8^+$ T cells or $CD4^+$ T cells, depending on the MHC molecule (MHC class I or MHC class II peptide) (Fig. 9) (Hedrick 2012).

On the other hand, the thymocytes can interact with an MHC molecule with a high affinity. Here, the thymocytes recognise self-antigen very efficiently and thus are deleted by apoptosis induction. This process is called negative selection (Starr et al. 2003; Palmer 2003; Stritesky et al. 2012). The orphan steroide receptor Nur77, an intracellular transcription factor, serves as a well-accepted marker for negative selection (Calnan et al. 1995; et al. 2003).

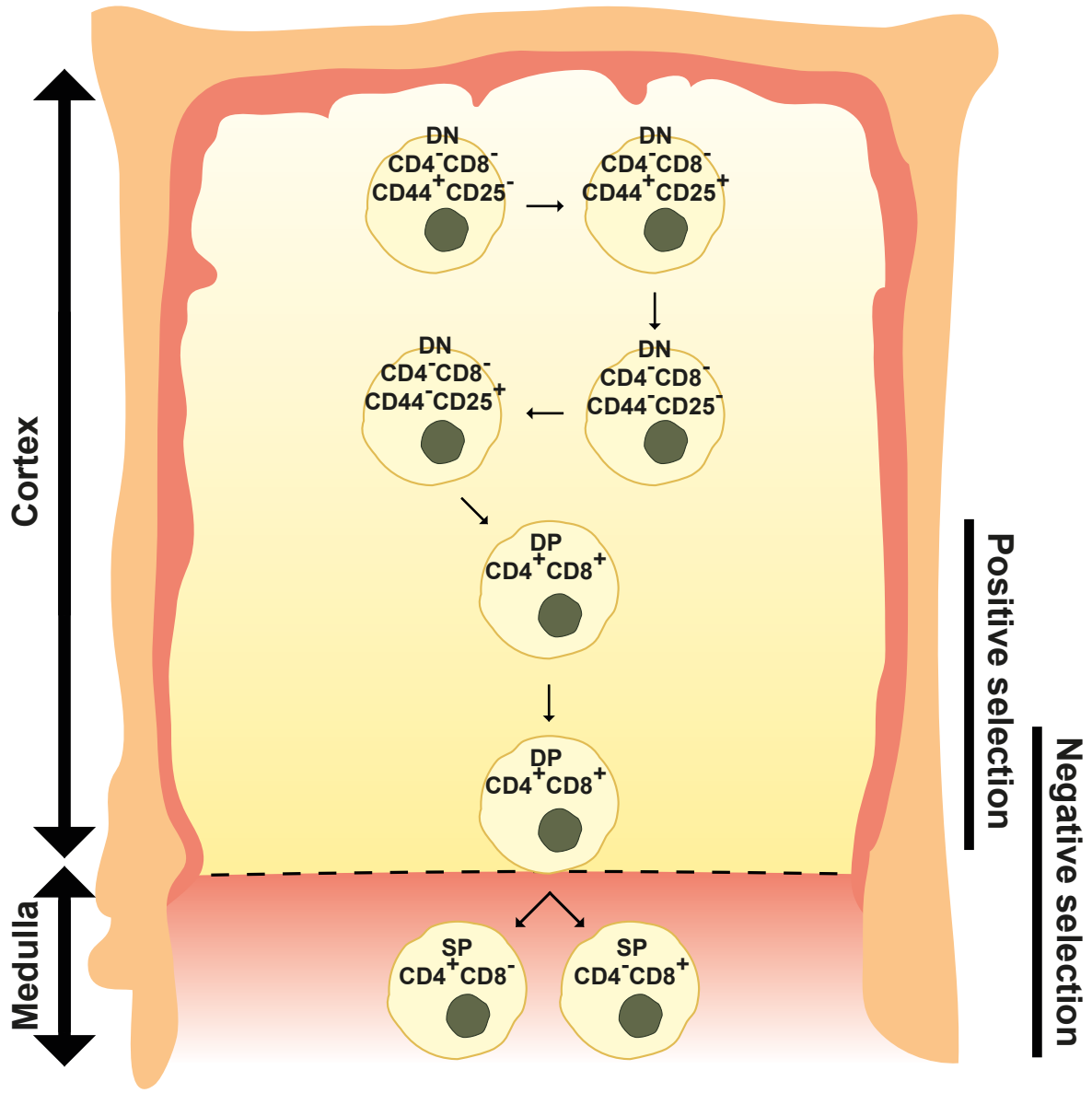


Figure 9: Thymocyte development in the thymus.
Depicted are the different stages of thymocyte development ranging from double negative (DN) to single positive (SP), their respective cell surface markers and their selection processes plotted against their location in the thymus.

Most of the thymocytes (roughly 98%) are deleted by these quality-control mechanisms. In order to be able to display all the necessary antigens, the medulla expresses specialised epithelial cells, the so called medullary thymic epithelial cells (mTECs) that are highly efficient APCs, are able to expose the entire self-peptide repertoire (Levine and Klionsky 2004; Klein and Kyewski 2000). Of note, the autoimmune regulator (Aire) is crucial for the ability of mTECs to present antigen via the MHC (Golstein and Kroemer 2007; Anderson et

al. 2002). A loss of Aire leads to the severe autoimmune polyendocrine syndrome type 1 (APECED) in men (Holler et al. 2000; Peterson et al. 1998; Vercammen et al. 1998).

Of note, some bacteria and viruses have boarded these mechanisms of antigen recognition in order to camouflage themselves from the immune system. In this regard, superantigens (SAgs) play an important role. SAgs are proteins of microbial origin and stimulate T cells via interaction with the V_β domain of the TCR. An example of such a SAg is the mouse mammary tumor virus (MMTV), is well characterized.

B cells present the SAg to indicidual T cell subsets upon MMTC infection. This leads to a T-B cell interaction, which in turn makes sure that infected B cells are preferentially clonally expanded. Along these lines, some of these B cells differentiate into long-lived memory cells, ascertaining a stable MMTV infection that can be passed on to offspring via maternal milk (Kroemer et al. 2009; Acha-Orbea and MacDonald 1995; Scherer et al. 1993).

1.3 The crosstalk between apoptosis and autophagy

1.3.1 General considerations about the interaction of apoptosis and autophagy

The idea of crosstalk existing between apoptosis and autophagy is relatively new, most likely due to the general understanding of these two distinct processes: Apoptosis is a – not to say the – cell death mechanism and a huge number of publications in the 1990s elucidated many important aspects (Thorburn 2007). In contrast, the understanding of autophagy is still incomplete in many ways and only recent publications aided in the further understanding of its functions. Autophagy is nowadays believed to be both – a cell death and a cell survival mechanism through its cytoprotective function (chapter 1.1) (Levine and Yuan 2005; Baehrecke 2005; Gozuacik and Kimchi 2007).

Two major crosstalk models between apoptosis and autophagy could be experimentally validated: One is the cooperation between the two, which leads to cell death. The other is the antagonism between autophagy and apoptosis, where autophagy blocks apoptosis

(Eisenberg-Lerner et al. 2009). The principal concept of the first model is either a backup mechanism where in the event one fails the other can fill the void, or a cooperative manner. Regardless of the mode of action, the ultimate goal remains the same: the death of the cell. The fact that one pathway is able to take over for both, points towards a coordination at the molecular level. This crosstalk could be shown for a multitude of different apoptosis-inducing agents, such as the activation of the TRAIL receptor-2 in cancer cells, ceramide in breast and colon carcinoma and with vitamin K2 in HL-60 cells (Park et al. 2007; Pattingre et al. 2009; Yokoyama et al. 2008). A study where $CD4^+$ T lymphocytes were infected with HIV, showed that autophagy enhances caspase-dependent cell death and as a result the blocking of autophagy led to the inhibition of apoptosis (Espert et al. 2006). Interestingly, the knock down of Atg7 or Beclin 1 suppressed caspase activation, whereas the inhibition of apoptosis accelerated autophagy induction. These two facts imply that autophagy acts upstream and independently of apoptosis (Eisenberg-Lerner et al. 2009). Autophagy intersects with the intrinsic pathway as well, as it can degrade catalase resulting in reactive oxygen species (ROS) accumulation and subsequent mitochondrial outer membrane permeabilization (MOMP) (Yu et al. 2006). This could be inhibited by the knockdown of Atg7 and Atg8 (Yu et al. 2006). All of the studies commonly showed that activation of apoptosis, autophagy or both together result in the killing of the cell, whether it being in a cooperative or in a mutually exclusive manner.

The second concept is the antagonistic model, where in principal apoptosis and autophagy are two discrete mechanisms that counteract one another. In this case autophagy is a cell survival mechanism, opposing the cell death mediated by apoptosis. The classical autophagy stimulus is starvation and a number of groups proved that an intact autophagosomal apparatus is essential for surviving periods of nutrient reduction, both *in vitro* (Boya et al. 2005; Lum et al. 2005) and *in vivo* (Komatsu et al. 2005; Kuma et al. 2004). As opposed to the role of apoptosis induction through catalase degradation (Yu et al. 2006), autophagy can also play a contradictory role in the intrinsic apoptosis pathway, namely through mitophagy (Novak 2012). During mitophagy, autophagy scavenges depolarised mitochondria and

prevents release of intracellular ROS and other pro-apoptotic molecules like cytochrome *c* and Diablo (Kubli and Gustafsson 2012; Kim et al. 2007). Taken together, the examples given demonstrate that autophagy can also be a potent inhibitor of cell death and is therefore an antagonist of apoptosis.

Of note, a third mode of action has been proposed in which autophagy enables apoptosis by providing sufficient levels of adenosine tri-phosphate (ATP) for its execution. The authors could show that a lack of autophagy-relevant proteins, such as Atg5, results in decreased phosphatidylserine exposure at the surface of apoptotic cells and reduced membrane blebbing (Inbal et al. 2002; Qu et al. 2007). The major hallmark of this proposed "enabling mechanism" is the lack of other autophagy-relevant cross talk events. How this is regulated and what mechanisms could be involved remains to be elucidated.

1.3.2 Beclin 1 acts as a hub connecting autophagy and intrinsic apoptosis

Beclin 1 could be identified via a yeast two-hybrid screen as an interaction partner of Bcl-2, the namesake protein of the Bcl-2 family of apoptosis-regulating proteins (Liang et al. 1998). Later studies demonstrated that Beclin 1 is a BH-3 only protein that interacts with other Bcl-2 family members, namely Bcl-xL, Bcl-w, Bim and Mcl-1 (Wirawan et al. 2012; Luo et al. 2012). This interaction is mediated through the BH3 domain of Beclin 1, and mutations in this domain result in a loss of interaction (Feng et al. 2007; Maiuri et al. 2007; Oberstein et al. 2007). Interestingly, Beclin 1 does not interact with other pro-apoptotic BH3 only proteins (Feng et al. 2007; Oberstein et al. 2007; Gordy and He 2012). Along these lines, only ER-localised Bcl-2 family members are able to inhibit starvation-induced autophagy by interaction with Beclin 1 (Pattingre et al. 2005; Maiuri et al. 2007). Recently, Chang and colleagues demonstrated that this interaction between Beclin 1 and Bcl-2 interaction at the ER is stabilised by the nutrient-deprivation autophagy factor-1 (NAF-1) (Chang et al. 2010). Via an siRNA knockdown of NAF1, the authors could also show the disruption of the Beclin 1/Bcl-2 complex that led to the induction of autophagy.

Assuming a more physiological setting, for instance intracellular stress due to starvation, Beclin 1 must disassociate from Bcl-2 and Bcl-xL in order to promote autophagy (Pattingre et al. 2005). Although numerous mechanisms have been described so far that involve proteins such as DAPK, HMGB1 or other BH3 only proteins (Luo and Rubinsztein 2007; Maiuri et al. 2007; Zalckvar et al. 2009; Zalckvar et al. 2009; Kang et al. 2010; Tang et al. 2010), Bcl-2 phosphorylation by JNK plays a pivotal role in the initiation of autophagy (Wei et al. 2008). The authors could demonstrate that JNK deficiency leads to a loss of starvation-induced autophagy and using a gain-of-function approach they demonstrated that autophagy is activated even under nutrient-rich conditions. This was accomplished by overexpression of a constitutively active JNK1-mutant. Interestingly, The Beclin 1/Bcl-2 complex is also sensitive to both Bad and Bax (Zhu et al. 2010; Maiuri et al. 2007). Both of these proteins are able to disassociate Bcl-2 from Beclin 1, but with very different outcomes. Bad removes the inhibitory effect of Bcl-2 and promotes autophagy, whereas Bax overexpression reduces autophagy (Maiuri et al. 2007; Luo and Rubinsztein 2010). However, the authors could prove that this reduction is not due to the disruption of the Beclin 1/Bcl-2 complex by Bax, but rather due to Beclin 1 being targeted for caspase cleavage (Luo and Rubinsztein 2010). Therefore, autophagy initiation together with the PI3K hVps34 was not occurring.

In contrast to the implications of the role of Beclin 1 in autophagy discussed above, the Beclin 1/hVps34 complex is not an essential component of autophagy in general. To look at this fact, the situation in T lymphocytes is best investigated. Although $CD4^{+}$ Beclin $1^{-/-}$ T cells show largely abolished autophagy and increased apoptosis, LC3 puncta formation could still be observed (Arsov et al. 2011; Kovacs et al. 2011). Along these lines, it was established that the Beclin 1-deficient T lymphocytes proliferated normally (Arsov et al. 2011). Another study was recently able to demonstrate that autophagy levels in T lymphocytes deficient for Vps34 are completely normal, although the T lymphocytes were clearly affected by the loss of Vps34 (Mcleod et al. 2011). Taken together, the data currently available do not allow for a comprehensive understanding of the crosstalk between apoptosis and autophagy mediated by Beclin 1.

1.3.3 Caspase-dependent autophagy regulation

Bcl-2 family members are not the only apoptosis-related proteins that are involved in autophagy. Caspases, essential components of both the extrinsic and the intrinsic apoptosis pathway have recently been found to play a role in autophagy as well.

The first work that was able to demonstrate the direct effect of a protein very similar in function to a caspase on another protein involved in autophagy was by Yousefi and colleagues (Yousefi et al. 2006). Although caspases and calpains differ in their cleavage-site specificity, they are quite similar among each other and a large number of proteins were identified that are equally susceptible to both (K. Wang 2000). They showed the cleavage of Atg5 by calpain 1 and calpain 2 in human neutrophils that were undergoing apoptosis (Yousefi et al. 2006). Interestingly, an amino-terminal cleavage product could be detected that translocated to the mitochondria where it associated with Bcl-x_L and thus induced cytochrome *c* release. Additionally, the overexpression of this cleavage product alone was sufficient to induce apoptosis (Yousefi et al. 2006). This finding was even more important when considering the fact that the overexpression of LC3 alone did not change apoptotic events, thus demonstrating that rather than autophagy as a whole, solely the cleavage product of Atg5 influenced apoptosis.

Later on, Beclin 1 was also identified as a target of cleavage by caspases. The identified caspases differed, depending on the experimental systems and cell types investigated. Caspases 3, 6, 9 and 10 have all been found to interact with Beclin 1 (Luo and Rubinsztein 2010; Cho et al. 2009; Wirawan et al. 2010; Zhu et al. 2010; Li et al. 2011; Rohn et al. 2011). Interestingly, one of these studies in Hela cells showed that the cleavage of Beclin 1 plays a role in autophagy, as non-cleavable Beclin 1 mutants were not able to increase autophagosome formation upon starvation (Zhu et al. 2010). In summary, these results prove the existence of a complex network between autophagy and apoptosis. The cleavage products of autophagosome-associated proteins seem to play a pivotal role not only in apoptosis, but also in autophagy by itself. The work introduced so far reveals only a small

part of this intricate network, a lot of work needs to be done to elucidate the exact molecular details that are still missing.

1.3.4 The interplay between the extrinsic apoptosis pathway and autophagy

As mentioned in section 1.2.3, extrinsic apoptosis is mediated by a death-inducing signalling complex (DISC) that is initiated by the triggering of an external cell surface receptor (Kischkel et al. 1995). Caspase 8 plays an important role in this DISC: When its pro-active form is cleaved, active caspase 8 is released and activates the effector caspase 3 (Peter and Krammer 2003). The FLICE inhibitory protein (c-FLIP) can interact with caspase 8 at the DISC and either inhibits or enhances caspase 8 activation (Thome and Tschopp 2001; Wilson et al. 2009; Ewald et al. 2011; Ueffing et al. 2008). Caspase 8-deficiency could be linked to excessive autophagy in fibroblasts, macrophages and T cells (Bell et al. 2008; Yu et al. 2004). Furthermore, a mutant of the Fas-associated protein with death domain (FADD) that did not contain a death effector domain (DED), was unable to mediate an interaction with either caspase 8 nor c-FLIP and showed the same phenotype (Bell et al. 2008; Thorburn et al. 2005). A recent study was able to provide an important link between the DISC and autophagy: FADD interacts with Atg5 through its DD (Pyo et al. 2005). Interestingly, a lack of FADD did not affect autophagosome formation, but rather rescued the increased cell death that resulted from Atg5. Therefore, the interaction of FADD with Atg5 seems to regulate apoptosis, not autophagy.

Recently, it could be proven that caspase 8 activation is not only restricted to the DISC, but rather that caspase 8 co-localises with Atg5, LC3 and p62 (Young et al. 2012). Moreover, FADD could be found on Atg5/Atg16L and LC3-positive cytosolic spots, indicating that FADD is also located on the autophagosomal membrane. The authors therefore concluded that the autophagosomal membrane serves as a platform for an intracellular DISC (iDISC) that recruits caspase 8 in order to initiate the intrinsic caspase 8/3 cascade (Young et al. 2012). However, c-FLIP was not found to be a part of this proposed iDISC complex, although it was identified as an important inhibitor of autophagy (Lee et al. 2009). In the same study, c-FLIP$_S$

and $cFLIP_L$ were also associated with the inhibition of rapamycin-induced autophagy (Lee et al. 2009). This phenotype could be rescued with a siRNA-mediated knockdown of c-FLIP. Interestingly, the authors could demonstrate that the anti-autophagic function of FLIP was independent of its anti-apoptotic function, since mutants that were unable to bind to FADD or TRAF2 and could not activate NF-κB could still inhibit autophagy.

Taken together, these studies clearly demonstrate that proteins that exhibit a specific function in DISC-mediated apoptosis have a different and independent function in autophagy. This is also true for autophagy-associated proteins and their cleavage products, which exert an all-together different function in the context of apoptosis. Importantly, many of the proteins that are associated with either apoptosis or autophagy seem to have dual roles and clearly demonstrate the close interaction between these two important pathways.

1.4 The Gadd45 family

1.4.1 General considerations about the Gadd45 family

The **G**rowth **A**rrest and **D**NA **d**amage-inducible gene 45 (Gadd45) family is comprised of 3 different genes, namely *Gadd45a*, *Gadd45b* and *Gadd45g*. As the name of the family implies, Gadd45 proteins have been implicated in cellular functions such as DNA repair (Vairapandi et al. 1996; Smith et al. 1994; Smith et al. 2000), cell survival (Smith et al. 2000; Gupta et al. 2006; Smith et al. 1996; De Smaele et al. 2001; Amanullah et al. 2003; Gupta et al. 2005), senescence (Tront et al. 2006), apoptosis (Takekawa and Saito 1998; Selvakumaran et al. 1994; Zhang et al. 2001; Yoo et al. 2003; Vairapandi et al. 2000; Azam et al. 2001; Harkin et al. 1999; Kojima et al. 1999; Hildesheim et al. 2002) and tumor development *in vivo* (Tront et al. 2006; Hollander et al. 1999). Gadd45 (Gadd45a), Myd118 (Gadd45b) and CR6 (Gadd45g), collectively referred to as the Gadd45 gene family, encode for small (18 kDa), evolutionary conserved proteins, which are highly acidic (pI= 4.0-4.2) and have a certain degree of similarity with each other (~55%) (Tamura et al. 2012; Liebermann and Hoffman 2008). The proteins exhibit their biological function via direct protein-protein

interaction (Tamura et al. 2012). The Gadd45 proteins are localised in the nucleus and the cytoplasm and are present at relatively low levels in normal, unstimulated cells (Vairapandi et al. 2002; Zhang et al. 1999; Abdollahi et al. 1991). Upon environmental stresses such as UV and γ irradiation or anisomycin treatment, the Gadd45 protein family members are rapidly induced (Takekawa and Saito 1998). In general, Gadd45 proteins are highly expressed during the G1 phase of the cell cycle and are significantly reduced during the S phase (Kearsey et al. 1995). In addition, the different Gadd45 family members are differentially expressed in distinct murine tissues (Table 1).

Tissue	Gadd45α	Gadd45β	Gadd45γ
Skeletal muscle	+	+/-	+
Kidney	+	-	+
Liver	+/-	+/-	+
Heart	+/-	-	-
Brain	+/-	-	-
Spleen	+/-	-	-
Lung	+/-	+	-
Testis	-	-	-

Table 1: Expression profile of the Gadd45 family in mouse tissue.
+ = high expression, +/- = moderate expression, - = low expression. Table set according to (Tattoli et al. 2012; Zhang et al. 1999; Knodler and Celli 2011).

Gadd45α was the first of the three proteins that was identified, thus the name. It was detected by the screening of a cDNA library of increased transcripts after ultraviolet (UV) irradiation of chinese hamster (CHO) cells (Fornace et al. 1988; Fornace et al. 1989). Gadd45α is also a target of p53 (Selvakumaran et al. 1994; Guillouf et al. 1995; Kastan et al. 1992). Gadd45β was transiently induced by interleukin-6 (IL-6) treatment in myeloid leukaemia cell lines (Abdollahi et al. 1991). It was also identified by TGF-β stimulation in hepatocytes and myeloid leukaemia cells and by T cell receptor (TCR) stimulation (Lu et al. 2001; Lu et al. 2004; Yoo et al. 2003; Selvakumaran et al. 1994; Schmitz et al. 2003).

Gadd45γ, also known as CR6, OIG37 or GRP17 was originally identified as an IL-2 inducible gene (Beadling et al. 1993; Nakayama et al. 1999; Kojima et al. 1999).

1.4.2 Protein-protein interaction and known binding partners

As mentioned earlier, Gadd45 proteins exert their biological function via a direct protein-protein interaction. How exactly this interaction is mediated is the focus of current research. For Gadd45α and Gadd45γ, the three-dimensional structure could be elucidated via nuclear magnetic resonance (NMR) and crystallography (Sánchez et al. 2008; Schrag et al. 2008). These two studies could show, that the two proteins share similar secondary structures, both having 5 α-helices and 5 β-sheets. The high levels of homology suggest similar functions, and indeed both proteins interact with overlapping interaction partners, including: CDK1 (Zhan et al. 1999; Jin et al. 2000; Yang et al. 2000), PCNA (Hall et al. 1995; Vairapandi et al. 2000; Azam et al. 2001), p21 (Yang et al. 2000; Zhao et al. 2000) and MTK1 (Takekawa and Saito 1998). All of these proteins are involved in cell cycle control, cell survival, senescence and tumor development.

Interestingly, Gadd45 proteins can interact with each other and form homo- or hetero-dimers. With the help of mutational and crystallography analyses, several regions crucial for the oligomerisation of Gadd45 proteins could be identified (Schrag et al. 2008). Moreover, some of these oligomerisation-impaired mutants showed a reduced interaction with p21, CDK1 and PCNA (Kovalsky et al. 2001; Schrag et al. 2008; Yang et al. 2000). Up to now, the structure of Gadd45β has not been resolved. However, as Gadd45β also interacts with a number of similar proteins, e.g. PCNA and p21 (Vairapandi et al. 1996; Smith et al. 1994; Smith et al. 2000; Vairapandi et al. 2000; Azam et al. 2001), and was shown to interact with Gadd45α (Kovalsky et al. 2001), it seems reasonable to assume an analogue mode of interaction. Additionally, a study that analysed the interaction of Gadd45β and MAP2K7, predicted a three-dimensional structure of Gadd45β and a similar mode of action as for both other Gadd45 proteins (Papa et al. 2007).

Interestingly, as Gadd45 proteins seem to exert similar functions and have a large pool of interaction partners in common. A redundant function, at least in part, was found by different groups (Liu et al. 2005): Young Gadd45$\beta^{-/-}$ and Gadd45$\gamma^{-/-}$ mice did not develope spontaneous signs of autoimmunity. However this was different when they reached a high age or were challenged with an induced autoimmune syndrome like experimental autoimmune encephalomyelitis (EAE) (Lu 2006). In contrast to this, mice deficient for both Gadd45 proteins show severe autoimmune lymphoproliferative syndrome and systemic lupus erythematosus.

1.4.3 Gadd45 proteins and cell cycle control

Ordinarily, the cell cycle is defined by the G1, S, G2 and M phases and is well regulated. This process is controlled in part but not exclusively by cyclin-dependent kinases (CDK) (Vermeulen et al. 2003). Between the G2 and the M phase, the CDK1/Cyclin1 B1 complex is a major checkpoint (Nurse 1996). The Gadd45 protein family members interact directly with CDK1, but not with cyclin1 B1 (Zhan et al. 1999). In this regard, the kinase activity of the whole complex is inhibited. Interestingly, Gadd45α and Gadd45β disrupt the whole complex, whereas Gadd45γ inhibits the complex without disrupting it (Vairapandi et al. 2002). Additionally, all three Gadd45 proteins interact with p21, which activates the G1/S checkpoint (Zhang et al. 1993; Vairapandi et al. 1996; Fan et al. 1999). As a consequence, the Gadd45 proteins indirectly lead to a cell cycle arrest (Zhang et al. 1993; Vairapandi et al. 1996; Fan et al. 1999).

1.4.4 Gadd45 proteins and MAPK signalling

In order to be able to process extracellular stimulations such as e.g. stresses, cytokines or growth factors, cells needed to develop mechanisms to receive those signals, transmit them and mount an appropriate response. These signal transduction events heavily rely on post-translational modifications of proteins, a key event of which is phosphorylation.

MAPK pathways are comprised of a three-tier kinase module in which a MAPK is activated upon phosphorylation by a mitogen-activated protein kinase kinase (MAPKK), which in turn is activated when phosphorylated by a MAPKKK (Dhillon et al. 2007). MAPKKK are serine-threonine kinases, which receive their activation signal via e.g. receptors on the cell surface. Their substrates, the MAPKK, which are threonine-tyrosine kinases phosphorylate the MAPK, which are serine-threonine kinases (Hagemann and Blank 2001).

To date, at least four important subfamilies of the MAPK could be distinguished: the extracellular signal-regulated kinases (ERK1/2), ERK5, the c-Jun amino terminal kinases (JNK1-3) and the p38 kinases (p38α, β and γ) (Han et al. 1994; Lee et al. 1994; Rouse et al. 1994; Freshney et al. 1994).

All three Gadd45 proteins directly interact with the upstream MAPKK kinase MTK1/MEKK4 in response to environmental stress (Takekawa and Saito 1998). This interaction results in a downstream activation of p38/JNK and the subsequent initiation of apoptosis (Takekawa and Saito 1998). For Gadd45β this interaction could be elucidated in great detail. Its N-terminal amino acid residues (13-132) bind to the auto-inhibitory domain of MTK1, and lead to a conformational change, resulting in dimerisation of the kinase, autophosphorylation and activation of the kinase domain (Takekawa and Saito 1998; Mita et al. 2002; Miyake et al. 2007). Gadd45α could also be identified as a direct interaction partner of the p38 MAPK (Bulavin et al. 2003).

p38 is directly activated via the MAPKKs, MKK3 and MKK6 (Enslen et al. 1998), whereas JNK gets phosphorylated via MKK4 or MKK7 (Haeusgen et al. 2011). Both MAPKs p38 and JNK have been reported to play a role in apoptosis, cell survival and autophagy (Xu et al. 2011; Kyriakis 2001; Keil et al. 2012; Chou et al. 2001). Along these lines, it was shown that Gadd45β directly interacts with MKK7/JNKK2, but not with MKK4/JNKK1, the other JNK kinase (Papa et al. 2004). Here, Gadd45β directly binds to MKK7/JNKK2 and thereby blocks its catalytic activity. Through this mechanism, JNK can no longer be phosphorylated and ultimately, apoptosis induction is inhibited (Papa et al. 2004).

Obviously, the interaction of the different MAPKs with the MAPKKs and the MAPKKKs needs to be tightly regulated in order to maintain cellular functionality. To this end, the phosphorylation events among each other are influenced by at least two independent methods: On the one hand the MAPKs need to be brought into close proximity to each other to allow for the phosphorylation event to take place. This is ascertained by scaffold proteins, which provide an insulated physical conduit, through which signals form the respective MAPK can be transmitted to the appropriate spatiotemporal cellular loci (Dhanasekaran et al. 2007). On the other hand, a so-called domain for versatile docking (DVD) at the MAPKK can determine specificity of the MAP3K docking partner (Takekawa et al. 2005). Additionally, a docking site in a non-catalytic region in ERK, p38 and JNK was identified which serves as an interaction domain for their corresponding MAPKKs (Tanoue et al. 2000; Tanoue et al. 2001). These common docking sites (CDs) increase the efficiency of their respective enzymatic activity and in this way represent another way of MAPK regulation (Tanoue et al. 2000).

1.4.5 Gadd45 proteins in apoptosis

The Gadd45 protein family has been implicated in many signalling processes, in which a deregulation can lead to an increase in apoptosis. Of these, the interaction with the MAPK p38 and the indirect regulation of JNK through MKK7 are considered to be the best described (Papa et al. 2004). For example, Gadd45α-deficient mouse skin tissue and keratinocyte cell lines show a reduced p38, JNK and p53 activation and are resistant towards UV-induced apoptosis (Hildesheim et al. 2002). TGF-β-dependent apoptosis is delayed in Gadd45β-silenced myeloid leukaemia and pancreatic cancer cell lines and in primary mouse hepatocytes (Takekawa et al. 2002; Yoo et al. 2003; Selvakumaran et al. 1994). This TGF-β-induced apoptosis is dependent on Smad 2, 3 and 4 (Yoo et al. 2003). Moreover, Gadd45β is 72-fold up-regulated in a mouse model of negative selection in the thymus upon stimulation of the TCR with high affinity ligands (Schmitz et al. 2003). Interestingly, the pro-apoptotic role of the Gadd45 proteins was not limited to the extrinsic apoptosis pathway, but

for Gadd45α could also be linked to Bax-mediated cytochrome *c* release into the cytoplasm in HeLa cells (Tong et al. 2005; Tamura et al. 2012).

In contrast to these observations, bone marrow cells from Gadd45β$^{-/-}$ mice were more susceptible towards apoptotic stimuli such as UV- irradiation and cytotoxic chemicals like chemotherapeutics, VP-16 and daunorubicin (Gupta et al. 2006; Gupta et al. 2006; Gupta 2003). Along these lines, Type1 T helper (TH1) cells in the synovial fluid of patients with rheumatoid arthritis express high levels of Gadd45β and are resistant to apoptosis (Du et al. 2008). This enhanced apoptosis resistance could be abolished by RNAi silencing of Gadd45β. Its overexpression in fibroblasts also elevated the apoptosis resistance in those cells, but MEFs from Gadd45β$^{-/-}$ mice showed no susceptibility to Tumor necrosis factor-α (TNF-α)– mediated apoptosis and had no differences in JNK activity (Engelmann et al. 2008; Amanullah et al. 2003). Furthermore it was recently shown that the induction of the pro-apoptotic JNK cascade downstream of the Tumor necrosis factor receptor (TNFR) was significantly increased in Gadd45β$^{-/-}$ mice after partial liver hepatectomy (Papa et al. 2008). After a partial liver hepatectomy, NF-κB is induced to counter the effects of JNK activity by induction of target genes and to allow for regeneration. As Gadd45β is an NF-κB target gene (De Smaele et al. 2001; Papa et al. 2004), Papa and colleagues conclude that Gadd45β represses JNK activity in hepatocytes after TNFR activation in response to partial liver hepatectomy (Papa et al. 2008). The same group was earlier able to identify the mechanism by which Gadd45β impairs JNK activation. Gadd45β interacts with MKK7/JNKK2, which is a specific and essential activator of JNK (Papa et al. 2004; Tournier et al. 2001). In summary, Gadd45β controls the activation of JNK.

1.4.6 Gadd45 proteins in methylation

The process of DNA methylation is an epigenetic modification to silence genes, which is an important mechanism of gene regulation in vertebrates (Reik 2007). A study in *Xenopus laevis* could show a Gadd45α-dependency in the process of active DNA demethylation

(Barreto et al. 2007). They concluded that Gadd45α relieves epigenetic gene silencing by promoting DNA repair, which erases methylation marks. Additionally, a recent study in *Danio rerio* elucidated that demethylation levels were *Gadd45*-dependent. Overexpression of *Gadd45α* increased demethylation, while silencing of the Gadd45 gene family reduced it (Rai et al. 2008). Gadd45 proteins have numerous implications in active DNA demethylation: A recent publication shows that *Gadd45a* and *Gadd45b* were both induced during epidermal differentiation, probably because of their interaction with base excision repair (BER) and nucleotide excision repair (NER) DNA demethylase complexes (Sen et al. 2010). The same study revealed that increased *Gadd45* expression induced premature differentiation of progenitors and inhibited their growth, both of which are hallmarks of demethylation activity (Sen et al. 2010). Moreover, enforced *Gadd45* expression led to the same increase in muscle differentiation genes as the depletion of DNA demethyltransferase 1 (DNMT1) (Sen et al. 2010). Along these lines, another publication showed the same effect for adult neurogenesis through DNA demethylation. Proliferation of neuronal progenitors and dendritic growth of new-born neurons in the adult hippocampus was severely impaired in Gadd45β-deficient mice (Ma et al. 2009; Wu and Sun 2009).

However, two different studies obtained contradictory results: Both groups showed that *Gadd45α*-deficiency did not have an impact on DNA demethylation (Engel et al. 2009; Jin et al. 2008). Thus, whether or not *Gadd45a*-deficiency has an impact on DNA demethylation is not clear yet. Interestingly, a group identified Gadd45β as a direct target of the forkhead box 3 transcription factor (Foxp3) in an array approach in hybridoma cells (Marson et al. 2007). Foxp3 is the main transcription factor regulating the induction of regulatory T cells (T_{reg}), a T cell subtype that has been shown to be a major contributor to peripheral tolerance (Sakaguchi et al. 1995). Here, the stability of so-called natural regulatory T cells ($nT_{reg}s$) is heavily dependent on the methylation status of a DNA region named the T_{reg}-cell specific demethylated region (TSDR) (Huehn, Polansky, and Hamann 2009). It was shown that T_{reg} stability is dependent on a demethylated TSDR/CNS2 (Floess et al. 2007; Polansky et al.

2008). Thus, there might be a connection between T_{reg} stability and Gadd45β. Whether this possible connection is of any relevance needs to be further elucidated in the future.

1.5 Aims

The aim of this thesis was to determine the effect of Gadd45β on different cellular mechanisms: On the one hand on autophagy and on the other hand on apoptosis in the context of negative selection in the thymus. As described above, autophagy has numerous and different implications in cellular biology and immunity, therefore its regulation needs to be tightly controlled. Previous work on this topic showed evidence of a Gadd45β-MEKK4-p38-dependent phosphorylation of Atg5 (Keil 2008). However, the physiological consequences and the proof of an *in vivo* phosphorylation remained unresolved. Recent publications could already demonstrate the influence of MAPKs on autophagy-related proteins (Wei et al. 2008; Wang et al. 2009). Nevertheless, the interactions studied so far were limited to the initiation phase of autophagy. Therefore, previous results of research on the MAPKs-dependent post-translational modification of the autophagic flux make up one part of this thesis.

On the other hand, Gadd45β was identified to be highly upregulated upon challenge of the TCR with high-affinity peptides in an *in vivo* model of negative selection (Schmitz et al. 2003). Additionally, Gadd45β-deficient thymocytes were protected from apoptosis compared with wildtype thymocytes in a fetal thymic organ culture model (FTOCs) (data not shown). The known interaction of Gadd45β with the MAPKKK MEKK4 led to the question if the TCR signalling in Gadd45β-deficient thymocytes is impaired (Takekawa and Saito 1998). Moreover, if the interaction is impaired, whether this is related to the defects in apoptosis and subsequently also to the negative selection process in the thymus.

2. Materials

2.1 Materials

2.1.1 Chemicals

If not particularly mentioned otherwise, all chemicals were obtained either from Sigma Aldrich (Munich, Germany), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany). The BMFZ (Düsseldorf, Germany) generated the peptides for the stimulation of the transgenic mice. The two peptides were: PCC (*pigeon cytochrome c*; aa 88-104; KAERADLIAYLKQATAK) and VSV8 (*vesicular stomatitis virus nucleoprotein*); aa 52-59; RGYVYQGL.

2.2 Cell Culture materials and devices

The cell culture Flasks, 10 cm dishes 35 mm, 6-well, 12-well, 48-well and 96-well plates were provided by NUNC/ Thermo Fisher Scientific (Rochester, USA). The 10 µl sterile pipette tips, as well as the 1.5 ml and 2 ml small reaction tubes, were obtained from Sarstedt (Nümbrecht, Germany). Sterile serological pipettes with volumes of 5 ml, 10 ml and 25 ml were supplied by Sterilin – Thermo Fisher Scientific. 15 ml and 50 ml reaction tubes were used from Greiner bio-one (Frickenhausen, Germany). Sterile 200 µl and 1 ml pipette tips were obtained from Starlab (Ahrensburg, Germany). 45 µm and 20 µm sterile syringe filters were provided by Becton, Dickinson (Heidelberg, Germany) and Millipore (Waltham, USA).
Cultivation conditions of 37°C at 5% CO_2 and 95% air humidity were maintained by a HERAcell 240i from Thermo Scientific. A 5810R centrifuge from Eppendorf (Hamburg, Germany) was used to settle down the cells, which were handled in a Sterile Guard III by The Baker Company (Sanford, USA).

2.2.1 Eukaryotic cell culture media and supplements

Reagent	Lot	Order-No.	Company
RPMI 1640	1003068	41965	GIBCO- life technologies (Grand Island, USA)
DMEM	1149983	41965-039	GIBCO- life technologies
Fetal Calf Serum (FCS)	A10108-2367	A15-101	PAA (Pasching, Austria)
Sodium-Pyruvate 100 mM	749750	11360	GIBCO- life technologies
Non-essential amino acids	930229	11140	GIBCO- life technologies
Penicillin/ Streptomycin 5 mg/ml	918582	15070	GIBCO- life technologies
β-Mercaptoethanol 50 mM	806672	31350	GIBCO- life technologies
L-Glutamine 200 mM	831221	25030	GIBCO- life technologies
MCSF BD 24232			Self purified

2.2.2 Prokaryotic media

Medium	Components
LB-medium	10 g/l Tryptone
	5 g/l Yeast extract
	10 g/l NaCl, pH 7.2

2.2.3 Medium for *in vitro* cultivation of primary T cells

Primary, murine T cells were cultured in RPMI1640, supplemented with 10% FCS, 1 mM Sodium Pyruvate, 1x non-essential amino acids, 50 mg/ml Penicillin/ Streptomycin and 50 µM β-Mercaptoethanol.

2.2.4 Medium for *in vitro* cultivation of mouse embryonic fibroblasts (MEFs)

If not suggested otherwise, mouse embryonic fibroblasts were cultivated in DMEM, complemented with 10% FCS and 50 mg/ml Penicillin/ Streptomycin.

2.2.5 Medium for *in vitro* cultivation of bone marrow derived macrophages (BMDMs)

Murine bone marrow derived macrophages were cultivated in DMEM, added with 10% FCS, 2 mM Glutamine, 50 µM β-Mercaptoethanol, 50 mg/ml Penicillin/ Streptomycin and 5% MCSF BD 24232.

2.2.6 Chemicals used for T cell stimulation

Reagent	Lot	Order-No.	Company
Phorbol12-Myristate 13-Acetate (PMA)	16561-29-8	P8139	Sigma Aldrich
Ionomycin (Iono)	56092-82-1	I-0634	Sigma Aldrich
Dexamethasone		D4902-100mg	Sigma Aldrich

2.2.7 Antibodies used in cell culture

Antibody	Lot	Clone	Order-No.	Company
Anti-Murine CD3		145-2C11		Self purified

Anti-Murine CD28	E18		Self purified
Anti-Murine CD95	Jo2	554254	Becton, Dickinson

2.3.1 Eukaryotic cell lines

Cell line	Origin
HEK 293T	Human embryonic kidney
MEF	Mouse embryonic fibroblasts
NIH/3T3	Mouse embryonic fibroblasts

2.3.2 Eukaryotic primary cells

Cells	Origin
Primary thymocytes	Mouse thymus
Bone marrow derived macrophages	Mouse bone marrow
$CD4^+$, $CD8^+$ T cells	Mouse spleen, peripheral and mesenchymal lymph nodes

2.4 Bacterial strains

E. coli strain	Genotype	Application	Reference source
Top10	F– *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1*ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Amplification of plasmid DNA	Invitrogen (Karlsruhe, Germany)

BL21 (DE3) gold	*E. coli* B F^- *ompT hsdS* (r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* (DE3) *endA* Hte	Expression of fusion protein	Stratagene (Heidelberg, Germany)

2.5 Mouse strains

If not mentioned otherwise, *in vivo* experiments were performed with wild type mice of the C57BL/6 strain. The transgenic strains were: N15 TCR transgenic rag2$^{-/-}$ (Ghendler et al. 1998), later referred as N15. The 5CC7 TCR transgenic rag2$^{-/-}$ on a C57BL/10 genetic background (Seder et al. 1992), later referred as 5CC7. The Gadd45$\beta^{-/-}$ mice on a C57BL/6 genetic background (M. Gupta et al. 2005). The pCAG-GFP-LC3 transgenic mouse (Mizushima et al. 2004), later referred as GFP-LC3$^{Tg/Tg}$. All mice were bred under specific pathogen free (SPF) conditions in the animal facility of the Helmholtz-Centre for Infection Research. If not specifically told otherwise, all experiments were done with littermate controls.

2.6 Materials and reagents for flow cytometry

2.6.1 Devices

Samples that were pre-labelled were analysed on a FACS Canto, LSR II and a Fortessa by Becton, Dickinson. For cell purification, the samples were sorted either on a FACS Aria II by Becton, Dickinson or on a Moflo by Beckman Coulter (Indianapolis, USA).

2.6.2 Fluorescent dyes

Reagent	Excitation	Emission	Oder-No.	Company
7-Aminoactinomycin D	546 nm	647 nm	A 9400	Sigma-Aldrich

APC Annexin V	650 nm	660 nm	550475	Becton, Dickinson
PE Annexin V	496 nm	578 nm	559763	Becton, Dickinson
FITC Annexin V	495 nm	521 nm	556419	Becton, Dickinson
LIFE/DEAD® Fixable Blue Cell Stain	305 nm	450 nm	L23105	Invitrogen
Lysotracker-Red DND-99	577 nm	590 nm	L7528	Invitrogen

2.6.3. Fluorochrome-labelled antibodies

Reactivity	Dye	Species	Notation	Order-No.	Company
CD4	Pacific Blue	Rat IgG_{2a}, κ	L3T4	100531	BioLegend
CD4	APC	Rat IgG_{2a}, κ	L3T4	100516	BioLegend
CD4	PE	Rat IgG_{2a}, κ	L3T4	553048	Becton, Dickinson
CD5	PE	Rat IgG_{2a}, κ	53-7.3	12-0051	eBioscience
CD8a	PerCP-eFluor 710	Rat IgG_{2a}, κ	53-6.7	46-0081	eBioscience
CD8a	Horizon V500	Rat IgG_{2a}, κ	53-6.7	560776	Becton, Dickinson
CD19	PerCP-Cy5.5	Rat IgG_{2a}, κ	1D3	45-0193	eBioscience

CD24	FITC	Rat IgG_{2b}, κ	M1/69	553261	Becton, Dickinson
CD25	APC	Rat IgG_1, λ	PC61.5	17-0251	eBioscience
CD25	PE	Rat IgG_1, λ	PC61.5	553866	Becton, Dickinson
CD62L	PE				
CD69	PE-Cy7	Hamster IgG	H1.2F3	25-0691	eBioscience
H57	APC	IgG_2, λ1	H57-597	561080	Becton, Dickinson
Vβ TCR Screening Panel (Vβ 2, 3, 4, 5.1/5.2, 6, 7, 8.1/8.2, 8.3, 9, 10[b], 11, 12, 13, 14, 17[a])	FITC			557004	Becton, Dickinson
Foxp3	PE	Mouse IgG_1	3G3	130-093-014	Miltenyi Biotech (Mönchengladbach, Germany)
Foxp3	APC	Mouse IgG_1	3G3	130-093-013	Miltenyi Biotech
F4/80	PE	Rat IgG_{2a}, κ	6F12	552958	Becton, Dickinson

2.7 Materials and Reagents used for immunoblot

PVDF membrane and photosensitive Hyperfilms were supplied by GE Healthcare (Buckinghamshire, UK). Tetra cell and criterion blotter for SDS-Page and protein transfer

were obtained from Bio-Rad Laboratories (Hercules, USA). ECL western blotting substrate was either supplied by Pierce/ Thermo Fisher or bought from Amersham/ GE Healthcare.

2.7.1 Primary antibodies

Antibody	Reactivity	Isotype	Species	Clone/ Notation	Company
Anti-ATG12	Human, Mouse, Rat (H, M, R)	IgG	Rabbit	EAW48955	ProSci (Poway, USA)
Anti-ATG16L1	H, M, R	IgG	Rabbit	D6D5	Cell Signalling (Boston, USA)
Anti-ATG5	H, M, R	IgG	Rabbit	D1G9	Cell Signalling
Anti-BIM	H, M	IgG_{2a}, κ	Rat	3C5	Alexis Biochemicals (New York, USA)
Anti-c-MYC	All	IgG	Rabbit	71D10	Cell Signalling
Anti-FLAG	H, M	IgG_1	Mouse	F 1804	Sigma-Aldrich
Anti-Gadd45β	M	IgG	Rabbit		Self Purified
Anti-GST	M	IgG	Goat	17-4577-01	Amersham Biosciences
Anti-HA	H, M	IgG_{2b}	Mouse	12CA5	Roche Applied Science
Anti-LC3B XP	H, M	IgG	Rabbit	D11	Cell Signalling
Anti-LC3A/B	H, M	IgG	Rabbit	4108	Cell Signalling
Anti-MEKK4	H, M, R	IgG_{2b}	Mouse	6C6	Sigma-Aldrich
Anti-Nur77	M	IgG_1	Mouse	12.14	Becton, Dickinson

Anti-p38	H, M	IgG	Rabbit	9212	Cell Signalling
Anti-p38 (P)	H, M	IgG	Rabbit	9211	Cell Signalling
Anti-Tubulin	H, M	IgG_1	Mouse	DM1A	Sigma-Aldrich
Anti-V5	H, M	IgG_{2a}	Mouse	R960-25	Invitrogen
Anti-β-Actin	H, M	IgG2a	Mouse	AC-74	Sigma-Aldrich

2.7.2 Horseradish peroxidase-conjugated secondary antibodies

Reactivity	Species	Clone/ Notation	Company
Mouse IgG1	Goat		Southern Biotechnology (Birmingham, USA)
Mouse IgG2a	Goat		Southern Biotechnology
Mouse IgG2b	Goat		Southern Biotechnology
Mouse IgG3	Goat		Southern Biotechnology
Mouse IgG	Goat		Dianova (Hamburg, Germany)
Rabbit IgG	Goat		Southern Biotechnology
Rabbit IgG	Goat		Dianova
Rabbit IgL	Mouse IgG1, κ		Jackson ImmunoResearch (Bar Harbor, USA)

2.8 Polymerase chain reaction (PCR)

All oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany), HPLC- purified and lyophilised. All PCR were performed on a PeqStar cycler provided by

PeqLab (Erlangen, Germany). The quantitative real-time PCR was done on a LightCycler 480 by Roche Applied Science (Basel, Switzerland).

2.8.1 Oligonucleotides for PCR

Name	Application	Sequence 5'➔3'
Gadd45β fwd	RT-PCR	CAC CCT GAT CCA GTC GTT CT
Gadd45β rev	RT-PCR	TTG CCT CTG CTC TCT TCA CA
Nur77 fwd	RT-PCR	TTC ATC CTC CGC CTG GCA TAC C
Nur77 rev	RT-PCR	GTC CGA AGC TCA GGC AGT TTG C
β-Actin fwd	RT-PCR	TGT TAC CAA CTG GGA CGA CA
β-Actin rev	RT-PCR	TCT CAG CTG TGG TGG TGA AG
FasL fwd	RT-PCR	CGG TGG TAT TTT TCA TGG TTC TGG
FasL rev	RT-PCR	CTT GTG GTT TAG GGG CTG GTT GTT
Bim fwd	RT-PCR	CTG AGT GTG ACA GAG AAG GTG G
Bim rev	RT-PCR	GTG GTC TTC AGC CTC GCG GT
Gadd45β qRT fwd	qRT-PCR	CTG CCT CCT GGT CAC GAA
Gadd45β qRT rev	qRT-PCR	TTG CCT CTG CTC TCT TCA CA

2.9 Materials and Reagents for confocal laser scanning microscopy

The fluorescent microscopic pictures were either taken with a Leica TCS SP5 II (Wetzlar, Germany) confocal laser scanning microscope (CLSM) or with a Nikon Eclipse Ti (Düsseldorf, Germany) microscope, additionally equipped with the spinning disc module UltraVIEW VoX by PerkinElmer (Waltham, USA).

2.9.1 Primary antibodies

Antibody	Reactivity	Isotype	Species	Clone/ Notation	Company
Anti-c-MYC	All	IgG	Rabbit	71D10	Cell Signalling
Anti-MEKK4	H, M, R	IgG_{2b}	Mouse	6C6	Sigma-Aldrich
Anti-V5	H, M	IgG_{2a}	Mouse	R960-25	Invitrogen
Anti-p38 (P)	H, M	IgG	Rabbit	9211	Cell Signalling
Anti-HA	H, M	IgG_{2b}	Mouse	12CA5	Roche Applied Science

2.9.2 Fluorochrome-labeled secondary antibodies

Antigen	Reactivity	Species	Dye	Application	Company
IgG	Mouse	Goat	AlexaFluor 405, 488, 594, 633	Immunofluorescence (IF)	Molecular Probes/ life technologies
IgG_1	Mouse	Goat	AlexaFluor 488, 594	IF	Molecular Probes/ life technologies
IgG_{2a}	Mouse	Goat	AlexaFluor 488, 594	IF	Molecular Probes/ life technologies
IgG_{2b}	Mouse	Goat	AlexaFluor 488, 594	IF	Molecular Probes/ life technologies
IgG	Rabbit	Goat	AlexaFluor 405, 488, 594, 633	IF	Molecular Probes/ life technologies

2.10 Frequently used buffers

Group	Buffer	Components
Cellular Buffers	PBS	138 mM NaCl
		8.1 mM Na_2HPO_4
		2.7 mM KCl
		1.5 mM K_2HPO_4, pH 7.4
	TBS	50 mM Tris
		150 mM NaCl, pH 7.4
	FACS Buffer	2% w/v BSA
		in PBS
	MACS Buffer	2% w/v BSA
		in PBS
	Annexin V Binding Buffer	10 mM Hepes/ NaOH pH 7.4
		140 mM NaCl
		2.5 mM $CaCl_2$
	CLSM Blocking Buffer	4% w/v BSA
		0.05% w/v Saponin
		in PBS
Lysis Buffers	TPNE	Final concentration 300 mM NaCl
		1% v/v Triton X-100
		2 mM EDTA
		in PBS
		pH 7.4
	DISC	30 mM Tris/ HCL pH 7.4
		150 mM NaCl
		10% v/v Glycerol
		1% w/v Triton X-100

		2 mM EDTA
		10 mM NaF
	RIPA	50 mM Tris pH 7.4
		1% NP40
		0.5% Sodium Deoxycholat
		0.1% SDS
		150 mM NaCl
		1 mM EDTA
		1 mM NaF
Immunoblot Buffers	5x Laemmli (Laemmli 1970)	50 mM Tris, pH 8.8
		10% w/v SDS
		25% v/v β-Mercaptoethanol
		50% v/v Glycerol
		0.25 mg/ml Bromphenolblue
	Running Buffer	25 mM Tris, pH 8.0
		192 mM Glycerol
		1% v/v SDS
	Transfer Buffer	25 mM Tris, pH 8.0
		192 mM Glycerol
		20% v/v Methanol
	Block Buffer	5% w/v non-fat dry milk
		0.2% v/v Tween-20
		in TBS
	Wash Buffer	0.05% v/v Tween-20
		in TBS
	100x protease inhibitors	100 µg/ml Apotinin

	100 µg/ml Leupeptin
	100 µg/ml Chymostatin
	100 µg/ml Pepstatin A

3. Experimental Procedures

3.1 Molecular Methods

3.1.1 Isolation of eukaryotic RNA

The RNA of eukaryotic cells was isolated from purified mammalian cells using a RNeasy Kit from Qiagen (Hilden, Germany). The cell lysis was performed with the QIAshredder columns, also from Qiagen. Both procedures were done according to manual.

3.1.2 Isolation of prokaryotic plasmid DNA

The preparation of plasmid DNA out of *E. Coli* was done with a NucleoBond® Xtra Maxi EF Kit, provided by Macherey-Nagel (Düren, Germany). The experimental procedure was performed according to the manufacturers manual.

3.1.3 Photometric determination of DNA/RNA concentration

DNA/RNA concentrations were determined by a Nanodrop 2000c (Thermo Scientific). Calculations were done according to the Lambert-Beer function:

$$E = c*\varepsilon*d \quad <=> \quad c = \varepsilon*d*E^{-1}$$

E = extinction

c = concentration [mol/l]

ε = coefficient of extinction [$M^{-1}*cm^{-1}$]

d = density of the cuvette [cm]

3.1.4 Reverse Transcription

Purified RNA was transcribed into copyDNA (cDNA) for subsequent PCR analysis with the help of the RevertAid™ Premium First Strand cDNA Synthesis Kit (Thermo Scientific). Poly(A) tailed mRNA was specifically transcribed using Oligo$(dT)_{18}$ primers.

3.1.5 Polymerase chain reaction

DNA fragments were amplified by PCR, using 2x KAPA ReadyMix (Peqlab). This was done either for subsequent analysis of cDNA (as described in 3.1.4) or genotyping. No proof-reading capability is required for both applications. For any other procedure, Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA) was used.

PCR components were mixed according to the following pattern:

Component	Amount
cDNA Template	1 µl (~50-100 ng)
2x ready-to-use mix	12.5 µl
Forward Primer 100 pM	1 µl
Reverse Primer 100 pM	1 µl
Dest H_2O	Up to total amount of 25 µl

The PCRs were incubated in a PeqSTAR thermocycler according to the template program shown below. The hybridisation temperature was chosen 3°C under the T_M of the primer combination.

Time	Temperature	Function	No. of Cyles
5 min	94°C	Initial denaturation	1
30 sec	94°C	Denaturation	25-34 cycles
30 sec	60°C (+/-x°C)	Hybridization	
30 sec	72°C	Elongation	
10 min	72°C	Terminal elongation	1

3.1.6 Analytic agarose gel electrophoresis

DNA fragments were separated according to their length in a 1-2% agarose gel in an electric field of 150mA. The horizontal gel-electrophoresis system perfectBlue M (PeqLab) was used. The run was done in TAE buffer (40 mM Tris pH 8.0, 20 mM acetic acid, 1 mM EDTA) with 0.5 µg/ml ethidium bromide. The intercalated ethidium bromide was visualised by UV-light (λ=254 nm). Sizes were checked against a low range 1-500 bp or 1 kB DNA ladder from PeqLab.

3.1.7 Quantitative real-time polymerase chain reaction

The quantitative real-time polymerase (qPCR) was used to both detect and quantify specific sequences in a DNA sample. In the context of this work it was used following reverse transcription. The non-specific fluorescent dye SYBR Green (Roche), which intercalates into the double-stranded DNA, is measured after each cycle, thus allowing the DNA concentrations to be quantified against a known standard.

Given the fact that SYBR green detects every double-stranded DNA, a subsequent melting point analysis was done to discriminate between the correct product of the qPCR and artefacts, such as primer-dimers. As the disassociation of short, double-stranded DNA is faster than the one of longer fragments, this is an easy way to assure the correct amplification product.

3.2 Biochemical Methods

3.2.1 Cell Lysis

For whole cell extracts $1x10^6$ to $5x10^6$ cell lysates were prepared in 50 µl of adequate lysis buffer, depending on the subsequent analysis (see 2.10). All buffers were freshly complemented with 1 mM PMSF and 1 µg/ml each of leupeptin, aprotinin, chymostatin and pepstatin A. If subsequent analysis of the phosphorylation state of a protein was important, 1 tablet of PhosSTOP Phosphatase Inhibitor Cocktail (Roche) was added.

subsequently, the cell extracts were kept for 20 min on ice and subsequently centrifuged for 15 min at 20,000 g. The supernatant was transferred into a pre-chilled tube and used for futher experiments.

3.2.2 Determination of total protein concentration

The total protein amount of lysates as generated in 3.2.1 was determined by bichinchoninic acid (BCA) protein assay reagent provided by Pierce/ Thermo Fisher according to the manufacturer's protocol. Here, the total protein concentration is detected by a colour change of the sample solution from green to purple, which correlates in linear way (P. K. Smith et al. 1985). This colour change was measured at a wavelength of 562 nm with a TECAN infinite 200M plate reader (Männedorf, Switzerland).

3.2.3 Immunoprecipitation

Immunoprecipitation was used to achieve a well-directed enrichment of a particular protein out of a whole-cell lysat (see 3.2.1) or to detect a particular protein-protein interaction. To this end, either Protein A Sepharose CL-4B (Sigma-Aldrich) in a 50% slurry or Protein G Dynabeads® Magnetic Beads (Invitrogen) were incubated for 4 h at 4°C under constant rotation with the designated antibody. Depending on the antibody, between 2-5 µg were used for each sample of beads (20 µl for Dynabeads®, 35 µl for Protein A Sepharose).

Subsequently, the beads were washed three times to get rid of unbound antibody. 200 µl of whole lysate was added to the beads and filled up to 750 µl with the corresponding buffer. The samples were rotated either for 4 h or over night at 4°C and then unbound protein was washed away three times with 500 µl of the buffer. The beads were taken up in 20 µl Laemmli buffer (see 2.10), incubated for 5 min at 95°C and subsequently analysed by SDS-PAGE and immunoblot.

3.2.4 SDS-PAGE

20 µg of total protein lysate was mixed with 5x Laemmli buffer (see 2.10) to a final concentration of 1x and heated for 5 min at 95°C. Then, the sample was loaded and separated in a polyacrylamide gel from 8-20%, according to the size of the protein of interest. To this end, a Biorad tetra cell was used. The gel was run at 33 mA per gel and 80 V until the separation was completed.

3.2.5 Immunoblot

The proteins that were separated in the gel were subsequently transferred to a Polyvinylidene Fluoride (PVDF) membrane (GE Healthcare), using a Biorad criterion blotter in 1x transfer buffer (see 2.10) at 500 mA at 80 V for 90 min. Then, the membrane was incubated in with blocking buffer (see 2.10) for at least 1 hour at room temperature under constant gentle shaking and afterwards placed in blocking buffer containing primary antibody diluted to the suppliers instructions and incubated over night at 4°C in an overhead mixer. After washing three times with immunoblot washing buffer (see 2.10), the membranes were subjected to the secondary antibody, diluted in 1x TBS for at least 1 hour at room temperature. After subsequent washing for three times with washing buffer the proteins were detected by SuperSignal West Dura substrate from Pierce/ Thermo Fisher Scientific or ECL Select™ from Amersham/ GE Healthcare.

3.2.6 Coomassie Blue staining

Proteins that were separated via SDS-PAGE were subsequently stained and fixed with the PageBlue Protein Staining Solution from Pierce/ Thermo Fisher Scientific. The solution contains the triphenylmethane dye Coomassie Brilliant Blue G250. After 30 min of staining, the gel was decoloured by rinsing the gel 2-3 times with millipore water under gentle shacking until the background was completely clear of the dye.

3.2.7 P^{33} detection in SDS-PAGE

MEFs were cultivated for 2 days in cell culture flasks and then washed twice with TBS. Subsequently, they were cultivated for 20-30 min in DMEM w/o PO_4^{3-}(Invitrogen). Following this starvation period, 2 mCi P^{33} (Hartmann Analytic, Braunschweig, Germany) were added to the medium and the cells were incubated for another 4 h. Then, the medium was discarded and the uptake was stopped with ice-cold PBS supplemented with NaF 10 mM, $NaVO_4$ 0.4 mM, EDTA 0.4 mM, $Na_4P_2O_7$ 10mM. The cells were lysed and subjected to immunoprecipitation (see 3.2.3) and subsequent SDS-PAGE in a NuPAGE® 4-20% SDS gel (Invitrogen). The gel was fixed (MeOH 20% v/v, acetic acid glacial 10% v/v) for 30 min and subsequently treated for 30 min with NAMP100 Amplify Solution (Amersham, GE Healthcare). Subsequently, the gel was dried and exposed to x-ray films where the irradiation blackened the film.

3.2.8 Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) was done to verify and quantify the specificity of a given antibody against its respective antigen. To this end, the antigen (100 µl in a 2 µg/ml solution in PBS) was bound overnight at 4°C to a 96-well flat-bottom plate (NUNC). Subsequently, unbound protein was washed away 3x with TBS-Tween (immunoblotblot washing buffer, see 2.10). The coated plate was incubated for 2 hours with

a blocking solution (2% (w/v) BSA in TBS-T) under gentle pivoting. By this means, the whole surface of the plate was saturated to prevent unspecific binding of the antibody later on.
The plate was then washed three times with the washing buffer and subsequently each well was covered with 100 µl of the diluted serum for 1 hour. Afterwards, the plate was again washed three times and then incubated for 45 min with a secondary, horseradish peroxidase coupled antibody against the respective first antibody. The antibody was diluted in a range from 1:2,000 to 1:5,000. Followed by three washing steps, 50 µl for each well of developing fluid (9 ml 0.11 M Na_2HPO_4, 1 ml 0.5 M citric acid, 10 mg OPTG, 10 µl 33% H_2O_2) was added. The colour change could usually be observed within minutes and then detected with a plate reader at a wavelength of λ=450 nm.

3.3 Cell biological methods

3.3.1 Transient transfection

Depending on the type of cell and the plasmid used, either jetPEI® by Polyplus-transfection (Illkirch Cedex, France), or Lipofectamine™ 2000 by Invitrogen were applied to transiently transfect the cells according to the manufacturers manual.

3.3.2 Production of Lentiviruses

Lentiviral transduction is an efficient way to incooperate genetic material stably into a given cell line. In order to produce the virus particles, HEK293T cells were seeded in 10 cm dishes. Three different preparations were made (each with 10 µg DNA per dish): The viral transfer vector including the target gene, the envelope plasmid and a third one encoding for viral structure proteins and the reverse transcriptase. 24 h 48 h post transfection the medium was exchanged and the viral containing medium was filtrated (0.45 µm, PVDF-membrane) and subsequently concentrated via ultracentrifugation (100000 g, 1 h, 4°C). The virus pellet was resuspendet thoroughly in PBS and stored at -80°C.

3.3.3 Transduction of cells with viral particles

For each approach of target cells, one batch of viral particles was used. Together with the cells, the virus particles were spun down for 1 h at 2000 rpm at 25°C. In order to enhance the effectiveness of the transduction, 8 µg/ml polybren were added. Subsequently, the cells were cultivated for 16 h under standard cell culture conditions. Then, the medium was changed and the cells were analysed for positive transduction.

3.4 Production and filtration of recombinant proteins

3.4.1 Expression of recombinant proteins

For the expression and subsequent filtration of the recombinant proteins, 500 ml of antibiotic containing LB medium (see 2.2.2) was inoculated with the respective Bl-21(DE3) strain of *E. Coli* and incubated at over night at 37°C at 220 rpm. The culture was then thinned with fresh LB medium to an optical density OD_{600} of 0.5 and incubated for another 30 min. Subsequently, the overexpression was started by addition of IPTG (Berkshire, UK). After induction, the bacteria were centrifuged for 10 min at 6000 rpm in a 5810R centrifuge from Eppendorf, the supernatant was then discarded and the pellet resuspended in 40 ml PBS. The bacteria were solubilised by ultrasonic (1 min, 70% power, Sonoplus Bandelin, Berlin, Germany) and subsequently centrifuged for 1 hour at 20000 rpm at 4°C. The supernatant was separated and according to the protein in question either the soluble or the unsoluble fraction was filtrated.

3.4.2 Dialysis

Dialysis allows to exchange the buffer of a given sample against another one, e.g. PBS. Prior to the procedure, the tube itself has to be cleared of heavy metal ions, which are a by-product of the manufacturing process. To this end, the tubes are heated for 2 hours at

60°C in a 1M $NaCO_3$ and 20 mM EDTA buffer solution, followed by a thorough flushing of the tube. The ratio of dialysed sample to dialysis buffer (PBS) must be 1:100, the process was done over night at 4°C under very gentle stirring.

3.4.3 Purification of polyclonal antibodies

In order to obtain a working anti-Gadd45β antibody, rabbits were immunised against full-length protein of Gadd4β. The sera were extracted after 120 days of immunisation and subjected to an ELISA analysis to verify specificity against the antigen. The sera were then diluted 1:10 with PBS and enriched with the help of a 5 ml protein G column, provided by Pierce/ Thermo Fisher Scientific in a Fast Protein Liquid Chromatography (FPLC) (UPC-900, GE-Healthcare). After FPLC purification, the samples were dialysed, the protein concentration was measured and the purity was confirmed with the help of a SDS-PAGE with subsequent coomassie staining.

3.5 Confocal laser scanning microscopy (CLSM)

3.5.1 Preparation of fixed samples

Adherent cells (mouse embryonic fibroblasts, MEFs) were seeded on a coverslip in a 12-well plate (NUNC) and then cultured over night for adherence. Depending on the experimental design, the cells were then subjected to the treatment in question. Subsequently, the cells were washed once with PBS and then fixed for 15-20 min with a freshly prepared 4% paraformaldehyde solution, diluted in PBS. After 3 washing steps with PBS for 5 min at room temperature, the cells were blocked and made permeable for 30 min with the CLSM blocking buffer (see 2.10). Afterwards, the cells were incubated over night, if necessary, with the first antibody at 4°C in CLSM blocking buffer. The manufacturer supplied the proper dilutions of the antibodies. After again 3 times washing with PBS, the corresponding, fluorochrome-labelled secondary antibodies (see 2.9.2) were applied for 45-120 min in CLSM

blocking buffer. Subsequently, the cells were washed again and if required stained with DAPI. The cover slip was then mounted with the help of mounting medium (Dako, Glostrup, Denmark) on a microscope slide and was kept at 4°C until usage. All steps from the instant of permeabilisation were performed under the exclusion of light.

3.5.2. *In vivo* imaging of primary T cells

LC3-GFP$^+$ T cells out of the thymus, spleen and peripheral lymph nodes were subjected to CLSM microscopy in an 8-well cell culture chamber (Sarstedt, Germany) pre-coated with Poly-L-Lysine (Sigma Aldrich) and kept at 37°C in a 5% CO_2 and 95% air humidity atmosphere during the whole experiment. The cells were stimulated with 20 ng/ml PMA and 1 µM Ionomycin and GFP-induction was observed in a time-dependent manner with a confocal microscope.

3.6 Cellular and mouse surgical methods

3.6.1 Isolation of lymphoid organs from mice

For the analysis of primary T cells, the thymus, spleen, mesenteric and peripheral lymph nodes were surgically removed from mice sacrificed either by cervical dislocation or by the exposition to high concentrations of CO_2. The organs were milled through a 45 µm filter and were suspended in 6.5 ml PBS. The cells were kept on ice at all times.

3.6.2 Flow cytometric analysis

$2*10^6$ cells, isolated as described in 3.5.1, were washed twice with 1 ml of FACS buffer (see 2.10) and resuspended in 250 µl of the same. Then, the cells were stained with the proper antibodies (see 2.6.3) with the dilutions specified by the manufacturer or with a self-determined concentration through at 4°C for 15-30 min. Subsequently, the cells were washed twice with 1 ml FACS buffer and then analysed in a LSR II Special Order System by

Becton, Dickinson. Intracellular staining of Foxp3 was done according to the manual either with a Kit from eBioscience or from Miltenyi Biotech.

3.6.3 Cell isolation via flow cytometry

The cells were collected as described in 3.5.1 and stained as described in 3.5.2 with the exception that PBS replaced the FACS buffer. Subsequently, they were sorted in a FACS Aria II (Becton, Dickinson) or a Moflo (Beckmann and Coulter). The cells were collected in a tube already containing the proper medium. The cells were sorted with the help of Dr. Lothar Gröbe from the sorting facility of the Helmholtz-Centre for Infections Research.

3.7 Quantification and Statistical analysis

3.7.1 Quantification of confocal laser scanning microscopy images

All confocal laser scanning microscopy images were quantified with the Volocity 3D Image Analysis Software by PerkinElmer. At least 50 cells were analysed for each quantification. Depending on the reporter construct that was used, either autophagosomes or autophagosomes and autophagolysosomes were counted. The software discriminated genuine signal against unspecific background via reconciliation of the intensity. To this end, all pictures of the same experiment were recorded with the same laser intensities. In the case of the Leica TCS SP5 II the settings of the photomultipliers were left untouched in the course of one experiment, with the Nikon Eclipse Ti equipped with the UltraVIEW VoX spinning disc module the exposure time of the CCD cameras staid the same.

The software used the watershed algorithm (Beucher 1992) to separate two signals that were closely adjoined and was set to reject every signal smaller than 0.15 μm^2. As a general guideline, it was programmed to look for round objects the size of 1.5 μm^2 to 3 μm^2.

3.7.2 Statistical analysis

All statistics were evaluated with the help of the statistical software Graph Pad Prism from Graph-Pad-Software Inc. (La Jolla, USA). Statistical significance was calculated by the two-tailed Mann-Whitney U test. Standard deviation (s.d.) and standard error of the mean (s.e.m.) were represented as error bars.

4. Results

4.1 The Role of Gadd45β in autophagy

4.1.1 Gadd45β together with MEKK4 overexpression results in an increased number of GFP-LC3 positive intracellular structures

Gadd45β has been reported to interact with the mitogen-activated protein kinase (MAP3K) MEKK4 to activate the MAPK p38 (Takekawa and Saito 1998). Although this finding was limited to classical p38 activation and apoptosis, it could be shown that the activation of p38 by Gadd45β/MEKK4 has an impact on autophagy by guiding the phosphorylated p38 to autophagosomes in the cytoplasm (Keil 2008). The MAPK p38α could also be linked to autophagy through the interaction with Atg9 (Webber and Tooze 2010). Additionally, p38 could be identified to positively regulate oridonin-induced autophagy, be involved in glial fibrillary acidic protein-dependent autophagy and in Atg9-dependent, starvation induced autophagy (Tang et al. 2008; Cui et al. 2007; Webber and Tooze 2010). Along these lines, the transfection of Gadd45β either alone or together with MEKK4 into NIH/3T3 cells stably expressing GFP-LC3 could significantly raise the numbers of intracellular GFP-LC3 spots 24 h post transfection (Fig. 10a). In order to quantify this finding, 50 cells of each transfection (Gadd45β alone, MEKK4 alone, Gadd45β and MEKK4 together and an empty vector control) were analysed by confocal microscopy and counted with the help of the Volocity imaging software.

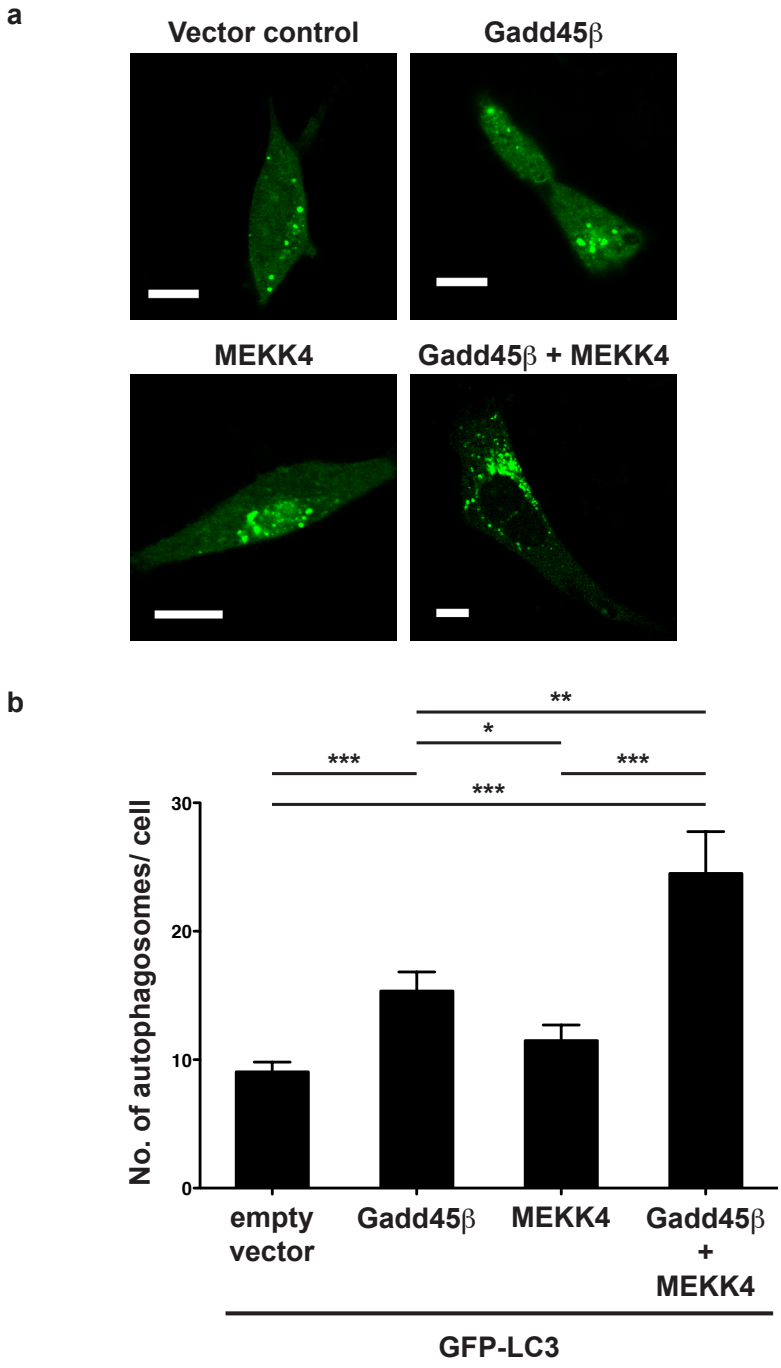


Figure 10: The influence of Gadd45β/MEKK4 overexpression on autophagosome formation in NIH/3T3 cells.
(a) NIH/3T3 cells stably expressing GFP-LC3 were transfected either with an empty vector, Gadd45β, MEKK4 or both of the latter. 24 h later confocal microscopy images were taken and the number of GFP-LC3 positive autophagosomes for each cell was counted in 50 cells per condition. Bars represent 10 µm. **(b)** The number of autophagosomes per cell in a statistic analysis. Significances were calculated by two-tailed Mann-Whitney tests (n=50, mean ± s.d., * P=0.0149, ** P=0.001, *** P<0.0001).

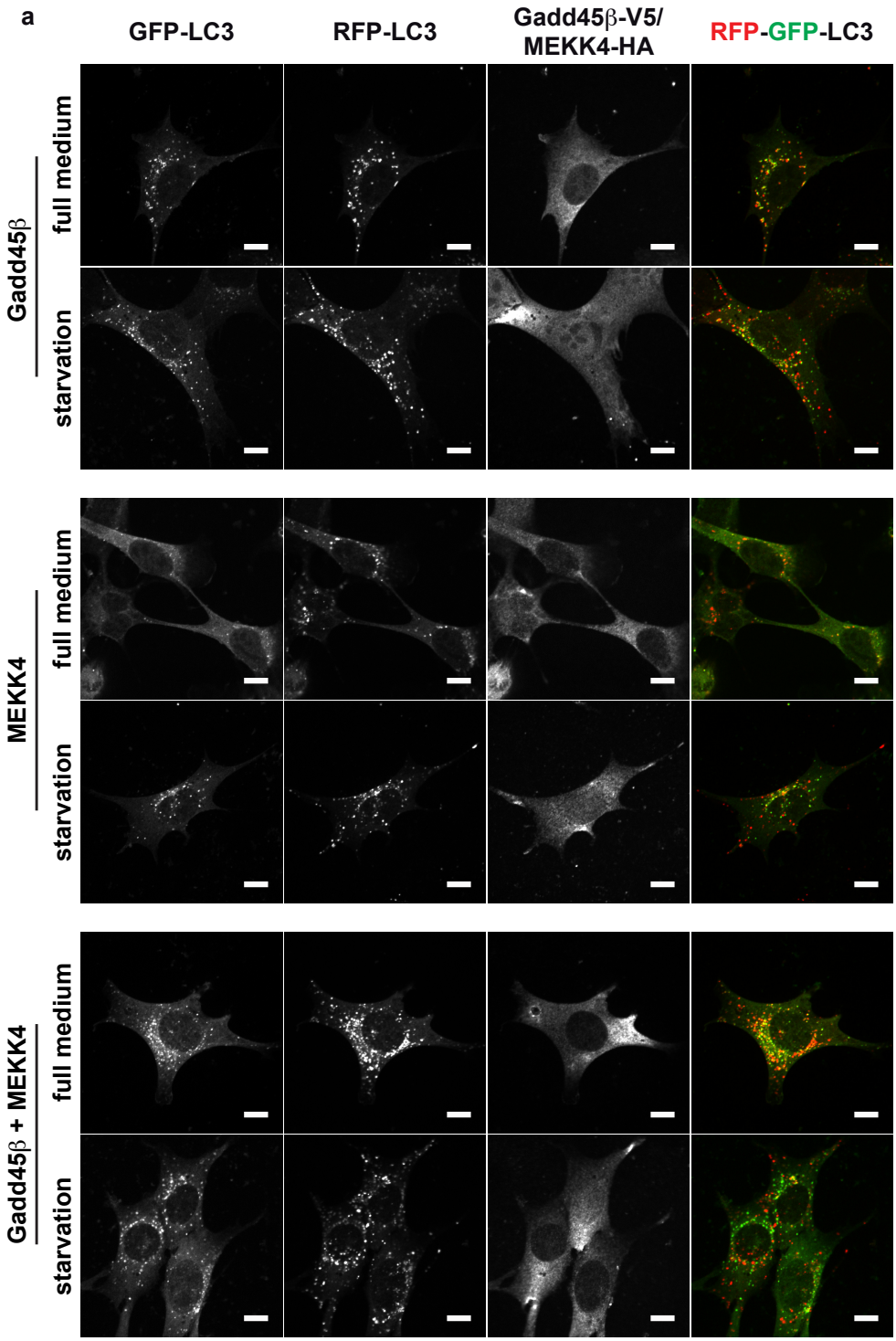
As a result it could be shown, that the co-expression of Gadd45β with MEKK4 significantly raised the amount of intracellular GFP-LC3 positive structures, i.e. autophagosomes. The overexpression of Gadd45β alone did also induce an increase in autophagosomal structures, but not to the same extend as both proteins did. Interestingly, MEKK4 transfection alone did not significantly raise the intracellular autophagy level. In summary, it could be shown that not only activated, phosphorylated p38 was directed to autophagosomes, but that p38 also seems to exert a functional role at these structures (Keil 2008).

4.1.2 The Gadd45β/MEKK4 pathway blocks starvation-induced autophagy

As could be shown in 4.1.1, Gadd45β together with MEKK4 transfection into NIH/3T3 cells stably expressing GFP-LC3 highly increased the number of GFP^+ cytosolic structures in the cell. However, in order to get a more detailed view of how p38 through the interplay of Gadd45β and MEKK4 influences autophagy, this method is insufficient. Thus, to quantify autophagy properly, it is important to differentiate between a higher amount of autophagosomes as a result of higher expression levels of autophagy-relevant proteins (in this case LC3) or because of a block in the autophagic flux. Both situations ultimately result in more GFP^+ cytosolic structures, i.e. autophagosomes (Klionsky et al. 2008). To be able to discriminate between the two different reasons of increased autophagosomes in the cytoplasm, the cells were transfected with an RFP-GFP-LC3 reporter construct (Kimura et al. 2007).

The function of the green fluorescent protein is more prone to acidic lysosomal degradation than the one of the red fluorescent protein, thus the emission of the green light is abbreviated earlier than the emission of the red light. A not-yet fused autophagosome would thereby be a RFP^+GFP^+ double positive structure in the cytoplasm, as opposed to a single positive RFP^+ spot that would mark an autophagosome already fused with a lysosome, an autolysosome. Thus, this method allows for monitoring of the autophagic flux.

NIH/3T3 cell line stably expressing RFP-GFP-LC3 were transfected with V5-tagged Gadd45β, MEKK4-HA or both and were subjected 24 h post-transfection to 4 h of HBSS-induced starvation (Fig. 11a).



b

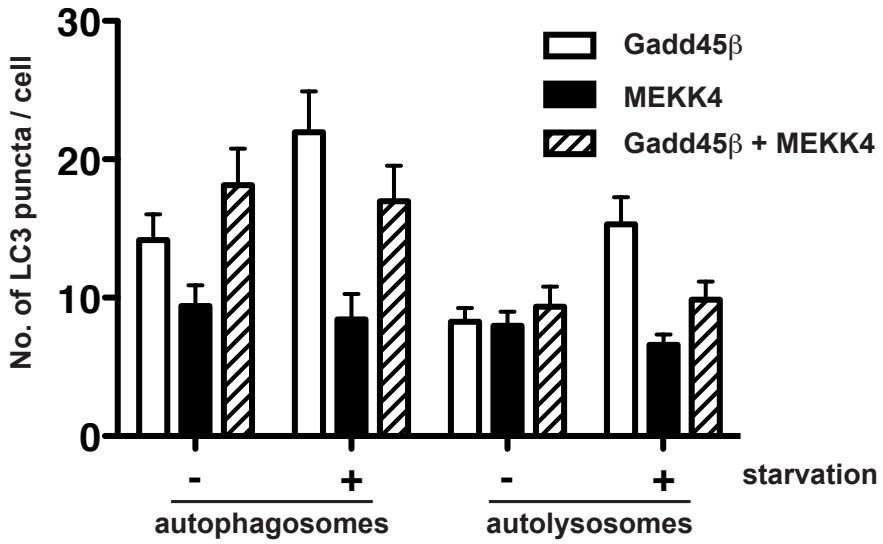


Figure 11: Gadd45β inhibits starvation-induced autophagic flux.
(a) NIH/3T3 cells stably expressing RFP-GFP-LC3 were transiently transfected with V5-tagged Gadd45β and HA-tagged MEKK4. 24 h post-transfection, the cells were fixed and stained either with anti-HA or anti-V5 antibody (AF633). Subsequently, samples were analysed by confocal microscopy. Representative images are shown. Bars, 10 µm. **(b)** Quantification of the experiment shown in (a). The number of autophagosomes (RFP^+GFP^+) and autolysosomes (RFP^+GFP^-) per cell was counted in 50 cells per condition ± s.e.m.

As observed with the GFP-LC3 reporter construct, the transfection of Gadd45β alone had a high effect on the rate of on-going autophagy. MEKK4 transfection alone did not alter the amount of either autophagosomes or autolysosomes, starved or under nutrient-sufficient conditions (Fig. 11b). Starvation had also no effect on the autophagic levels in Gadd45β transfected cells. Moreover, the co-transfection of Gadd45β and MEKK4 led to an increase in autophagosome numbers but not in autolysosomes. Again, starvation did not significantly alter the dynamics of the autophagic flux. Both experiments together show the influence of Gadd45β and MEKK4 on cellular macroautophagy.

4.1.3 p38-deficiency leads to a highly increased amount of autophagosomes but only to a minor increase of autolysosomes

(p)p38 colocalisation with autophagosomes could be identified via confocal microscopy and phosphorylated, thus activated, (p)p38 could be found in lysates cotransfected with Gadd45β and MEKK4 without further stimulation. To identify the role that p38 plays in the autophagy

pathway, the effect of a p38α gene knockout was determined by analysing confocal microscopy images of p38α$^{-/-}$ (Adams et al. 2000) and the corresponding wild type MEFs (Fig 12a). To quantify these numbers in a statistical adequate approach, pictures of 50 cells per condition were made by confocal microscopy and subsequently analysed with the help of imaging software.

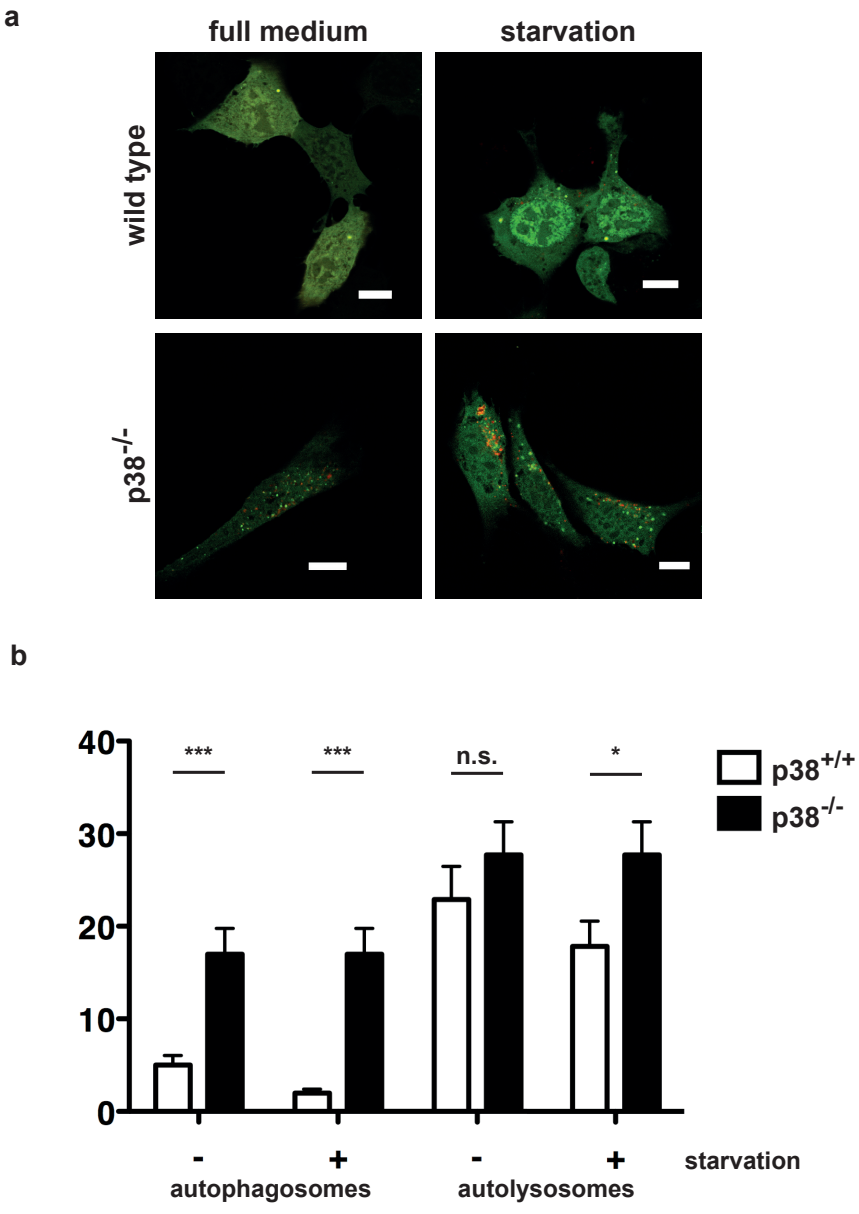


Figure 12: p38-deficiency severely decreases the amount of autophagosomes but not autolysosmes under starvation conditions.
(a) p38$^{+/+}$ and p38$^{-/-}$ MEFs were transfected with the RFP-GFP-LC3 reporter constructed. 24 h post-transfection the cells were starved for 4 h in HBSS or left untreated. Subsequently, the cells were fixed and analysed by confocal microscopy for GFP-RFP localisation. Bars represent 10 µm. **(b)** Statistical significances were calculated by two-tailed Mann-Whitney tests (n=50, mean ± s.d., * P=0.0142, *** P<0.0001, n.s. = not significant).

After quantitative analysis and statistic evaluation, the result showed that in p38 wildtype MEFs the number of autophagosomes (RFP^+GFP^+) as well as the number of autolysosomes (RFP^+) decreased upon starvation, whereas in the p38-deficient situation the number of both stayed the same (Fig. 12b). Comparing the results of both genotypes amongst each other, the most striking differences were observed in the number of autophagosomes. Irrelevant of the nutrition conditions, both results showed a highly significant increase in autophagosome numbers (P<0.0001) in the p38-deficient MEFs. The statistical analysis was calculated using the two-tailed Mann-Whitney test (n=50, mean ± s.d.). Further downstream of the autophagic flux, the tendency still remained the same but only to a minor extent (P=0.0142 for starvation conditions, n.s. for non-starvation conditions). Taken together, these results indicate that p38-deficiency results in a severe deregulation of the autophagic flux, irrespective of an acutely stimulated situation or the basal autophagy levels in an unstimulated condition.

4.1.4 Atg5 is a substrate of p38

The previous results show the impact that Gadd45β and MEKK4 exert on cellular macroautophagy through p38. To qualify as a target of MAPKs, a protein has to have a specific conserved S/T-P motif (Shaul and Seger 2007), meaning that every serine or threonine which can be phosphorylated has to be followed by a proline. Through numerous studies in yeast, many of the important molecules and their homologues in mouse and men could already be identified (Levine and Deretic 2007). Of the most prominent autophagy related proteins, an alignment showed Atg5 to have two such potential phosphorylation sites, at T75 and T249 (Fig. 13a). Atg5 has previously been shown to have a critical role in macroautophagy (Kuma et al. 2004). Of those two potential phosphorylation sites, only the TP binding site at position 75 is conserved through all species from men to yeast, the TP binding site at location 249/250 is not conserved in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Rattus novegicus*. Those three species also show the lowest overall homology of Atg5 as compared to mouse (Fig. 13b). Not surprisingly, *Homo sapiens* showed the highest homology to murine Atg5, 96.7% as opposed to *Saccharomyces cerevisiae* with

a homology of only 23.4%. Interestingly, *Danio rerio*, being a member of the class of the Actinoptygii, shows an Atg5 homology of 79.4% to mice as opposed to *Rattus norvegicus* with a homology of only 62.3%. Therefore, although being more closely related to the mouse in respect to the degree of kinship, the autophagic machinery is not as nearly related in the rat.

a

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                    1         10        20        30        40        50        60
                    |         |         |         |         |         |         |
      Mus musculus  MTDDKDVLRDVWFGRIPTCFTLYQDEI---TEREAEPYYLLLPR----VSYLTLVTDKVK
      Homo sapiens  MTDDKDVLRDVWFGRIPTCFTLYQDEI---TEREAEPYYLLLPR----VSYLTLVTDKVK
        Bos taurus  MTDDKDVLRDVWFGRIPTCFTLYQDEI---TEREAEPYYLLLPR----VSYLTLVTDKVK
        Sus scrofa  MTDDKDVLRDVWFGRIPTCFTLYQDEI---TEREAEPYYLLLPR----VSYLTLVTDKVK
     Gallus gallus  MTDDKDVLRDVWFGRIPTCFTLYQDEI---TEREAEPYYLLLPR----ISYLTLVTDKVK
       Danio rerio  MADDKDVLRDVWFGRIPACFTLSPDET---TEREAEPYYLLLPR----VSYLTLVTDKVK
  Rattus norvegicus MTDDKDVLRDVWFGRIPTCFTLYQDEI---TEREAEPYYLLLPR----VSYLTLVTDKVK
    D. melanogaster MAHDREVLRMIWEGQIGICFQADRDEI---VGIKPEPFYLMISR----LSYLPLVTDKVR
      S. cerevisiae MNDIKQLL---WNGELNVLVSIDPSFLMKGSPREIAVLRIRVPRETYLVNYMPLIWNKI-

      Mus musculus  KHFQKVMRQEDVS-EIWFEYEGTPLKWHYPIGLLFDLL-ASSSALPWN-----------I
      Homo sapiens  KHFQKVMRQEDIS-EIWFEYEGTPLKWHYPIGLLFDLL-ASSSALPWN-----------I
        Bos taurus  KHFQKVMRQEDIS-EIWFEYEGTPLKWHYPIGLLFDLL-ASSSALPWN-----------I
        Sus scrofa  KHFQKVMRQEDIS-EIWFEYEGTPLKWHYPIGLLFDLL-ASISALPWN-----------I
     Gallus gallus  KHFQKVMRQEEVN-EIWFEYEGTPLKWHYPIGLLFDLH-ASNTALPWS-----------I
       Danio rerio  KHFLKVMKAEDVE-EMWFEHEGTPLKWHYPIGVLFDLH-ASNSALPWN-----------V
  Rattus norvegicus KHFQKVMRQEDVS-EIWFEYEGTPLKWHYPIGLLFDLL-ASSSALPWN-----------I
    D. melanogaster KYFSRYISAEHQDGAVWFDFNGTPLRLHYPIGVLYDLLHPEEDSTPWC-----------L
      S. cerevisiae KSFLSFDPLTDSEKYFWFEHNKTPIPWNYPVGVLFDCLAGKSATFTTSFENQVKDVLTFL

      Mus musculus  TVHF---KSFPEKDLLHCPSKDAVEAHFMSCMKEAD-ALKHKSQVINEMQKKDHKQLWMG
      Homo sapiens  TVHF---KSFPEKDLLHCPSKDAIEAHFMSCMKEAD-ALKHKSQVINEMQKKDHKQLWMG
        Bos taurus  TVHF---KSFPEKDLLHCPSKDVIEAHFMSCVKEAD-ALKHKSQVINEMQKKDHKQLWMG
        Sus scrofa  TVHF---KSFPEKDLLHCPSKDVIEAHFMSCVKEAD-ALKHKSRVISDMQRKDHKQLWMG
     Gallus gallus  TVHF---KNFPEKDLLHCHSKDVIEAHFMACIKEAD-ALKHKSQVINEMQKKDHKQLWMG
       Danio rerio  TVHF---KNFPEQDLLHCSTNSVIEAHFMSCIKEAD-ALKHKGQVINDMQKKDHKQLWMG
  Rattus norvegicus TVHF---KSFPEKDLLHCPCKDAVEAHFMSCVKEAD-ALKHKSQVINEMQRKDHKQLWMG
    D. melanogaster TIHF---SKFPEDMLVKLNSKELLESHYMSCLKEAD-VLKHRGLVISAMQKKDHNQLWLG
      S. cerevisiae RIHLVMGDSLPPTIIPIASSKTQAEKFWFHQWKQVCFILNGSSKAIMSLSVNEARKFWGS

      Mus musculus  LQNDRFDQFWAINRKLMEYPPEENGFRYIPFRIYQTTTERPFIQKLFRP-VAADGQLHTL
      Homo sapiens  LQNDRFDQFWAINRKLMEYPAEENGFRYIPFRIYQTTTERPFIQKLFRP-VAADGQLHTL
        Bos taurus  LQNDRFDQFWAINRKLMEYPAEENGFRYIPFRIYQTTTERPFIQKLFRP-VSTDGQLHTL
        Sus scrofa  LQNDRFDQFWTINRKLIEYPPEENGFRYIPFRIYQTTTERPFIQKLFRP-VAADGQLHTL
     Gallus gallus  LQNDKFEQFWAINRKLMEYPPEDSGFRYIPFRIYQATTERPFIQKLFRP-IASGGQLHTL
       Danio rerio  LQNDKFDQFWAMNRKLMEYPTEEGGFRYIPFRIYQTMSDRPFIQTLFRP-VSSEGQALTL
  Rattus norvegicus LQND-LTSFG---------PSTGNSWNTLQKKMDFVISLLEYI----RP---------QL
    D. melanogaster LVNEKFDQFWAVNRRLMEPYGDLESFKNIPLRIY-TDDDFTYTQKLISP-ISVGGQKKSL
      S. cerevisiae VITRNFQDFIEISNKI-----SSSRPRHIPLIIQTSRTSGTF--RISQPTISMTGVNPTL

      Mus musculus  GDLLREVCPSAVAPEDGEKRSQVMIHGIEPMLETPLQWLSEHLSYPDNFLHISIVP-Q--
      Homo sapiens  GDLLKEVCPSAIDPEDGEKKNQVMIHGIEPMLETPLQWLSEHLSYPDNFLHISIIP-Q--
        Bos taurus  GDLLKEVCPSAVAPEDGEKKNQVMIHGIEPMLETPLQWLSEHLSYPDNFLHISIIP-Q--
        Sus scrofa  GDLLREVCPSAVAPEDGEKKSQVMIHGIEPLLETPLQWLSEHLSYPDNFLHISIVP-Q--
     Gallus gallus  GDLLKDVCPSAITPEDGEKTTQVMIHGIEPMLETPLQWLSEHMSYPDNFLHISIIP----
       Danio rerio  GDLLKELFPAAI--EDEPKKFQVMIHGIEPLLETPIQWLSEHLSHPDNFLHISIIP----
  Rattus norvegicus NGLSFRSCS-------------------------VLWPQMDSCIP---LEIS-------
    D. melanogaster ADLMAELSTPV------RRAVGCRTHGIDLHEETQLQWMSEHLSYPDNFLHLSVDY-K--
      S. cerevisiae KDIEGDILD--VKEGINGNDVMVICQGIEIPWHMLLYDLYSKLRSFDGFLYITLVPIKGG

      Mus musculus  ----PTD
      Homo sapiens  ----PTD
        Bos taurus  ----PTD
        Sus scrofa  ----PTD
     Gallus gallus  ---RPTD
       Danio rerio  ---APSD
  Rattus norvegicus  -------
    D. melanogaster  -----DV
      S. cerevisiae  DKASSEL
```

b

	M. musculus	H. sapiens	B. taurus	S. scrofa	G. gallus	D. rerio	R. norvegicus	D. melanogaster	S. cerevisiae
Mus musculus		96.7%	96.0%	95.3%	88.8%	79.4%	62.3%	47.5%	23.4%
Homo sapiens	96.7%		97.8%	94.2%	89.5%	80.9%	61.6%	47.5%	22.7%
Bos taurus	96.0%	97.8%		94.9%	89.2%	81.2%	61.6%	47.8%	23.4%
Sus scrofa	95.3%	94.2%	94.9%		86.6%	79.1%	60.5%	46.8%	23.1%
Gallus gallus	88.8%	89.5%	89.2%	86.6%		79.7%	57.6%	46.2%	22.1%
Danio rerio	79.4%	80.9%	81.2%	79.1%	79.7%		53.6%	48.0%	22.1%
Rattus norvegicus	62.3%	61.6%	61.6%	60.5%	57.6%	53.6%		33.6%	15.6%
D. melanogaster	47.5%	47.5%	47.8%	46.8%	46.2%	48.0%	33.6%		18.8%
S. cerevisiae	23.4%	22.7%	23.4%	23.1%	22.1%	22.1%	15.6%	18.8%	

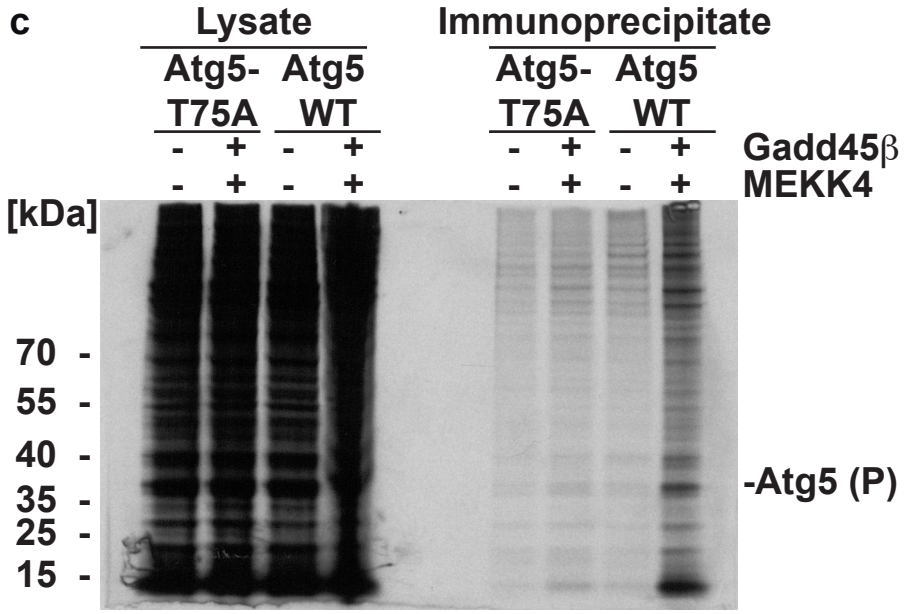


Figure 13: *In vivo* phosphorylation of Atg5 at threonine T75.
(a) Alignment of the protein sequences of Atg5 of the indicated species. Highlighted in grey are the potentially conserved phosphorylation sites. **(b)** Homology analysis of the Atg5 protein sequences of the same species as in (a) **(c)** $Atg5^{-/-}$ MEFs were stably reconstituted either with wildtype Atg5 or a mutant in which the threonine T75 is substituted against an alanine. Then, the cells were cultivated for 30 min in a phospho-deficient medium and subsequently in a $^{33}PO_4^{3-}$-containing one. After 4 h the cells were harvested, lysed and Atg5 was immunoprecipitated for 4 h. Subsequently, the beads were subjected to SDS-PAGE and autoradiography.

The phosphorylation of Atg5 by p38 was already shown *in vitro* (Keil 2008). To substantiate these *in silico* and *in vitro* findings and to verify a phosphorylation of Atg5 at the TP binding site 75 by p38, an *in vivo* phosphorylation approach was performed. To this end, reconstituted $Atg5^{-/-}$ MEFs (Kuma et al. 2004) either with MYC-tagged wildtype Atg5 or with an Atg5 mutant, in which threonine 75 was substituted against an alanine, were used. These cells were previously generated (Keil 2008). As expected, the transfection with Gadd45β and MEKK4 did not result in a phosphorylation of the phosphorylation-defective Atg5 T75A mutant, whereas in the wildtype reconstituted MEFs the incooperated ^{33}P darkened the x-ray film at a molecular weight of 35 kDa, the size of free Atg5 (Fig. 13c). Thus, the direct phosphorylation of Atg5 by the Gadd45β/MEKK4/p38 pathway could be proven.

4.1.5 Atg5 phosphorylation at threonine 75 blocks the autophagic flux

Overexpressing Gadd45β and MEKK4 in NIH/3T3 cells could clarify the role of the two proteins and their connection to the autophagic flux. To further strengthen the point that the phosphorylation site T75 of Atg5 is the target of p38 and its phosphorylation exerts a direct effect on the autophagic flux, again the Atg5$^{-/-}$ MEFs reconstituted either with wildtype Atg5, a T75A mutant with a substitution of threonine against alanine or a T75E mutant, where the threonine is substituted against a glutamic acid, were analysed (Fig. 14a).

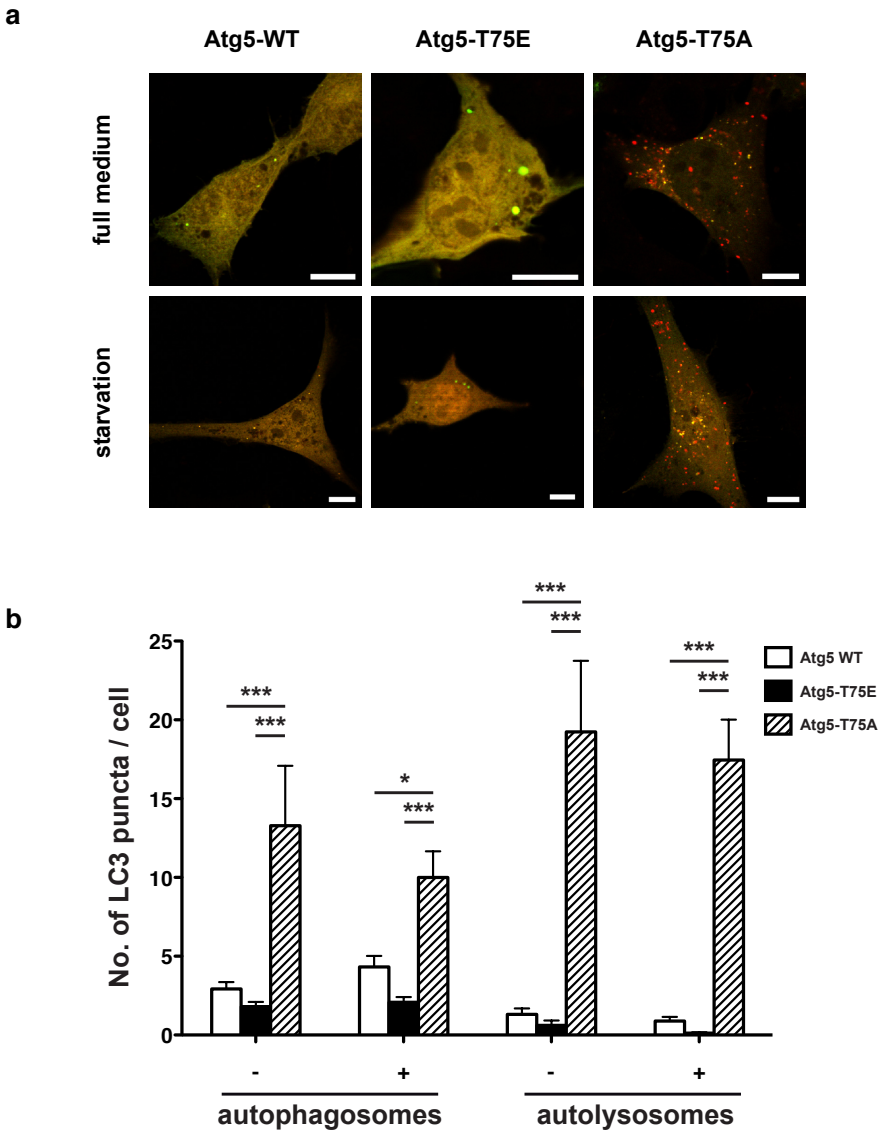


Figure 14: The effect of loss and gain of function of threonine 75 in Atg5 in MEFs under starvation.
(a) Atg5$^{-/-}$ MEFs were stably reconstituted with wildtype Atg5 or a mutant in which the threonine T75 is substituted against a glutamic acid or an alanine, respectively. The cells were transfected with the RFP-GFP-LC3 reporter construct and 24 h later either starved in HBSS for 4 h or left untreated. Then, the cells were fixed and subjected to confocal microscopy. Bars represent 10 µm. **(b)** Statistical significances were calculated by two-tailed Mann-Whitney test (n=50, mean ± s.d., * P=0.0166, *** P<0.0001).

The alanine substitution mutant T75A served as a negative control, which could no longer be phosphorylated. The T75E mutant mimicked a constant phosphorylation by providing a negative charge to Atg5 that imitated its phosphorylated state. With these two controls, the cells were transfected with RFP-GFP-LC3 and 24 h post-transfection either starved for 4 h with HBSS or left untreated in full medium. Subsequently, the cells were subjected to confocal microscopy and 50 pictures per cell type and condition were analysed for autophagosome (GFP^+) and autolysosome formation (GFP^+RFP^+) (Fig. 14b). In the wildtype situation a slight increase in the number of autophagosomes upon starvation could be detected, whereas the number of RFP^+ spots stayed the same. As expected, the starvation had no effect on the T75E mutant, demonstrating that the phosphorylation of Atg5 or in this case its substitution, led to a loss of sensitivity to nutrient conditions *in vitro*. Not surprisingly, the Atg5 T75A mutant showed completely inverted results. Regardless of the nutrition state of the cell, the number of autophagosomal (RFP^+GFP^+) spots was increased in a highly statistical relevant manner in contrast to the Atg5 T75E reconstituted cells ($P<0.0001$) and remained that way after starvation. The statistical analysis was calculated with the two-tailed Mann-Whitney test (n=50, mean ± s.d.). The same was even more true for the number of autolysosomes (RFP^+). These numbers were elevated to an even higher level than the autophagosomes, being statistical highly significant ($P<0.0001$), both against the wildtype reconstituted and the T75E mutant. Starvation, however, had no significant effect on the number of RFP^+ spots, as expected.

In line with the previous results, the T75E mutant also stayed the same, regardless of the nutrition state of the cells. However, the absolute numbers were even lower as compared to the wild type reconstituted cells. Taken together, these results confirmed the previous experiments and proved the function of the phosphorylation site threonine 75 in Atg5.

4.1.6 Phosphorylation of Atg5 at threonine 75 does not affect downstream localisation of the protein related to lysosomes

Preceding experiments could identify the phosphorylation of Atg5 at the threonine 75 as a key modulator of autophagic flux. Atg5 is known to form a complex with Atg12 and Atg16 and this complex mediates the lipidation of Atg8/LC3 during autophagosome formation (Mizushima et al. 2001; Komatsu et al. 2001). Upon completion of this task, this Atg5-Atg12-Atg16 complex is thought to be recycled from the autophagosomal membrane. To confirm or rule out that the phosphorylation of Atg5 at threonine 75 has an impact on the localisation of Atg5 after the lipidation of LC3 at the autophagosomal membrane, the localisation of Atg5 was determined via confocal microscopy. Again, $Atg5^{-/-}$ MEFs that were either reconstituted with wildtype Atg5, the T75A or the T75E mutant were starved for 4 h with HBSS or left untreated. Subsequently, the lysosomes were coloured with Lysotracker red and the MYC-tagged Atg5 was labelled with an α-MYC antibody in Alexafluor488. As controls, $Atg5^{-/-}$ MEFs were treated the same and wildtype reconstituted MEFs were stained without the primary antibody but with the secondary Alexafluor488 coupled (Fig. 15b). Neither in wildtype reconstituted, Atg5-T75A nor Atg5-T75E reconstituted MEFs a colocalisation between Atg5 and Lysotracker red^{+} spots could be observed (Fig.15 a,c,d). This result was consistent regardless of the nutrition state of the cell. These findings strongly suggest that the phosphorylation of Atg5 by p38 has no effect on the subcellular localisation of the same prior or after exerting its function of lipidation of Atg8/ LC3 at the autophagosomal membrane.

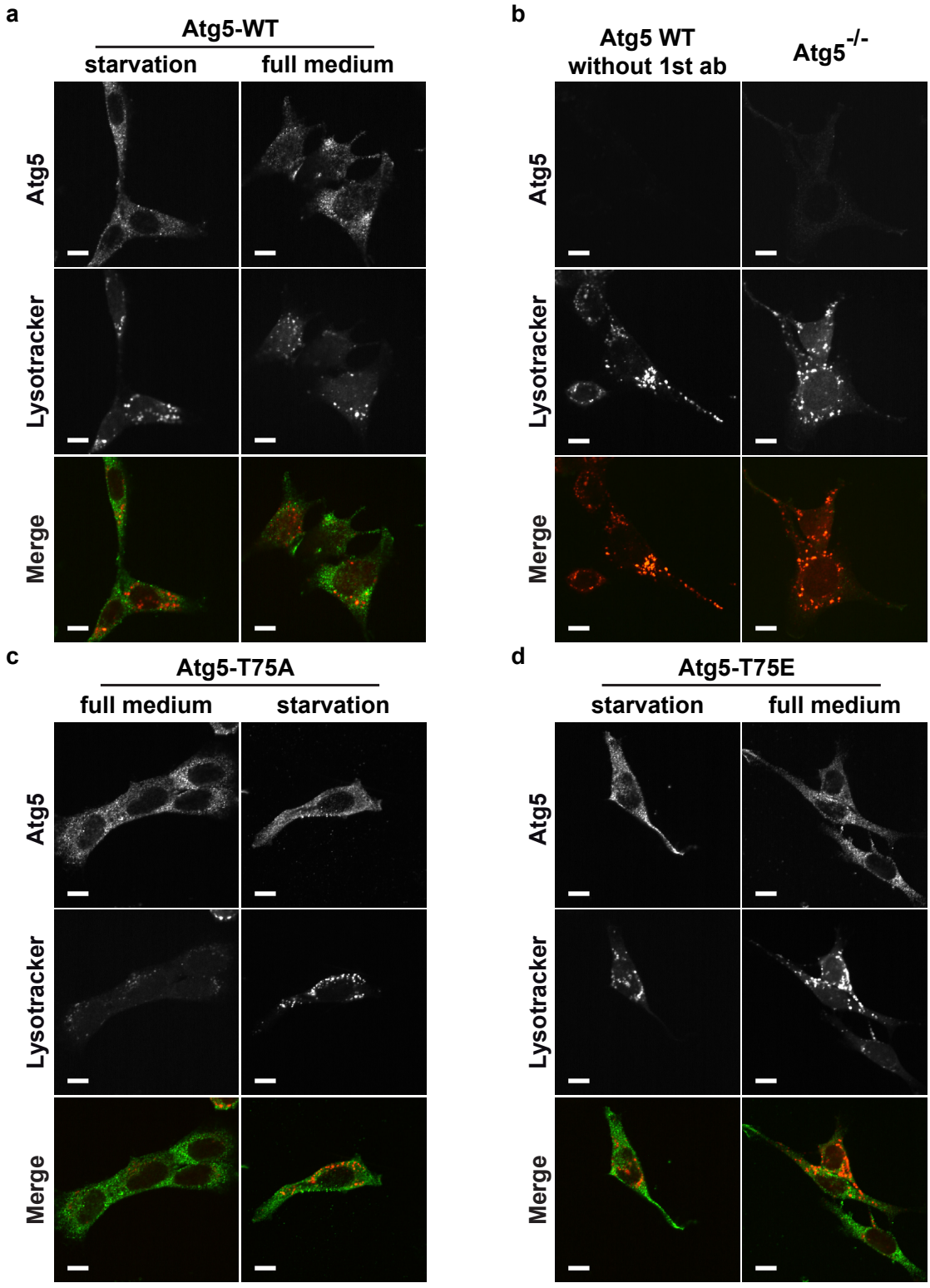


Figure 15: The phosphorylation state of threonine 75 does not alter the localisation of Atg5 against lysosomes.
(a) Atg5$^{-/-}$ mouse embryonic fibroblasts (MEFs) stably reconstituted with MYC-tagged Atg5 were either starved or left untreated for 4 h prior fixation. The tag was stained with an anti-MYC antibody (AF488) and lysosomes were coloured with Lysotracker red. Subsequently, samples were analysed by confocal microscopy. **(b)** MEFs as in (a) were treated the same but without the anti-MYC antibody. Atg5$^{-/-}$ MEFs were treated as in (a) as a control. **(c)** Atg5$^{-/-}$ MEFs stably reconstituted with an alanine for the threonine at amino acid 75 in Atg5 were treated as in (a). **(d)** Atg5$^{-/-}$ MEFs stably reconstituted with a glutamic acid for a threonine at amino acid 75 in Atg5 were treated as in (a).

4.1.7 Increased autophagy in Gadd45β-deficient cells upon lipopolysaccharide (LPS) stimulation

A recent publication showed that Gadd45β was induced upon stimulation with LPS (Zhang et al. 2005). Additionally, it was reported that autophagy initiation is mediated by Toll-like receptor 4 (TLR4) activation (Xu et al. 2007; Sanjuan et al. 2007; Delgado et al. 2009). In order to analyse a potential physiological role of the Gadd45β/MEKK4/p38 signalling event in the TLR4 pathway, MEFs that were generated from Gadd45β-deficient and their corresponding wildtype mice were stimulated with 100 ng/ml LPS for 0.5, 2, 4, 8 and 24 h or left untreated, both in the absence and presence of 100 nM bafilomycin A1 for the last 2 h of stimulation.

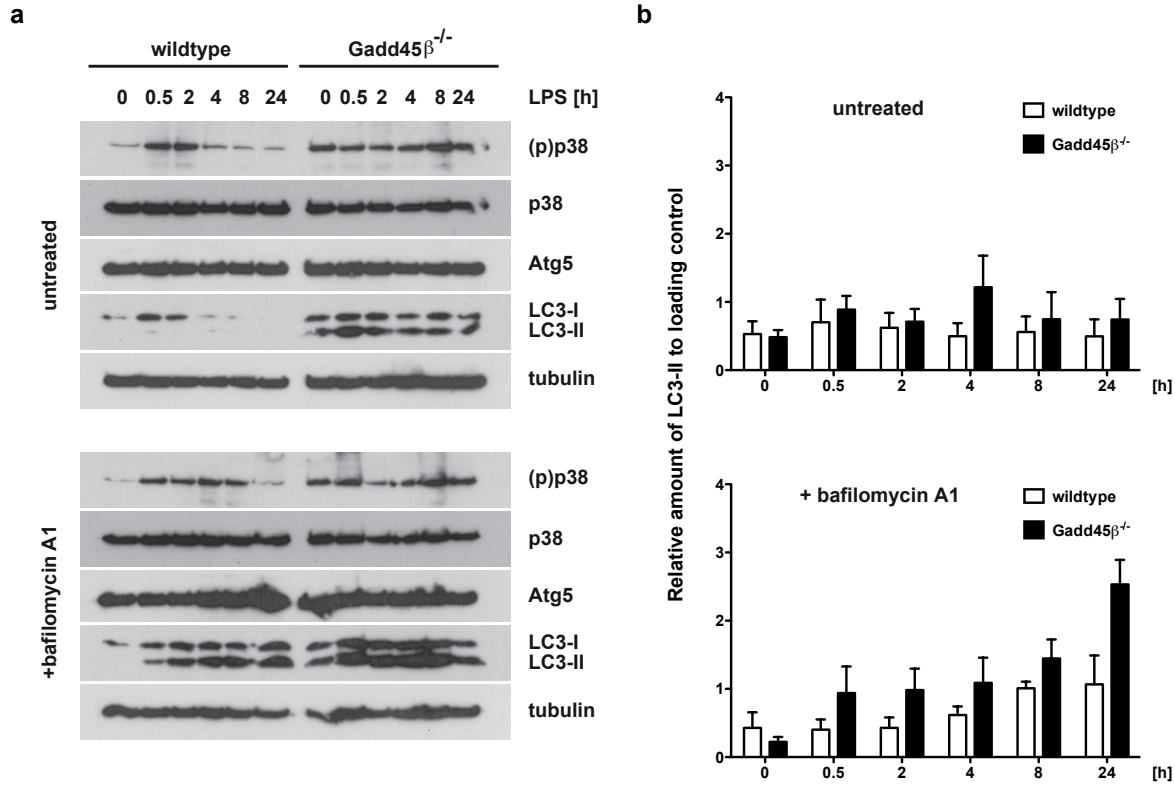


Figure 16: Gadd45β suppresses autophagy upon LPS treatment in MEFs.
(a) MEFs from wildtype and Gadd45β deficient animals were stimulated in vitro with 100 ng/ml LPS for the illustrated time periods. 2 h prior to harvesting, the cells were additionally treated with either 100 nM bafilomycin A1 or left untreated. Subsequently, cellular lysates were prepared and subjected to SDS-PAGE and immunoblotting. The blots were probed for phospho-p38, total p38, Atg5 and LC3. Tubulin served as a loading control. Results shown are representative for four independent experiments. **(b)** Quantification of the results shown in (a). The intensities of the LC3-II bands were normalised to the tubulin loading control. Data are represented as mean of three independent experiments ± s.e.m.

Bafilomycin A1 is an inhibitor of vacuolar type H^+-ATPases (V-ATPases) in eukaryotic cells and blocks the acidification of autophagosomes, thereby preventing autophagosomal degradation (Bowman et al. 1988; Yamamoto et al. 1998). Then, the protein extracts were subjected to SDS-PAGE and subsequent immunoblot analysis. In the absence of bafilomycin A1, almost no Atg8/LC3-I and no phosphatidylethanolamine (PE) conjugated LC3-II was detectable. The conversion of LC3-I to PE-conjugated LC3-II marks the membrane association of the protein to the autophagosome. By these means, the ratio of LC3-I to LC3-II and from LC3-II to a loading control can be used as an indicator of autophagic flux. As a consequence, autophagosomes are prone to lysosomal fusion and degradation, a block of this step is necessary to draw any valid conclusions (Klionsky et al. 2008).

In the Gadd45$\beta^{-/-}$ MEFs, the levels of membrane-associated LC3-II did already increase upon LPS stimulation without the inhibition of acidification by bafilomycin A1 treatment (Fig. 16a, upper panel). Moreover, when acidification was inhibited, the levels of LC3-II were highly increased in Gadd45$\beta^{-/-}$ MEFs upon stimulation with LPS. Already after half an hour of stimulation, the Gadd45β–deficient MEFs showed a high accumulation of LC3-II in contrast to the wildtype situation, where larger quantities of the protein only started to pile up after 4 to 8 h. By correlating the amount of LC3-II protein as determined by semi-quantitative immunoblot with the amount of a loading control, e.g. tubulin, it was possible to obtain quantitative information about the amount of LC3-II (Fig. 16b). Quantification of the abundance of LC3 was measured in three independent experiments and confirmed the observed increase in autophagic flux in the Gadd45$\beta^{-/-}$ situation upon stimulation with LPS.

The LPS stimulation of MEFs could be shown to have a direct connection to the Gadd45β/MEKK4/p38 pathway. Because the mouse embryonic fibroblasts are a model system, they do have certain drawbacks. These specific MEFs for example were immortalised by the transfection with the Simian Vacuolating Virus 40 Tag (SV40 large T antigen). In order to circumvent the possibility that the process of immortalisation has influence on the results, primary bone marrow-derived macrophages (BMDMs) were generated from wildtype and Gadd45β-deficient mice. To this end, bone marrow cells were

isolated out of the tibia and femur and differentiated for 7 days in DMEM containing M-CSF (see 2.2.5), (McWhirter et al. 2009). The BMDMs were analysed by FACS with the help of an αF4/80 antibody prior to LPS treatment against an unstained control to ascertain the differentiation into BMDMs (Fig. 17b).

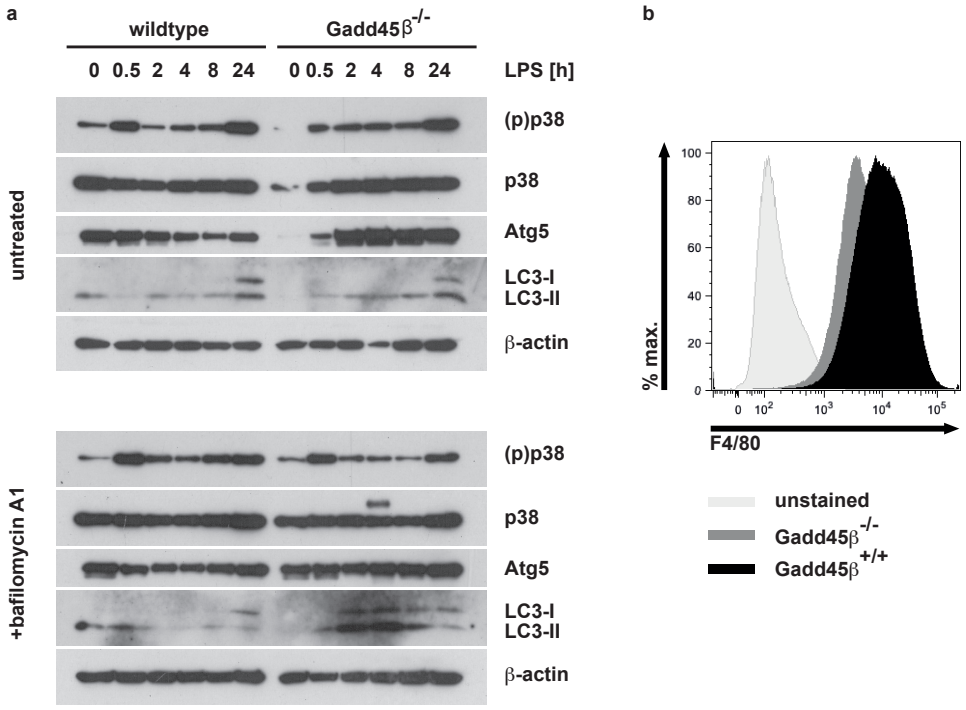


Figure 17: Gadd45β-deficiency enhances LPS-induced autophagy in bone marrow-derived macrophages.
(a) Bone marrow-derived macrophages (BMDMs) from wild type and Gadd45β-deficient mice were stimulated *in vitro* with 100 ng/ml LPS for the indicated time periods. In order to prevent the acidification of the autophagosomes and thus LC3-II degradation, half of the cells (lower panel) were treated with 100 nM bafilomycin A1 2 h prior to harvesting. Subsequently, cellular lysates were prepared and analysed by SDS-PAGE and immunoblotting. The blots were probed for phospho-p38, total p38, Atg5 and LC3. β-actin served as a loading control. The results are representative for two independent experiments. **(b)** F4/80 expression of BMDMs prior to LPS treatment, stained with a specific, FITC-labelled antibody and subsequent FACS analysis. Grey is the unstained control.

The F4/80 protein is the mouse homologue of the human EGF-like module-containing mucin-like hormone receptor-like 1 protein, a member of the adhesion-GPCR family and serves as a well-accepted marker for mature macrophages, which derive out of the myeloid lineage.

Subsequently they were stimulated with LPS (see 4.1.7), subjected to SDS-PAGE and analysed by immunoblotting. Without blocking the lysosomal degradation with the help of bafilomycin A1, neither wildtype nor Gadd45β-deficient BMDMs accumulated LC3-II to a

large extent. In both cases a significant amount of the protein could be detected only after 24 h of stimulation (Fig. 17a). Importantly, with the use of bafilomycin A1, an accumulation of LC3-II could already be detected after 2 h of stimulation in Gadd45β-deficient BMDMs, whereas the wild typical cells did not accumulate LC3-II to any significant extent. The data is representative for one out of two independent experiments with BMDMs.

Taken together, the results show that the Gadd45β-MEKK4-p38 pathway controls at least in part the TLR4-mediated autophagy in fibroblasts and in macrophages.

4.1.8 Gadd45β supresses αCD3-mediated autophagy in primary thymocytes

Schmitz and colleagues could show a high induction of *Gadd45b* transcript upon TCR stimulation in the N15 mouse model for negative selection in the thymus (Schmitz et al. 2003). As TCR signalling events have recently been shown to exert an impact on autophagy (Salmond et al. 2009), thymocytes were stimulated with αCD3 for the indicated time points.

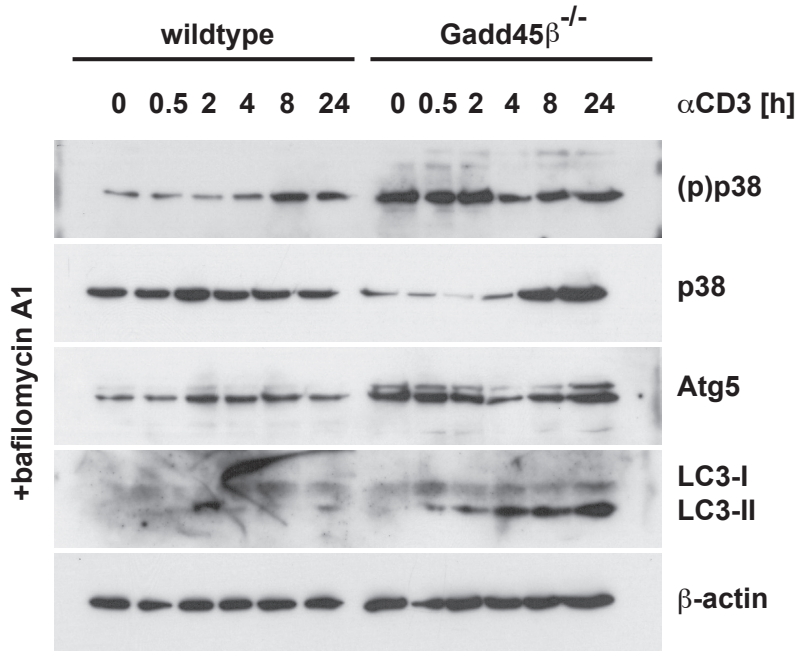


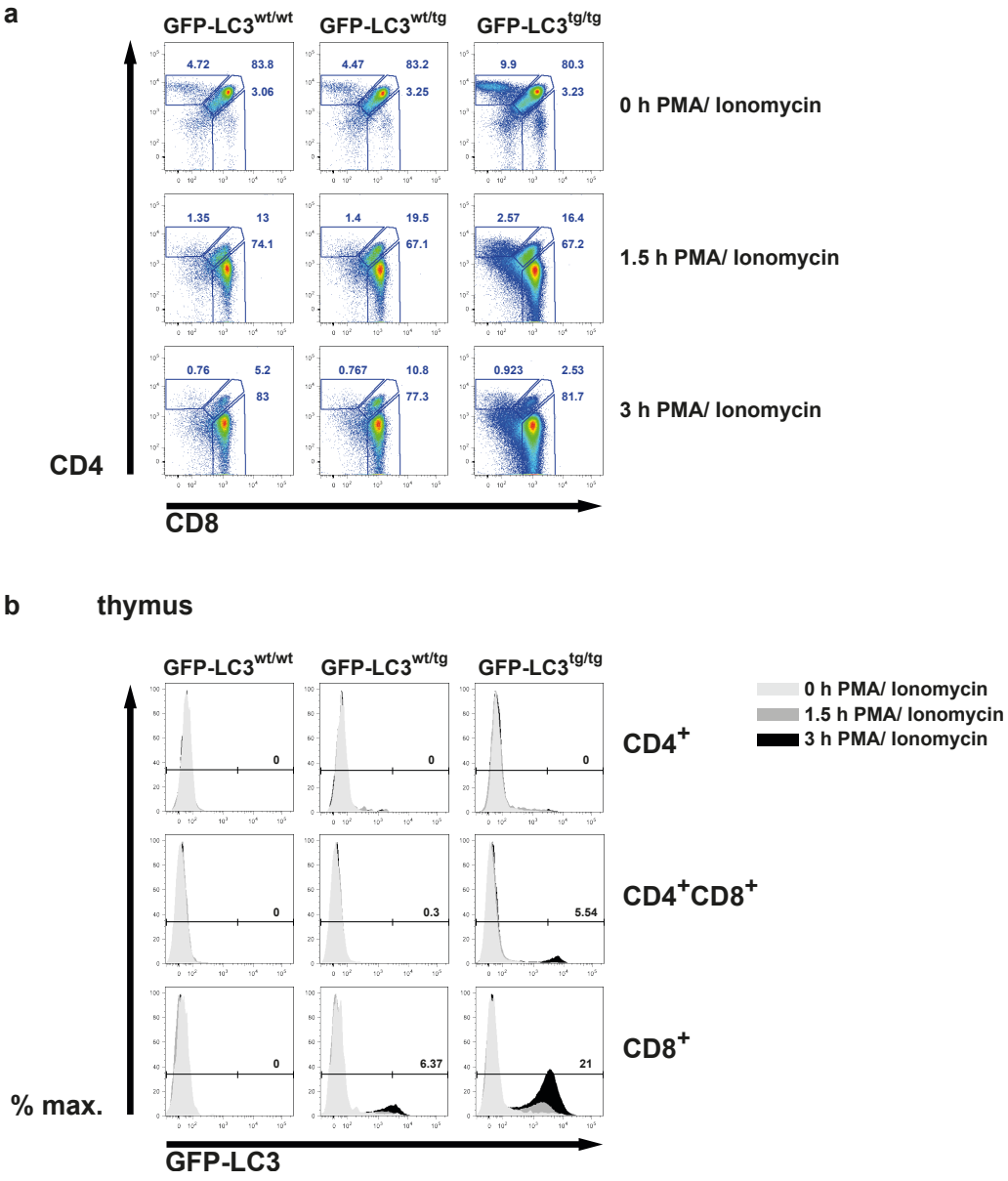
Figure 18: Gadd45β suppresses αCD3-mediated autophagy in primary thymocytes.
Thymocytes of wildtype and Gadd45β$^{-/-}$ mice were prepared and subjected to αCD3 treatment for the indicated time points with a concentration of 10 µg/ml. 2 h before harvesting, the cells were treated with bafilomycin A1 to prevent acidification of the autophagosomes and thus protein degradation. Subsequently, cellular lysates were prepared and analysed by SDS-PAGE and immunoblotting. The blots were probed for phospho-p38, total p38, Atg5 and LC3. β-actin served as a loading control. The mice were littermates and 3-4 weeks of age.

The stimulation was done either in the presence or absence of bafilomycin A1 for the same reasons mentioned in figure 16 and 17. Depicted is the data with bafilomycin A1. Upon prolonged stimulation, the Gadd45β-deficient thymocytes showed an increased abundance of LC3-II protein as compared to the wildtype situation (Fig. 18). Here, no accumulation of LC3-II could be detected at any timepoint. On the contrary, the Gadd45β-deficient thymocytes showed a high increase in LC3-II levels, already apparent after 4 h of stimulation and steadily increased up to 24 h of stimulation. Taken together, this experiment demonstrated that the Gadd45β-MEKK4-p38 pathway of autophagy downregulation is not restricted to LPS stimulation, but is also relevant to other stimuli and cell types.

4.1.9 $CD8^+$ T cells and thymocytes show *in vitro* an upregulation of GFP-LC3 upon PMA/Ionomycin stimulation

Autophagy is arguably foremost a cell survival mechanism. Recent reports show that both, T cell homeostasis and function, also heavily rely on autophagy and its capability of degrading unneeded intracellular content (Jia and He 2011) and surface receptor expression (Mcleod et al. 2011). Additionally, autophagy was found to regulate energy metabolism during effector T cell activation (Hubbard et al. 2010). In order to assess the role of autophagy in T cell activation in more detail, thymocytes of GFP-$LC3^{wt/wt}$, GFP-$LC3^{wt/tg}$ and GFP-$LC3^{tg/tg}$ mice were stimulated *in vitro* with 20 ng/ml PMA and 1 µM Ionomycin for 1.5 or 3 h (Fig. 19a). Subsequently, the samples were analysed by FACS and subgroups of thymocytes were set according to the $CD4^+$/$CD8^+$ distribution, to distinguish $CD4^+$ against $CD4^+CD8^+$ and $CD8^+$ thymocytes. The stimulation-dependent reduction of $CD4^+CD8^+$ positive thymocytes could be observed in all 3 genotypes. The frequency of GFP^+ cells of all 3 subgroups and genotypes are shown in Fig. 19b. As expected, wildtype thymocytes exerted no GFP signal in either subgroup of thymocytes. In heterozygous GFP-LC3 mice (GFP-$LC3^{wt/tg}$), a GFP^+ group, was also absent in $CD4^+$ and $CD4^+CD8^+$ double positive thymocytes, but a fraction of 6.3% $CD8^+$ cells showed an induction of a GFP signal after 3 h of PMA/Ionomycin stimulation. Induction of GFP-LC3 in the $CD4^+$ single positive cells were

also absent in the GFP-LC3 homozygous mice (GFP-LC3$^{tg/tg}$). However, 5.5 % of the CD4^{+}CD8^{+} double positive thymocytes up regulated GFP-LC3 after 3 h of stimulation and, more pronounced, 21.0% of the single positive CD8^{+} thymocytes induced GFP-LC3 after 3 h of stimulation. The gating for the spleen was accordingly done to the thymus with the exception of double positive cells (data not shown). Here, a very small fraction of CD4^{+} T cells of GFP-LC3$^{wt/tg}$ and GFP-LC3$^{tg/tg}$ mice became GFP^{+} after 1.5 and 3 h of stimulation, 2.3% and 4.4%, respectively (Fig. 19c).



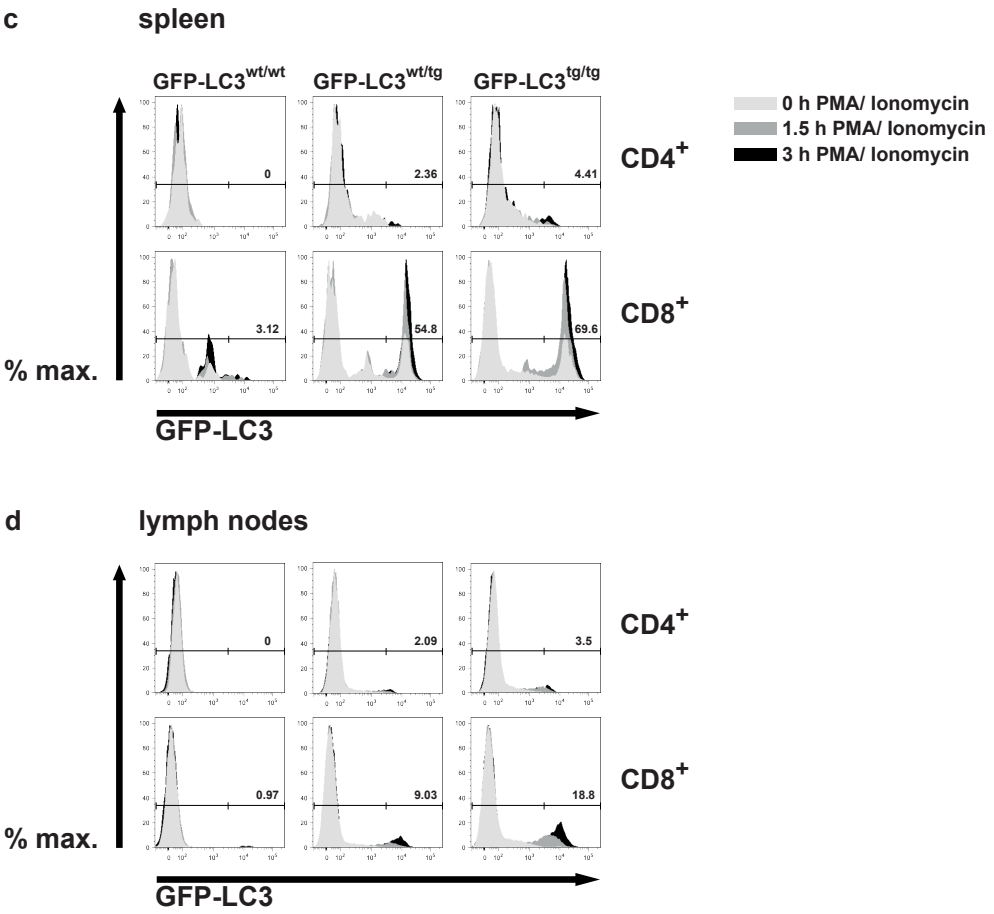


Figure 19: CD8$^+$ but not CD4$^+$ T cells and thymocytes highly upregulate GFP-LC3 *in vitro* upon PMA/Ionomycin stimulation.
(a) CD4$^+$CD8$^+$ profile of *in vivo* stimulated thymocytes of wild type and GFP-LC3 transgenic mice. The cells were stimulated with 20 ng/ml PMA and 1 µM Ionomycin for the indicated time periods and subsequently analysed by FACS. **(b)** Depicted are the same cells and gatings as in (a) for either CD4$^+$ single positive (upper panel), CD4$^+$CD8$^+$ double positive (middle panel) or CD8$^+$ single positive cells (lower panel). The indicated percentages do reflect the amount of GFP-LC3 positive thymocytes after 3 h of stimulation with PMA/ Ionomycin. **(c)** The same settings as in (b) for the splenocytes of the same mice. **(d)** A similar panel as in (b) and (c) for the lymph nodes of the same mice. Panel (a)-(d) show a representative analysis for two independent experiments. **(e)** A representative CD8$^+$ splenocyte of a GFP-LC3 transgenic mouse after 4 h stimulation with PMA/Ionomycin. Bar represents 10 µm.

Interestingly, a large population within the $CD8^+$ T cells became GFP positive, 54.8% in the GFP-$LC3^{wt/tg}$ mice and 69.8% in the GFP-$LC3^{tg/tg}$ mice. As already observed in the thymus, the homozygous mice showed a higher increase in the amount of GFP^+ T cells as compared to the heterozygous ones. This seems to argue in favour of a gene-dose dependency of GFP-LC3 induction in heterozygous T cells compared to homozygous ones. For the peripheral lymph nodes the same gating strategy was applied as for the spleen and, although in reduced numbers the same pattern of GFP^+ T cells emerged (Fig. 19d). Again, only very few of the $CD4^+$ T cells did show an induction of GFP signal, 2.0% for the heterozygous mice and 3.5% for the GFP-$LC3^{tg/tg}$ mice. In line with the previously obtained results of the $CD8^+$ T cells population, a larger fraction did become GFP^+, 9.0% of the $CD8^+$ T cells of GFP-$LC3^{wt/tg}$ mice and 18.8% of the homozygous GFP-LC3 transgenic mice.

Due to the concept of flow cytometry, it is only possible to determine the amount of GFP signal per cell, but not its localisation. To proof that the observed increase in GFP signal is not due to an increase in autofluorescence but to an accumulation of GFP^+ spots in the cytoplasm of T cells, splenocytes were sorted according to their expression profile in regard to CD4 and CD8 expression. Subsequently, those cells were plated in a live cell-imaging chamber pre-coated with Poly-L-Lysine and stimulated under surveillance by a confocal microscope. While no $CD4^+GFP^+$ splenocytes could be detected upon stimulation, it was possible to make pictures of $CD8^+GFP^+$ T cells (Fig 19e). Although the cytoplasmic compartment is relatively small in T cells, two GFP^+ autophagosomal spots could be clearly identified within the cell.

These experiments demonstrate, that upon activation of T cells with PMA/ Ionomycin, $CD8^+$ but not $CD4^+$ T cells form autophagosomal spots in the cytoplasm.

4.2 The Role of Gadd45β in apoptosis during negative selection in the thymus

4.2.1 General characterisation of Gadd45β$^{-/-}$ mice in comparison to wildtype animals

Currently there are three different Gadd45β$^{-/-}$ mouse lines (Gupta et al. 2005; Papa et al. 2004; Lu et al. 2004). The Gadd45β$^{-/-}$ mouse generated in the laboratory of Dan Liebermann was used in this work to elucidate the role of Gadd45β in apoptosis and autophagy (Gupta et al. 2005). So far, no publication has analysed the lymphoid compartment of these knockout mice in detail. Thus, in order to ascertain that the knock out of the Gadd45β gene had no unknown side effects and to characterise the compartment properly, the composition of the T cell compartment in the mouse was analysed in detail.

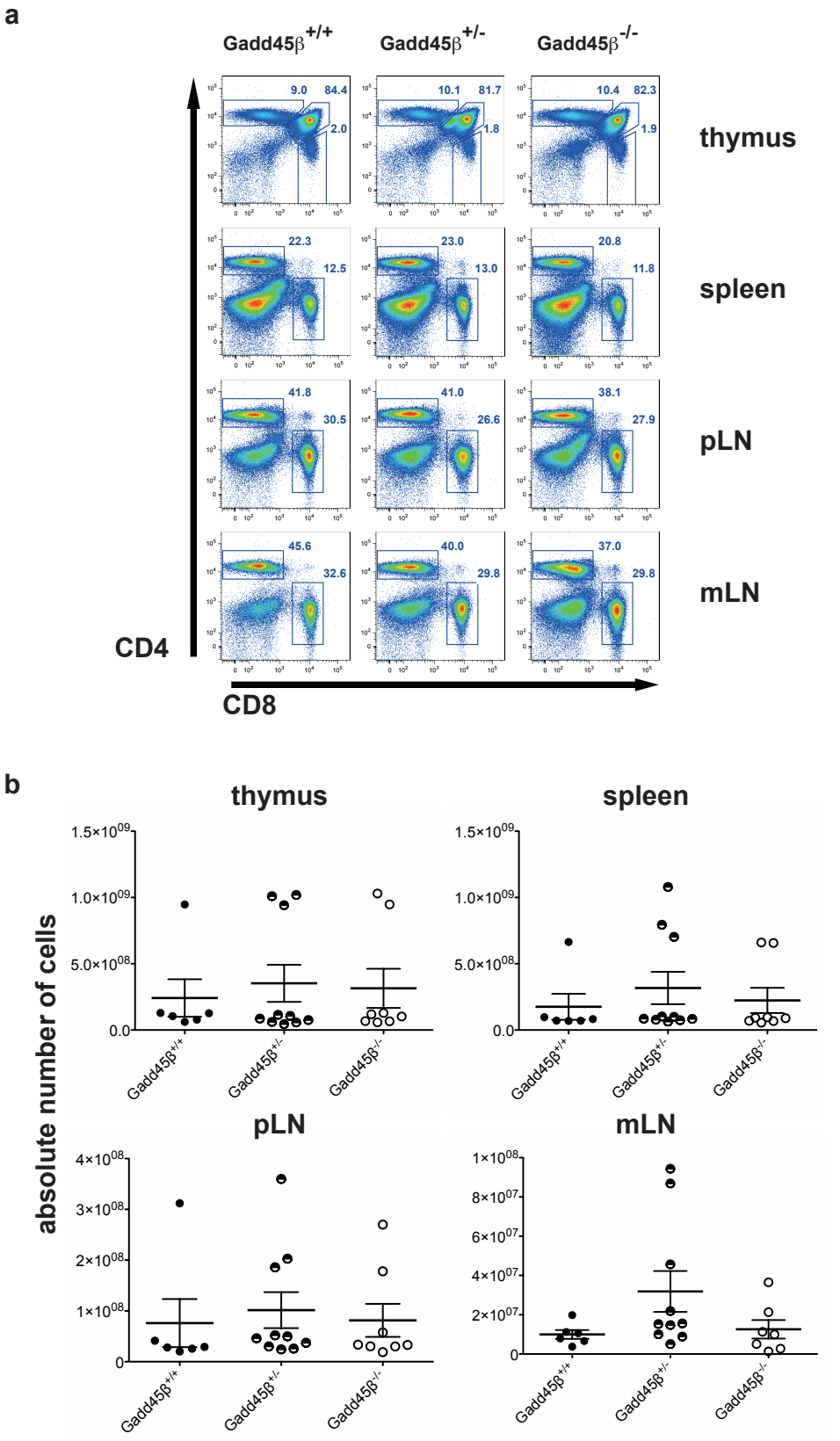


Figure 20: General analysis of thymocytes from wildtype, Gadd45β$^{+/-}$ and Gadd45β$^{-/-}$ mice.
(a) Representative pseudo-colour dot blots of wildtype, Gadd45β$^{+/-}$ and Gadd45β$^{-/-}$ mice. Depicted are all three genotypes and the distribution in a CD4^{+}/CD8^{+} profile in the thymus, spleen, peripheral lymph nodes and mesenteric lymph nodes. **(b)** Scatter dotblot diagrams of the absolute number of cells of the different lymphatic organs depicted in (a). All mice were littermates and 6 weeks of age. Data from 4 independent experiments are shown. Statistical significances were calculated by two-tailed Mann-Whitney tests (n=3, mean ± s.d.).

In figure 20a representative FACS analysis of major lymphatic organs: thymus, spleen, peripheral and mesenteric lymph nodes are depicted. No alterations in the $CD4^+/CD8^+$ profile could be found in any of the depicted organs. The total number of T cells in the lymphatic organs is shown in figure 20b. Here, no statistical significant differences could be observed as well. In order to rule out any misbalances in the amount of total $CD4^+$ and $CD8^+$ T cells, these values were calculated for each organ as well. Again, no statistically significant differences could be observed in the amount of total $CD4/8^+$ T cells (Figure 21).

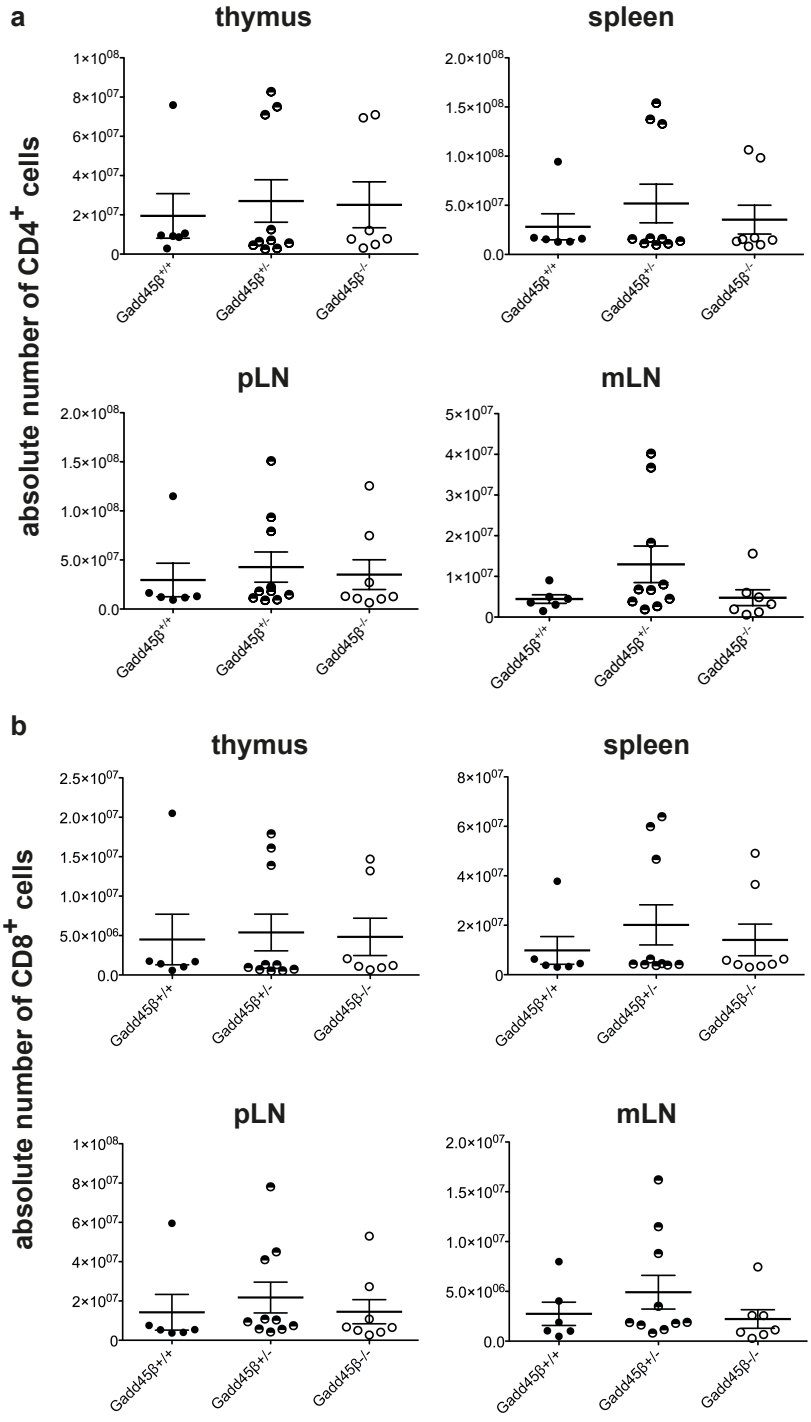


Figure 21: Absolute numbers of cells in respect to their cell surface marker expression in different lymphatic organs.
(a) Scatter dotblot diagrams of the absolute number of $CD4^+$ cells of the different lymphatic organs depicted in figure 11. **(b)** Scatter dotblot diagrams of the absolute number of $CD8^+$ cells of the different lymphatic organs depicted in figure 11. All mice were littermates and 6 weeks of age. Data from 4 independent experiments are shown. Statistics were calculated by two-tailed Mann-Whitney test (n=3, mean ± s.d.).

Along these lines, the percentages of $CD4^+$ and $CD8^+$ T cells relative to the whole population of T cells were analysed (Fig. 22 a, b).

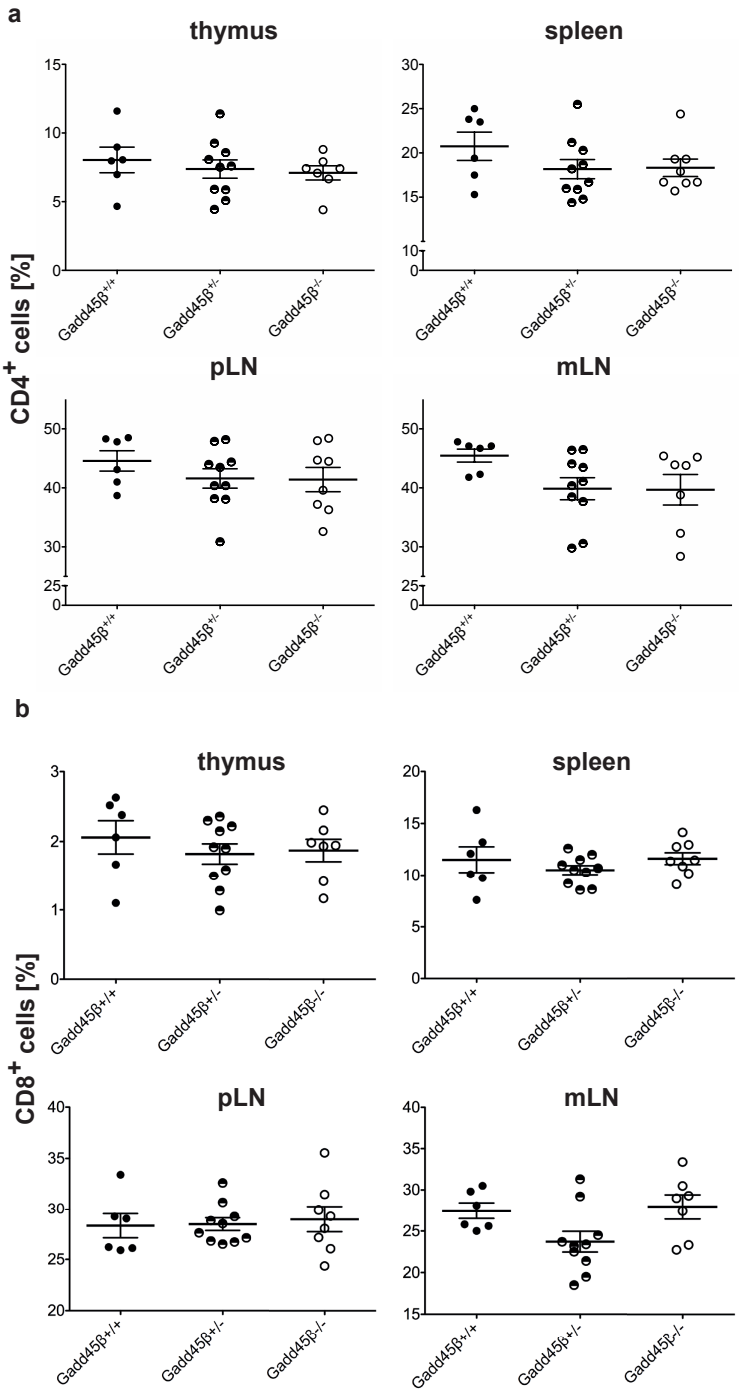


Figure 22: Percentages of cell surface marker expression in different lymphatic organs.
(a) Scatter dotblot diagrams of the percentage of $CD4^+$ cells of the different lymphatic organs depicted in figure 11. **(b)** Scatter dotblot diagrams of the percentages of $CD8^+$ cells of the different lymphatic organs depicted in figure 11. All mice were littermates and 6 weeks of age. Data from 4 independent experiments are shown. Statistics were calculated by two-tailed Mann-Whitney test (n=3, mean ± s.d.).

Interestingly, there were no general tendencies consistent in the $CD8^+$ compartment, whereas the percentage of $CD4^+$ T cells was decreased in all lymphatic organs of Gadd45$\beta^{+/-}$ and Gadd45$\beta^{-/-}$ mice analysed, although not statistically significant.

4.2.2 Gadd45$\beta^{-/-}$ mice have a misbalance in the ratio of $CD4^+$ effector versus regulatory T cells

The whole Gadd45 protein family is strongly associated with autoimmunity. Gadd45β deficiency for instance is described to exacerbate experimental autoimmune encephalomyelitis (EAE) and also to play a role in the appearance of autoimmune lymphoproliferative syndrome and systemic lupus erythematosus in a Gadd45β/γ double deficient situation (Liu et al. 2005). Regulatory T cells were first described in 1971 and are subject of intense research today (Gershon and Kondo 1971).

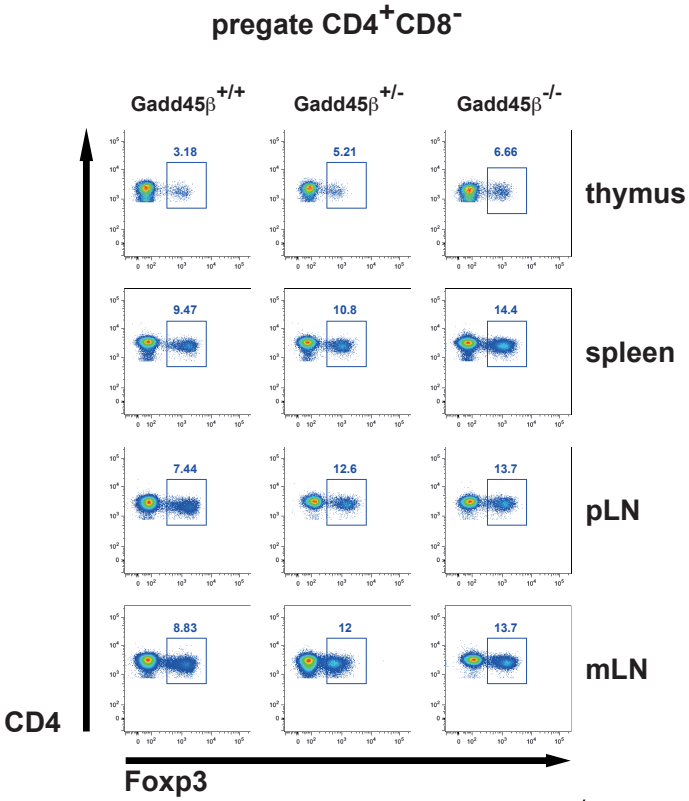


Figure 23: Intracellular Foxp3 staining in wildtype and Gadd45$\beta^{-/-}$ mice in different lymphatic organs.
Representative pseudo-colour dot blots of the same wildtype, Gadd45$\beta^{+/-}$ and Gadd45$\beta^{-/-}$ mice shown in 11. Depicted are all three genotypes and the distribution in a $CD4^+$/$Foxp3^+$ profile in the thymus, spleen, peripheral lymph nodes and mesenteric lymph nodes.

To assess the possible role of Gadd45β in autoimmunity, the absolute number and composition in terms of frequency of the $CD4^+$ T cell repertoire of Gadd45$\beta^{+/+}$, Gadd45$\beta^{+/-}$ and Gadd45$\beta^{-/-}$ mice in the thymus, spleen, peripheral lymph nodes and mesenteric lymph nodes was analysed via FACS. The frequency of regulatory T cells as determined by Foxp3 staining in the $CD4^+$ compartment was altered. Figure 23 shows a representative FACS profile of pregated $CD4^+$ T cells, intracellularly stained for Foxp3. In all four organs, the frequency of $Foxp3^+$ T cells was highly statistically significantly elevated in the Gadd45$\beta^{-/-}$ mice (Fig. 24a).

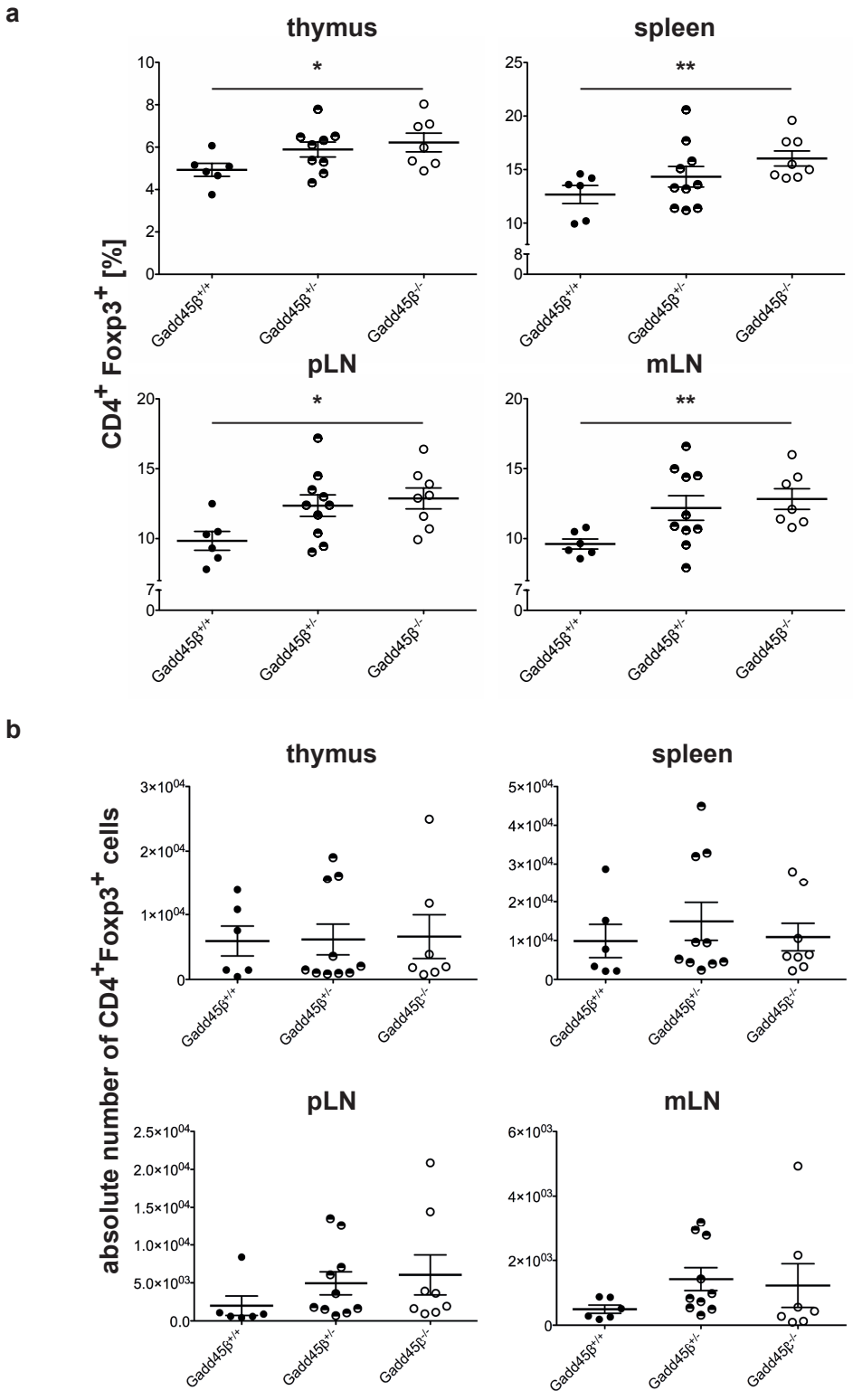


Figure 24: The percentages but not the absolute numbers of CD4$^+$Foxp3$^+$ regulatory T cells are increased in Gadd45β$^{-/-}$ mice.
(a) Scatter dotblot diagrams of the percentage of CD4$^+$Foxp3$^+$ cells of the different lymphatic organs of the mice depicted in figure 11. **(b)** Scatter dotblot diagrams of the absolute numbers of CD4$^+$Foxp3$^+$ cells of the different lymphatic organs depicted in figure 11. All mice were littermates and 6 weeks of age. Data from 4 independent experiments are shown. Statistics were calculated by two-tailed Mann-Whitney test (n=3, mean ± s.d.).

Interestingly, the absolute numbers of these cells were not elevated in the same way (Fig. 24b). As already stated in chapter 4.2.1, the frequency of $CD4^+$ T cells also showed a slight decrease in all organs, though not statistically significant (Fig. 22a). This experiment shows, that the number of $CD4^+$ effector cells is reduced in Gadd45$\beta^{-/-}$ mice and in this way, the number of $Foxp3^+$ regulatory T cells lay out a larger piece of the overall composition of $CD4^+$ effector T cells in the Gadd45β-deficient situation.

4.2.3 Gadd45β-dependent differences in apoptosis of double positive ($CD4^+CD8^+$) thymocytes

In an array analysis of a model organism of negative selection in the thymus, Gadd45β was identified as the most highly induced gene upon triggering of the TCR (Schmitz et al. 2003). Then, previous work could show that Gadd45β expression in a double positive ($CD4^+CD8^+$) thymocyte cell line is strongly connected to apoptosis. Upon overexpression with a retroviral vector containing Gadd45β, these double positive cells were more sensitive to αCD3 induced apoptosis than their uninfected control group (Keil 2008). To confirm this data in an approach more closely related to the *in vivo* situation, primary thymocytes from Gadd45β-deficient animals and their wildtype littermates were stimulated with different apoptotic stimuli to induce cell death. The double positive thymocytes of this *in vitro* approach were subsequently stained with AnnexinV/7AAD to discriminate between living and apoptotic cells (Fig. 25a).

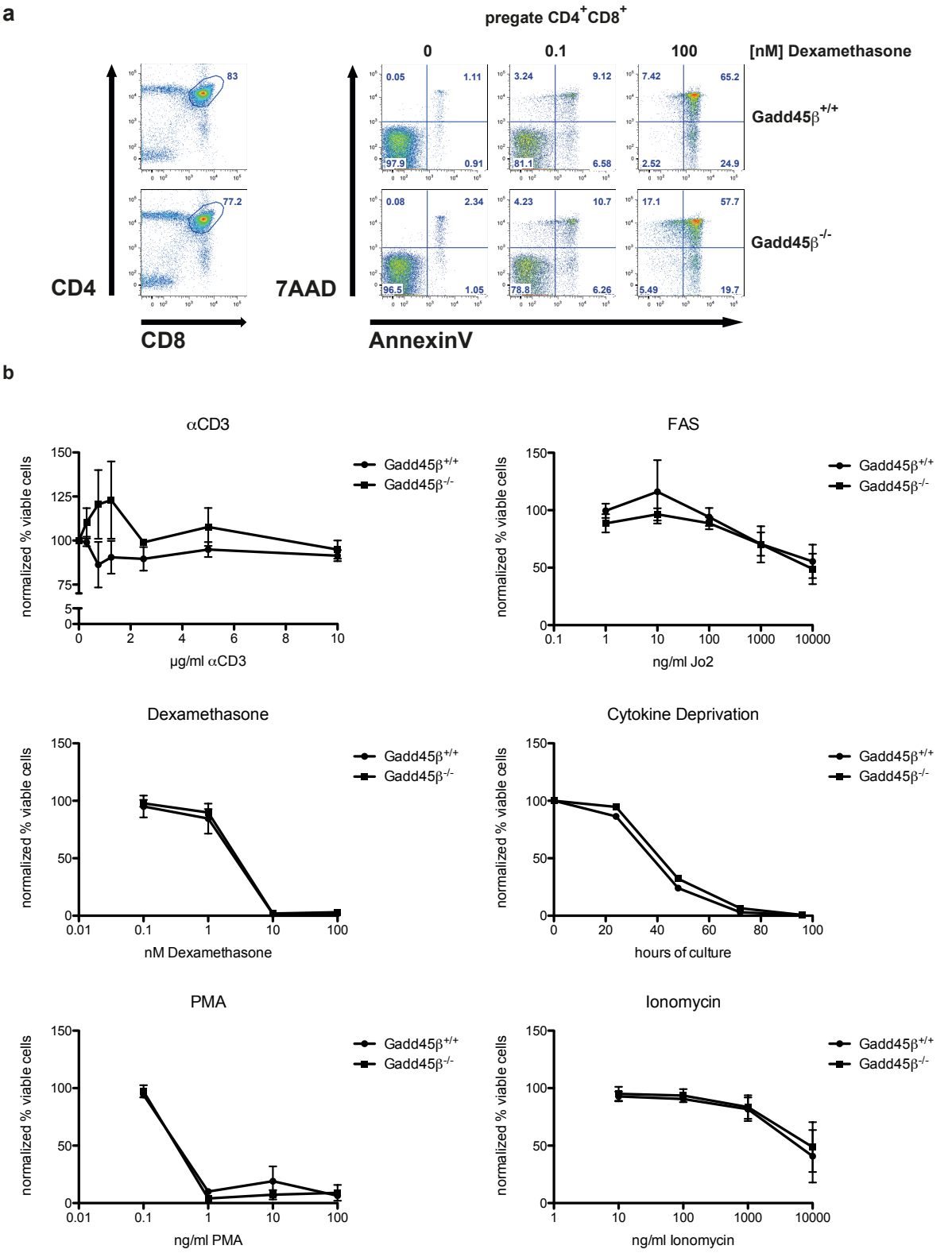


Figure 25: Double positive ($CD4^+CD8^+$) $Gadd45\beta^{-/-}$ thymocytes show different apoptotic behaviour upon T cell receptor stimulation, but not to other stimuli.
(a) $1x10^6$ thymocytes were *ex vivo* cultured for 16 h with the indicated concentrations of dexamethasone. Subsequently, they were stained for CD4, CD8 and apoptosis was determined with an AnnexinV/7AAD staining. **(b)** Statistic analysis of the specific apoptosis rate of the results determined in (a). Additional stimuli were applied with the indicated concentrations. For cytokine deprivation, the cells were cultured for the indicated time period without any stimulus. All mice were littermates and 6 weeks of age. Statistical significances were calculated by two-tailed Mann-Whitney tests (n=3, mean ± s.d.).

The analysis is representative for all apoptotic stimuli applied. The viability of the $CD4^+$ $CD8^+$ thymocytes towards different stimuli, being it intrinsic ones like dexamethasone or the phorbol ester PMA or extrinsic, like the αFas antibody, showed no significant differences (Fig. 25b). Also the complete lack of stimulation by cytokine deprivation led to comparable results between wildtype and Gadd45$\beta^{-/-}$ mice. When stimulated with αCD3, the $CD4^+CD8^+$ double positive thymocytes showed a deviant behaviour. In the Gadd45$\beta^{-/-}$ situation, the cells seemed to be protected against apoptosis. Especially at lower concentrations of under 2 µg/ml, which simulate endogenous stimulation by MHC much closer than high concentrations of 10 µg/ml, the viability of double positive Gadd45$\beta^{-/-}$ thymocytes was increased.

4.2.4 The V_β repertoire of Gadd45$\beta^{-/-}$ mice shows no statistical significant difference to wildtype mice

Viral superantigens in association with MHC class II molecules specifically bind to certain V_β elements of the TCR and induce apoptosis in large fractions of immature $CD4^+CD8^+$ double positive thymocytes bearing these V_β elements (Fairchild and Rosenwasser 1993). Analysing the V_β repertoire of Gadd45$\beta^{-/-}$ against Gadd45$\beta^{+/+}$, it is possible to assess the influence of these virals superantigens on the process of negative selection.

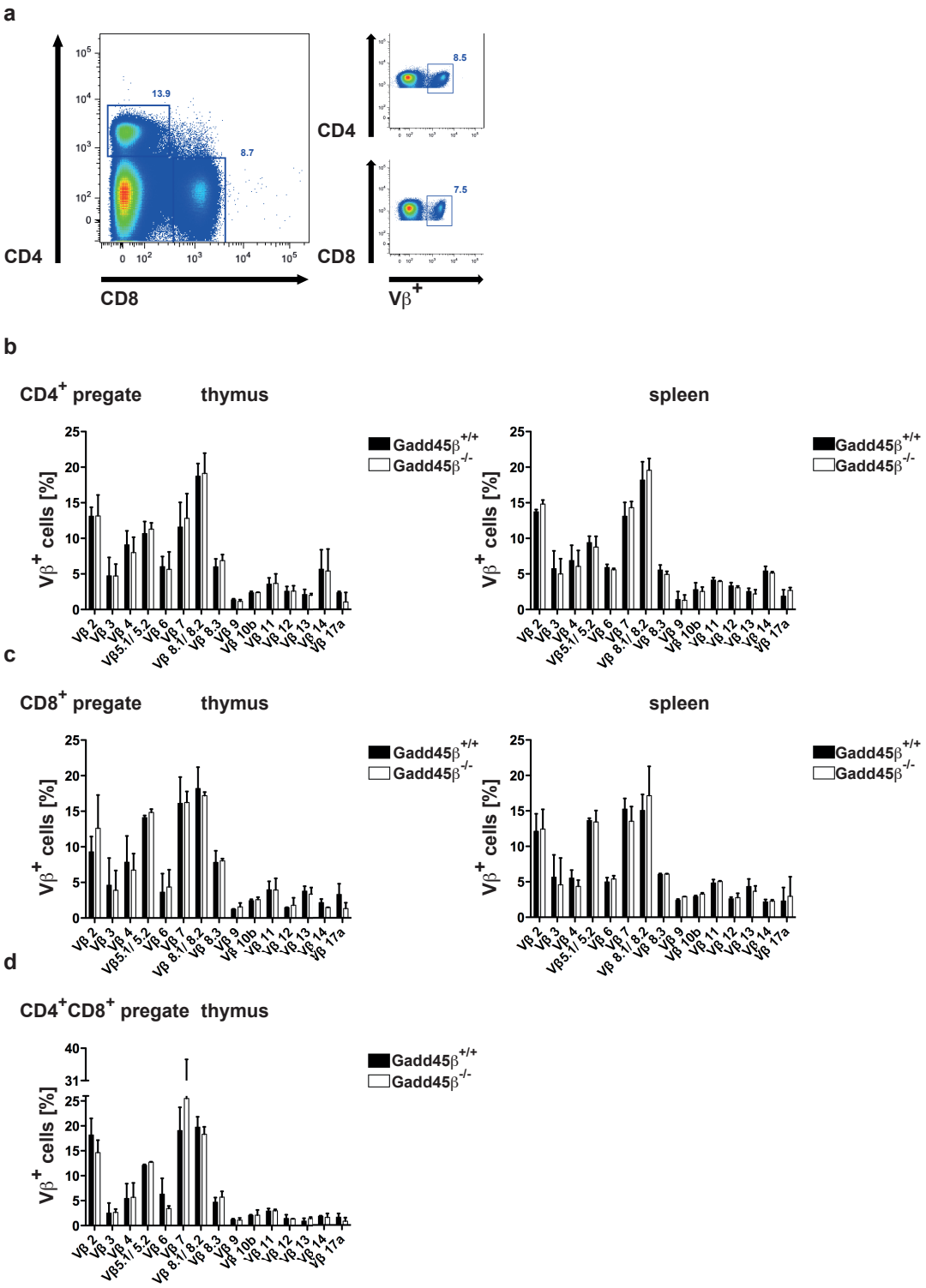


Figure 26: T cell receptor V_β repertoire in Gadd45β-sufficient and deficient mice shows no coherent differences.

(a) Representative pseudo-colour dotblots of a CD4/CD8 staining with subsequent analysis of the respective subset with a V_β-specific, FITC labelled antibody. **(b)** Thymi of 5 week old littermates were stained for the most prominent V_β subtypes and were subsequently analysed by FACS. The upper panel shows a pregate on $CD4^+$ T cells. **(c)** The middle panel shows the same experiment as in (a), but with a pregate on $CD8^+$ T cells. **(d)** The lower panel shows the same experiment as in (a) but with a pregate on $CD4^+CD8^+$ double positive thymocytes **(b)-(d)** Statistics were calculated by two-tailed Mann-Whitney test (n=3, mean ± s.d.), graphs show two independent experiments.

To test for differences in the V_β repertoire of the TCR in these mice, an array of antibodies against different β-chains was used for flow cytometrical analysis: the whole panel is listed in chapter 2.6.3. In the thymus, $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ thymocytes were analysed separately, in the spleen the $CD4^+$ and the $CD8^+$ compartment (Fig. 26a-c). In no compartment of any of the two organs a consistent difference could be found, arguing that viral superantigens do not seem to play a role in the negative selection in these mice. Therefore, it can be concluded that superantigen-induced negative selection is independent of Gadd45β.

4.2.5 Decreased negative selection *in vivo* in Gadd45β$^{-/-}$ mice

αCD3 injection into Gadd45β$^{-/-}$ and wildtype mice suggested an *in vivo* role of Gadd45β in negative selection in the thymus (Keil 2008). However, as αCD3 is not found under physiological conditions in an organsim and can also induce a cytokine storm, this approach is a rather crude model for negative selection. Thus, another stimulus, which can trigger exclusively the TCR, is necessary to confirm this data. In order to generate such a model system, an N15 TCR transgenic RAG2$^{-/-}$ H-2^b (N15) mouse was bred with the Gadd45β$^{-/-}$ one in order to generate a mouse with a monoclonal TCR repertoire, which can be easily triggered and is deficient for Gadd45β. Subsequently, this mouse was injected the VSV8 peptide, triggering specifically the TCR and inducing negative selection (Ghendler et al. 1998). N15 mice served as a control group, which were Gadd45β-proficient (Fig. 27a). The data shown is representative for at least 3 animals per group. Upon injection with PBS, both CD4/CD8 profiles did remain identical, whereas 24 h post injection with VSV8 peptide, the Gadd45β-proficient animals showed a high increase in $CD4^-CD8^-$ double negative thymocytes (61.9%) and a decrease in $CD4^+CD8^+$ double positive thymocytes (16.5%), whereas in the Gadd45β$^{-/-}$ animals a large pool of $CD4^+CD8^+$ double positive thymocytes (56.1%) remained. 48 h post injection of the animals, the frequency of $CD8^+$ single positive thymocytes in the Gadd45β$^{+/+}$ mice had risen from 11.3% after 24 h to 25.4%. In the same

time this compartment had grown to 38.4% in the Gadd45β$^{-/-}$ animals. In order to determine whether the CD8^{+} thymocytes were mature ones and able to leave the thymus afterwards, the expression of heat-stable antigen (HSA, CD24) was controlled (Kishimoto and Sprent 1997) (Fig. 12e). Both, in the Gadd45β-proficient as in the Gadd45β–deficient situation, the maturity of the CD8^{+} thymocytes was increased 24 and 48 h post injection as compared to CD4^{+}CD8^{+} double positive thymocytes.

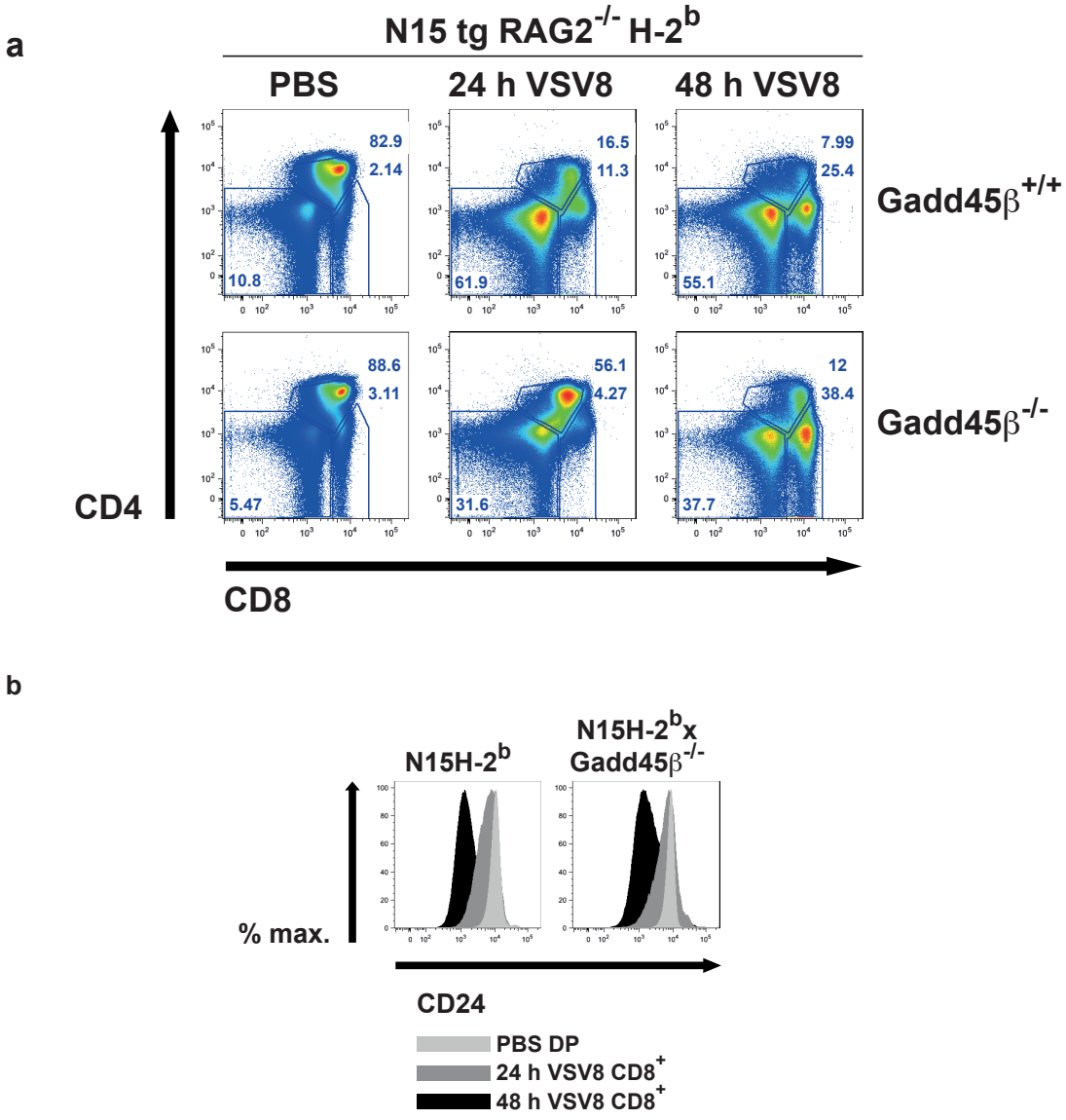


Figure 27: Gadd45β-deficiency in thymocytes results in increased positive selection.
(a) N15H-2^{b} mice and N15H-2^{b}xGadd45β$^{-/-}$ mice were injected into the tail vain with 25 µg VSV8 peptide for 24, 48 h or with PBS as a control. Representative pseudo-colour dotblots of CD4/CD8 profiles are depicted. Numbers indicate CD4^{+}CD8^{+} double positive, CD8^{+} single positive or CD4^{-}CD8^{-} double negative thymocytes. The mice were 3 weeks old. **(b)** Representative histogram analyses of the expression of CD69 on CD4^{+}CD8^{+} double positive thymocytes upon challenge with VSV8 peptide.

This observation was true for all animals, as can be seen in the statistical evaluation (Fig. 28). These observations could also be validated in absolute numbers of $CD8^+$ thymocytes, where interestingly only 48 h post injection with VSV8 peptide the numbers were higher than in the wildtype (Fig. 28b).

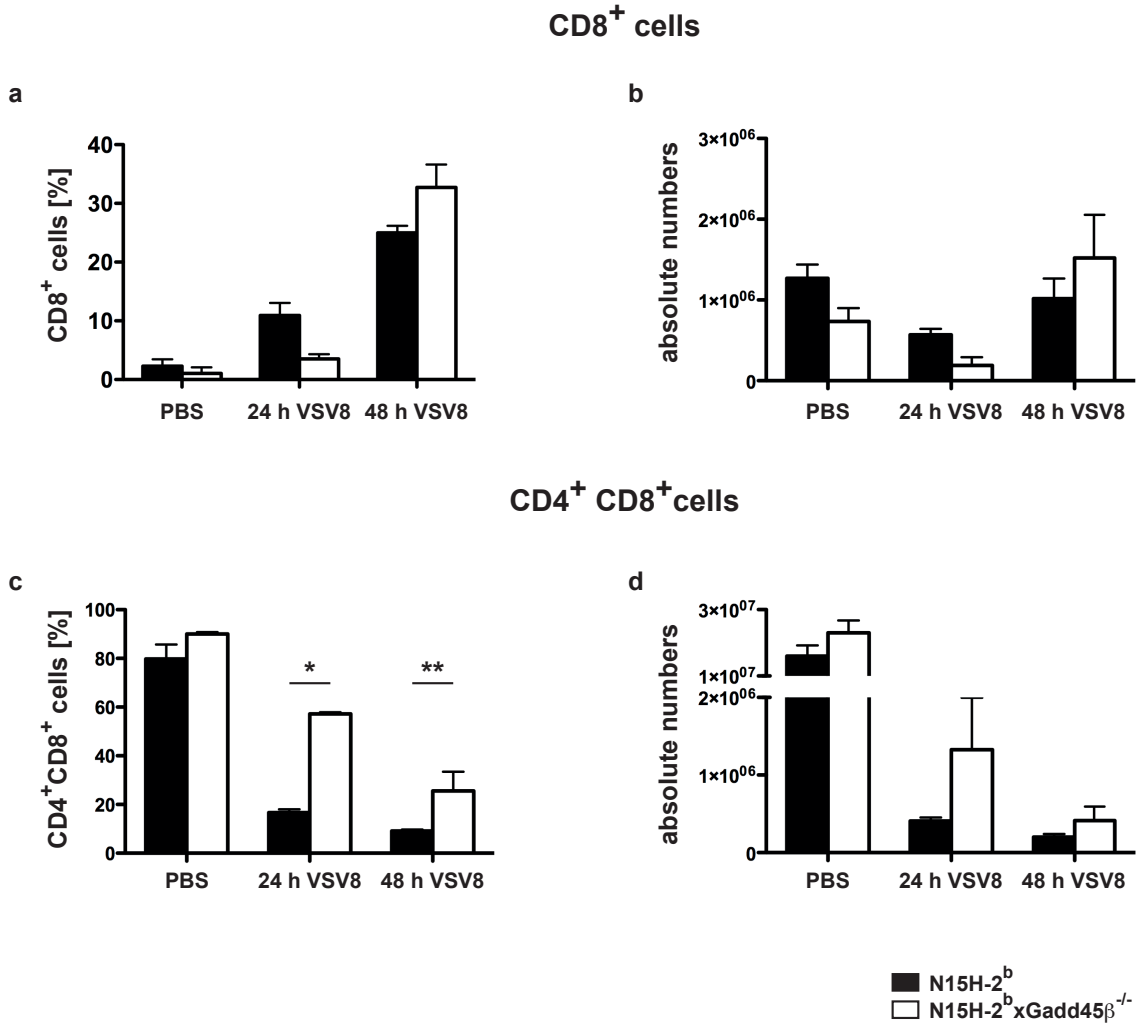


Figure 28: Statistic analysis of frequencies and absolute numbers of wildtype and Gadd45β-deficient mice upon VSV8 challenge.
(a) Statistical analysis of $CD8^+$ thymocyte frequencies. **(b)** Statistical analysis of absolute numbers of $CD8^+$ thymocytes. **(c)** Statistical analysis of $CD4^+CD8^+$ thymocyte frequencies. **(d)** Statistical analysis of absolute numbers of $CD4^+CD8^+$ thymocytes. (a)-(d) show statistics of the same experiment as in Figure 18. Statistical significances were calculated by two-tailed Mann-Whitney tests (n=3-7, mean ± s.d., * P=0.0106, ** P<0.0079).

The total numbers of $CD4^+CD8^+$ double positive thymocytes were also higher in the Gadd45β-deficient situation. Here, 24 h post stimulation with VSV8 peptide, an increased population of thymocytes retained a double positive phenotype (Fig. 28d).

The comparison of the maturation status of Gadd45β-deficient with wildtype thymocytes revealed no differences, showing that wildtype and Gadd45β-deficient thymocytes are equally mature when they migrate to the periphery (Fig. 29a)

Of note, CD69 is not only described as an activation marker of T cells, but also to be induced during the process of negative selection (Brändle et al. 1994). Indeed, a slight increase in CD69 expression in Gadd45β-deficient double positive thymocytes could be verified (Fig. 29b).

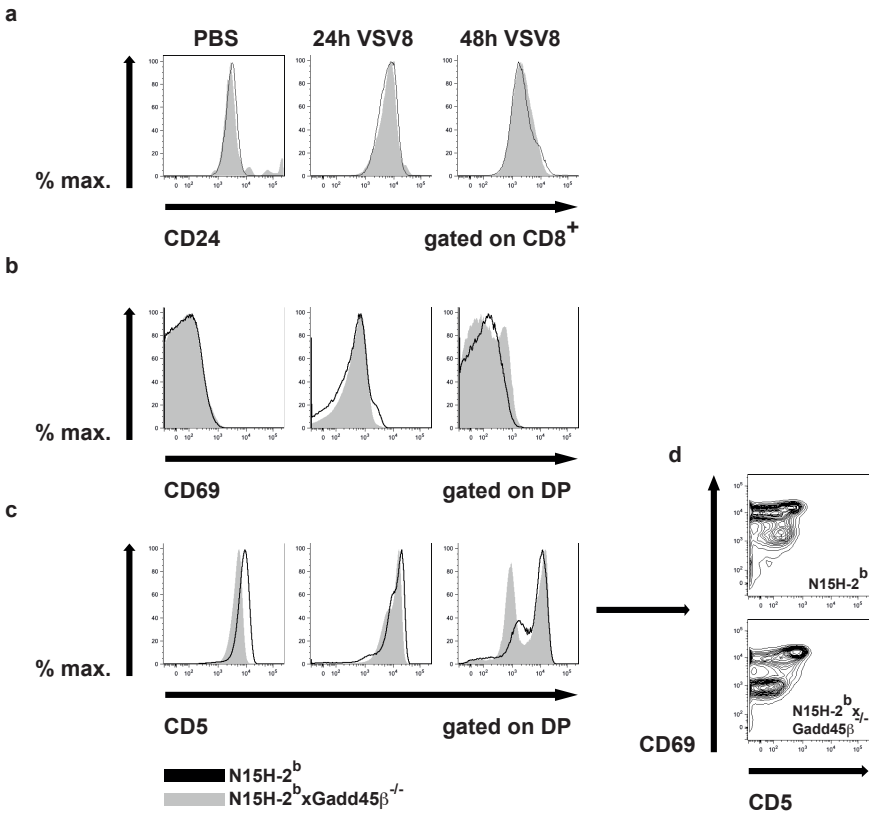


Figure 29: Expression of surface markers on T cells of N15H-2^b and N15H-2^bxGadd45β$^{-/-}$ mice.
(a) Representative histogram analyses of the expression of CD24 on $CD8^+$ thymocytes in N15H-2^b and N15H-2^bxGadd45β$^{-/-}$ mice upon different time points of VSV8 peptide stimulation. **(b)** Representative histogram analyses of the expression of CD69 on $CD4^+CD8^+$ double positive thymocytes upon challenge with VSV8 peptide. **(c)** Representative histogram analyses of the expression of CD5 on $CD4^+CD8^+$ double positive thymocytes upon challenge with VSV8 peptide. **(d)** CD5/CD69 contour-blots of 48 h VSV8 peptide stimulated thymocytes shown in (c).

Additionally, the expression of CD5 in immature, $CD4^+CD8^+$ double positive thymocytes is reported to be associated with positive selection (Li and Page 2001). The FACS analysis for this marker could show a second population in $CD4^+CD8^+$ thymocytes, which have downregulated the marker substantially (Fig. 29c). A contour-blot of CD69 plotted against CD5 expression revealed a significant second population in the Gadd45β-deficient thymocytes, which are less positive for CD69 and CD5.

To verify the expression levels of Gadd45β in the thymus during the process of negative selection, samples of the thymi were taken and analysed via quantitative real-time polymerase chain reaction (qRT-PCR). As described in the literature, Gadd45β was highly up regulated (72-fold) after TCR stimulation (Schmitz et al. 2003).

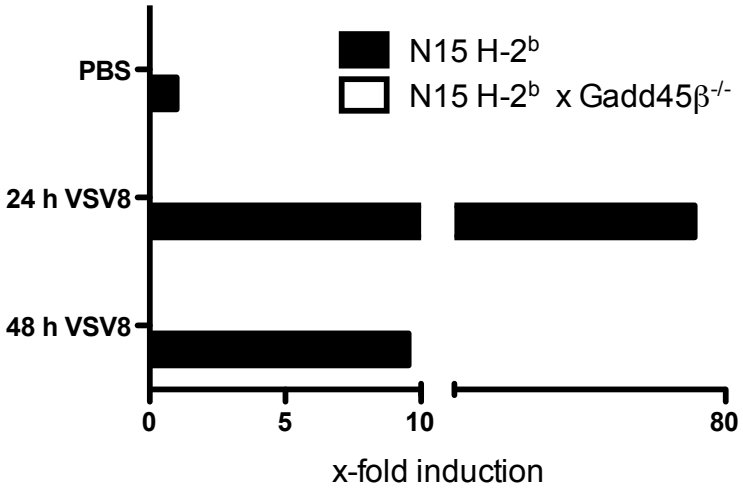


Figure 30: qRT-PCR of VSV8-stimulated N15 H-2^b and N15 H-2^b x Gadd45$\beta^{-/-}$ mice.
Bar graph of a qRT-PCR of Gadd45β-induction upon VSV8 stimulation of Gadd45β-deficient and wildtype N15 H-2^b mice. The samples were taken from animals of the same experiment as in figure 27-29.

As a consequence from this experiment, it can be stated that Gadd45β–deficiency leads to defect in negative selection in the thymus, resulting in increased numbers of mature, $CD8^+$ single positive T cells.

5. Discussion

5.1 Autophagy-regulation by Gadd45β

5.1.1 Molecular aspects of the Gadd45β-MEKK4-p38 pathway

Mitogen-activated protein kinases (MAPKs) are important regulators of cellular events, such as proliferation, differentiation, immunity and cell death (Krishna and Narang 2008). Post-translational modifications in general allow fast reactions to alter physiological settings, as the time necessary for translation and transcription can be disregarded. In respect to autophagy, the role of post-translational modifications has already been reported for a number of different kinases and their targets, for example ULK1/2, Vps34 and mTOR (Sarbassov et al. 2005; Ganley et al. 2009; Proikas-Cezanne et al. 2004). The c-Jun N-terminal protein kinase (JNK) for instance was shown to phosphorylate Bcl-2. Consequently, Beclin 1 is released from the complex and is able to initiate autophagy in unison with Vps34 (Pattingre et al. 2009; Wei et al. 2008). The extracellular-signal-regulated kinase (ERK) was also found to interact with a major autophagy-regulating protein, mTOR (Wang et al. 2009). The actual details of ERK, which acts on mTOR, are not as well elucidated as for JNK. Although these and other studies have already provided a great deal of insight into post-translational modifications in the initiation phase of autophagy, little is known about their role at later time points during the autophagic flux.

As mentioned earlier, Gadd45β was found to directly interact with the MAPK/ERK kinase kinase (MEKK4) and MAP kinase kinase (MKK)7 (Papa et al. 2004; Takekawa and Saito 1998). In this regard, Gadd45β was shown to exert opposing functions: on the one hand the interaction with MKK7 inhibits JNK activation (Papa et al. 2004), while on the other hand the interaction with MEKK4 activates JNK and p38 (Takekawa and Saito 1998). This dual role of Gadd45β proofs the importance of MAPK signalling.

Previous work in our lab already showed a Gadd45β-dependent translocation of phosphorylated p38 to distinct cytosolic spots, that were identified as autophagosomes (Keil 2008). Therefore, one aim of this study was to proof the direct interaction of p38 with its target protein, Atg5. Indeed, upon overexpression of Gadd45β together with MEKK4 in mouse embryonic fibroblasts (MEFs), the number of GFP-LC3 positive dots was highly statistically significantly increased (Fig. 10a+b). Interestingly, overexpression of MEKK4 alone did not elevate the number of GFP-LC3 puncta at all, as compared to the empty vector control. This can be explained by the fact that Gadd45β was found to be only minimally expressed under steady-state conditions in most tissues (Zhang et al. 1999). Therefore, in the absence of Gadd45β, MEKK4 is in a closed confirmation and is, thus, unable to exert its function (Mita et al. 2002). The fact that GFP-LC3 puncta formation is increased in Gadd45β-transfected MEFs, further adds proof to this theory. As expected, overexpression of both Gadd45β and MEKK4 resulted in the highest number of intracellular GFP^+ dots.

The results from this experiment clearly demonstrated that Gadd45β is the limiting factor in this newly found connection between Gadd45β, MEKK4 and p38. Along these lines, the double transfection of Gadd45β and MEKK4 resulted in increased autophagosome numbers, though this was starvation-independent, and no increase in autolysosome numbers (Fig 11). These results strongly argue in favour of a block in the autophagic flux and rules out the possibility of an increased autophagosome formation. The analysis of p38α–deficient MEFs further supports the suggested Gadd45β-MEKK4-p38 pathway (Fig. 12).

It remains unknown why the p38 wildtype MEFs show a decrease in autophagosome as well as in autolysosome numbers. The MEFs were obtained from Dr. Angel Nebrada, (Porras et al. 2004) and were immortalised either by retroviral infection or by simian virus 40. This may be a reason for the aberrant reaction to HBSS-induced starvation. Still, the fact that p38α-deficient MEFs show such a clear difference compared to wildtype cells undermines the importance of p38 in autophagy.

The fact that the relevant phosphorylation site in Atg5 at the threonine T75 is conserved from yeast to men suggests that the phosphorylation of Atg5 is an important mechanism in limiting autophagy in all species (Fig. 13a+b). The *in vivo* phosphorylation of Atg5 in wildtype but not in phosphorylation- defective mutant MEFs by overexpression of Gadd45β and MEKK4 could be shown by a radioactive labelling *in vivo* (Fig. 13c). Using this method, the phosphorylation of an Atg protein, involved in the Atg12 conjugation system, was shown for the first time. The importance of T75 in the Atg5 protein was observed by mutation of the phosphorylation site: A T75A-expressing mutant, in which the site in question could no longer be phosphorylated, which resulted in a drastic increase in autophagosome and autolysosome abundance with and without starvation conditions (Fig. 14a+b). Interestingly, the phosphorylation state of Atg5 did not have any consequences for the cellular localisation of Atg5, thus a secondary spatial effect due to an altered Atg5 localisation can be ruled out (Fig. 15). Surprisingly, a recent report showed that a protein called tectonin beta-propeller repeat containing 1 (TECPR1) co-localises with the Atg12-Atg5 conjugate after autophagosomal completion (Chen et al. 2012). Along these lines TECPR1 was also reported to be crucially involved in the maturation of autophagosomes to autolysosomes. If TECPR1 is a key factor in Atg12-Atg5-dependent fusion of autophagosomes with lysosomes, it could be the mechanism by which phosphorylated Atg5 is inhibiting the autophagosomal fusion. However, it has long since been established that Atg12-Atg5 disassociates from the autophagosomal membrane directly after membrane closure, which is in accordance with the results of this thesis (Fig. 15) (Mizushima et al. 2001). Thus, the molecular details and a possible connection to the Gadd45β-MEKK4-p38 pathway has to be investigated. Moreover, Chen and colleagues used an U_2OS-TR inducible cell line with a shRNA knockdown of TECPR1 (Chen et al. 2012). Whether the observed differences in autophagic behaviour are also relevant in other cell lines or, most interestingly, in primary cells, remains to be analysed.

Taken together, these results demonstrate that Gadd45β and MEKK4 act together on p38 and direct it to the autophagosomal membrane. There, it phosphorylates Atg5 at the threonine 75, resulting in the block of autophagosomal and lysosomal fusion (Fig. 31). Along

these lines, autophagosomes accumulate and this results in the increased number of GFP-LC3 dot-like structures that could initially be identified.

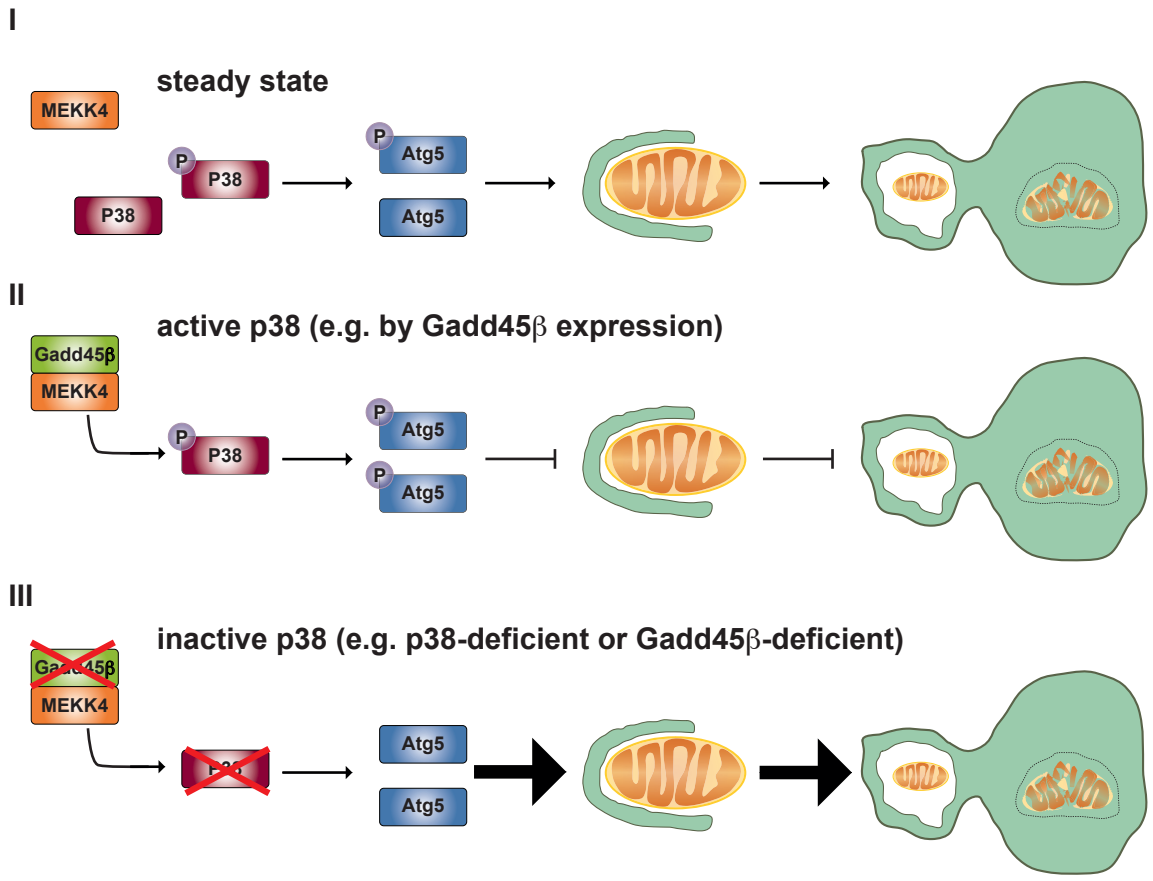


Figure 31: Schematic representation of the role of Gadd45β-MEKK4-p38 pathway in Atg5 phosphorylation and autophagic flux.
I. Steady state conditions: A fraction of the intracellular Atg5 pool is phosphorylated, and a normal autophagic flux is established. **II.** Gadd45β and MEKK4 act on p38 and guide it to the autophagosomal membrane, where it phosphorylates Atg5, thus inhibiting autophagosomal maturation. **III.** In a Gadd45β-deficient situation p38 does not phosphorylate Atg5, which results in an increased autophagosomal activity.

Recently, Webber and colleagues could identify p38-interacting protein (p38IP) as an interaction partner of Atg9. They demonstrated that active p38 sequesters p38IP away from Atg9 and in this way indirectly downregulates autophagy (Webber and Tooze 2010). However, Webber and colleagues did not look for the kinase activity of p38 and, as a consequence, were not able to rule out the possibility of a competition between p38 and Atg9. Therefore, the post-translation modification of Atg5 by Gadd45β-MEKK4-p38 is the first direct modification of an Atg-protein described so far that is not involved in the initiatory phase of autophagy but in its progression.

5.1.2 The physiological role of the Gadd45β-MEKK4-p38 pathway

The discovery of this new post-translational modification of an Atg-protein involved in autophagosomal maturation leads to the question whether this new pathway has any physiological relevance. Previous reports have shown that Gadd45β is induced *in vivo* upon injection of lipopolysaccharide (LPS) into mice (Zhang et al. 2005). Additionally, autophagy is beginning to emerge as a topic also relevant for innate immunity. In a number of publications, it was shown that triggering of the Toll-like receptor (TLR)4 resulted in increased levels of autophagy (Xu et al. 2007; Sanjuan et al. 2007; Delgado et al. 2009). Along these lines, Gadd45β–deficient and wildtype MEFs were stimulated with LPS with or without bafilomycin A1. In the Gadd45β$^{-/-}$ MEFs, LC3-II was increased compared to the wildtype controls. This was true for untreated cells as well as for cells that were subjected to bafilomycin A1 treatment, although the increase in LC3-II abundance was much more apparent in the treated cells (Fig.16). In order to rule out the possibility that the differences between wildtype and Gadd45β-deficient cells was an artefact of the immortalisation process of the MEFs, the experiment was repeated with bone marrow-derived macrophages (BMDMs) (Fig. 17). As shown in MEFs, the Gadd45β-deficient BMDMs also showed an increase in LC3-II protein abundance after treatment with LPS and bafilomycin A1. The increase in autophagy due to LPS stimulation is not without reason, as LPS is part of the bacterial outer membrane. It functioned as a substitute for a gram-negative bacterium, such as *Shigella* or *Salmonella,* in this experiment. Among others, these bacteria serve as well-characterised model pathogens (Ogawa et al. 2005; Birmingham et al. 2008). However, whether autophagy is able to help in bacterial clearance or not is also largely dependent on the pathogen itself. With *Listeria monocytogenes*, Rich and colleagues could already demonstrate in 2003 that autophagy plays a pivotal role in the intracellular clearance of bacteria in macrophages (Rich et al. 2003). Although this pathogen is gram-positive another group could demonstrate Atg5-dependency of autophagosomal clearance of *Streptococcus pyogenes* (Nakagawa et al. 2004). Other bacteria, such as *Mycobacterium tuberculosis,* were found to be subjected to autophagosomal degradation (Gutierrez et al. 2004), indicating that autophagy is a major

defence mechanism against bacterial invasion. Along these lines, the Gadd45β-MEKK4-p38 pathway could be implicated in the downregulation of autophagy after the clearance of the pathogen. The importance of autophagy downregulation after an acute activation phase is just as important as the upregulation of autophagy at the beginning, in order to prevent the cell from self-digestion (Poels et al. 2012).

Schmitz and colleagues could identify Gadd45β in a screening experiment of negative selection in the thymus (Schmitz et al. 2003). Here, Gadd45β was the most upregulated gene. Interestingly, recent studies were able to link autophagy to T cell receptor (TCR) signalling (Paul et al. 2012). The study could show the selective sequestration of the adapter protein Bcl-10 to autophagosomal degradation, resulting in a weakened NF-κB activation.

Upon triggering of the TCR, Gadd45β-deficient thymocytes showed increased levels of LC3-II in comparison to wildtype thymocytes (Fig 18). This was only true after bafilomycin A1 treatment: unmanipulated thymocytes did not show such increase in the expression levels of LC3-II (data not shown). These results demonstrate that the inhibiting effects of the Gadd45β-MEKK4-p38 pathway are not limited to certain stimuli or cell types, but seem to influence the autophagic machinery generally and in all cell types. This implication could become even more important when considering the emerging role of autophagy in T cells. As discussed earlier, recent studies could show that autophagy has numerous implications in the homeostasis, immunity and energy metabolism of T cells (Hubbard et al. 2010; Pua et al. 2007; Leavy 2008; Jia and He 2011; Walsh and Bell 2010). Why autophagic levels, as measured by GFP-LC3 fluorescence, differ between diverse T cells subsets (Fig. 19) is not yet clear and further experiments need to be performed. Additionally, the GFP-LC3 reporter mice that were used for this experiment were created with an EGFP-LC3 cassette whose expression is driven by a cytomegalovirus immediate-early (CMVie) enhancer and chicken β-actin (CAG) promoter (Mizushima 2009). Thus, although the expression of the transgene should be equal in all tissues due to the β-actin promoter, a promoter-driven effect cannot be totally disregarded. Therefore, additional experiments need to be performed, which do not

rely on the transgene as a means to detect autophagy, such as SDS-PAGE and immunoblotting for LC3-conversion.

Interestingly, these results are in contrast to a study of Atg5$^{-/-}$ T cells, where Pua and colleagues were able to find an overall decrease in thymic cellularity, but no differences between the individual T cell subsets (Pua et al. 2007). However, CD8^{+} effector T cells are known to undergo dramatic metabolic changes upon activation, such as enhanced glycolysis, glucose and glutamine uptake. This would confirm the role of autophagy in T cells upon activation. In a recent study of activated Atg7$^{-/-}$ T cells, the autophagosomal cargo was mainly comprised of cytosolic contents, delivering energy for the T cell as opposed to resting T cells, where also mitochondria were included into the degradation (Hubbard et al. 2010).

Interestingly, these changes in T cell metabolism are also MAPK dependent (Carr et al. 2010; Marko et al. 2010). These findings emphasise the link of MAPK signalling and T cell metabolism, where the Gadd45β-MEKK4-p38 signalling might also play an important role. Moreover, recent studies could link mTOR activation to TCR signalling (Salmond et al. 2009). Thus, the breeding of Gadd45β-deficient mice to GFP-LC3 reporter mice could help to further elucidate the function of Gadd45β specifically in autophagy in T cells.

So, apparently the Gadd45β-MEKK4-p38 pathway exerts an important physiological role in dampening the autophagic response after activation by a variety of different stimuli.

5.2 Gadd45β and apoptosis of developing T cells

5.2.1 The impact of Gadd45β–deficiency on autoimmunity

Gadd45β was found to have numerous implications in apoptosis induction and also in its inhibition (Papa et al. 2007; Gupta et al. 2006; Takekawa and Saito 1998). However, a possible role of Gadd45β-deficiency in T cells in regard to apoptosis was not yet characterised for the Gadd45β$^{-/-}$ mouse generated in the laboratory of Dan Liebermann (Gupta et al. 2005). Although there are reports that proof the Gadd45β-MEKK4 pathway to

be required for adequate cytokine production by $CD4^+$ helper T cells, no coherent analysis of T cell populations in Gadd45β-deficient mice compared to wildtype has been done (Yang et al. 2001; Liu et al. 2005; Chi et al. 2004). Surprisingly, this is also true for the $CD4^+Foxp3^+$ regulatory T cells, although Gadd45β and Gadd45γ double-deficiency was demonstrated to have a severe autoimmune phenotype (Liu et al. 2005), and Gadd45β has been shown to be a target of the master transcription factor of regulatory T cells (Tregs), Foxp3 (Marson et al. 2007). The steady-state observations of Gadd45β-deficient mice compared to wildtype littermates revealed a statistically relevant increase of $CD4^+Foxp3^+$ T cells in terms of frequency (Fig 24a). However, this could not be confirmed by absolute numbers of these cells (Fig 24b), so that the higher frequency of these cells in Gadd45β-deficient mice is due to a reduction in $CD4^+$ effector T cells (Fig 22a). At first glance this observation is a contradiction to the results of other groups: If Gadd45β-deficiency leads to a decrease in $CD4^+$ effector T cells, than why are Gadd45$\beta^{-/-}$ mice more prone to autoimmunity? The study of Liu and colleagues only determined the absolute number of splenocytes of the Gadd45$\beta^{-/-}$ and Gadd45$\beta^{-/-}$/Gadd45$\gamma^{-/-}$ mice and not the absolute numbers of the different subsets (Liu et al. 2005). Moreover, Liu and colleagues did not include a Foxp3-staining into their analysis of infiltrated leukocytes (Liu et al. 2005). Therefore, they declared $CD4^+CD25^+$ T cells as activated effector cells. However, these cells show a reduced $CD4^+$ expression when compared to $CD4^+CD25^-$ T cells, a characteristic for Tregs (Sakaguchi et al. 1995). Additionally, the observations were made in a different knockout mouse than the one that led to the observations in this work. Most importantly however, Liu and colleagues used a peptide derived of the myelin oligodendrocyte glycoprotein (MOG) to trigger an immune response that leads to experimental autoimmune encephalomyelitis (EAE) as opposed to the steady-state conditions that were used in this work. In order to proof a functional relevance of the decreased absolute numbers of $CD4^+$ effector T cells and the resulting increased frequencies of $CD4^+Foxp3^+$ Tregs, comparable conditions have to be applied. Along these lines, the Gadd45β-deficient mouse used in this thesis should be subjected to MOG and resulting EAE. Subsequently, the same analysis has to be performed again. This should

reveal whether the autoimmune phenotype is due to a more rapid expansion of Gadd45β$^{-/-}$ T cells compared to wildtype ones or not. Of note, the concentration of anti-nuclear antibodies (ANAs) in the serum of Gadd45β-deficient mice of high age (10 months) was comparable to wildtype mice (data not shown). Additionally, the Gadd45β-dependent demethylase activity could be the reason why Gadd45β-deficient mice are more prone to autoimmunity than the corresponding wildtype animals. The involvement of Gadd45β in demethylation is well described: An overexpression of Gadd45α led to increased demethylation in *Danio rerio*, while the silencing of the whole Gadd45 gene family reduced it (Rai et al. 2008). As described earlier, the methylation status of the Treg specific demethylated region (TSDR) in Tregs is important for maintenance of the immunosuppressive phenotype of these cells (Floess et al. 2007; Polansky et al. 2008). Thus, Tregs and conventional CD4$^+$ T cells from Gadd45β-deficient mice were analysed for the methylation state of the TSDR. These results were then compared to the results from wildtype animals (data not shown). The TDSR of Gadd45β$^{-/-}$Foxp3$^+$ Tregs was normally demethylated and the methylation of naïve CD4$^+$CD62L$^+$ T cells was comparable to the wildtype animals. Therefore, the reported demethylation activity of Gadd45β seems to have no impact on the Foxp3 locus and, thus, on Treg lineage stability (Sen et al. 2010).

In conclusion it can be said that Gadd45β-deficiency alone seems not to have a severe impact on the autoimmune-phenotype of mice. Both studies of methylation and of autoimmunity discussed earlier also had a look at mice deficient for at least two different Gadd45 family members (Liu et al. 2005; Rai et al. 2008). When taking into consideration that the Gadd45 family members are conserved among each other (Ashford and Porter 1962; Tamura et al. 2012; Liebermann and Hoffman 2008), a certain degree of redundancy seems to play a role. Thus, in order to analyse the impact of Gadd45-deficiency in respect to autoimmunity, a situation of wildtype against double- or triple-deficiency of the Gadd45 family needs to be analysed.

5.2.2 The role of Gadd45β in apoptosis in the context of negative selection in the thymus

Gadd45β-deficiency could be linked to an abbreviated phosphorylation of p38 *in vivo* and thus to a reduced cleavage of the effector caspase 3 (Höcker et al., in preparation). In order to ascertain that the reduced cleavage of caspase 3 is TCR-dependent, the apoptotic response of Gadd45β-deficient thymocytes to well-known apoptotic stimuli was analysed (Fig. 25). Interestingly, Gadd45β-deficiency led to an altered apoptotic response only upon αCD3 stimulation. Here, only low concentrations of αCD3 had opposing effects in wildtype and Gadd45β$^{-/-}$ thymocytes. Although αCD3 does not occur naturally, the huge differences in cell survival upon treatment with low concentrations can be interpreted as follows: Only physiological relevant concentrations of a given TCR stimulus lead to different outcomes in apoptosis. Specifically this correlation is well-described: the outcome of thymic selection processes is highly dependent on the strength of the TCR signal the T cells receives (Stritesky et al. 2012; Singer et al. 2008).

This protection against apoptotic events upon a weak TCR stimulation would match the results obtained earlier in the laboratory using fetal thymic organ culture (FTOCs) (data not shown): Double positive thymocytes are protected against TCR-stimulation-dependent apoptosis. FTOCs have an advantage over αCD3 stimulation of complete mice: The potential side effects, namely a cytokine storm that is originating in the periphery, are eliminated (Martin and Bevan 1997). In order to show a relevance of these results *in vivo*, a Gadd45β-deficient mouse was bred to a N15H-2^{b} TCR transgenic mouse on a RAG2$^{-/-}$ genetic background. Utilising this mouse, a cytokine storm could be excluded as an interference and thus, the obtained results were only due to downstream signalling events of the TCR that resulted from Gadd45β-deficiency. The analysis of these mice showed a pronounced increase in mature CD8^{+} T cells 48 h post TCR stimulation in terms of frequency as well as in absolute numbers (Fig. 28a+b). These cells have severely decrease of CD24 expression, showing that they are mature T cells ready to leave the thymus and are not in an intermediated state of T cell development (Fig. 27). That this increased population of CD8^{+}

thymocytes is due to a defect in negative and not positive selection can be seen in the $CD4^+CD8^+$ double positive population: CD69 is comparable between wildtype and Gadd45β-deficient mice and expression of CD5 is more pronounced in wildtype T cells (Fig. 29). If anything, than this would imply more T cells in the wildtype and not in the Gadd45β-deficient situation.

Of note, p38, JNK and the MEK5-ERK5 pathway have already been implicated in negative selection (Starr et al. 2003; Sohn et al. 2008). Thus, the interaction of Gadd45β and MEKK4 with p38 is linked to negative selection by the MAPK. Importantly, Bim and Nur77 were shown to be crucial for negative selection (Bouillet et al. 2002; Calnan et al. 1995; Zhou et al. 1996; Kuang et al. 1999). For Bim the means of acting on negative selection are self-evident: Being a pro-apoptotic Bcl-2 family member and its activity is regulated by MAPK-mediated posttranslational modification (Hübner et al. 2008). However, studies also showed a Bim-independent way of negative selection (Hu et al. 2009; Kovalovsky et al. 2010). Nevertheless, Kovalovsky and colleagues could not totally rule out Bim-dependency in negative selection and Hu and colleagues found a Bim-dependency in double-positive thymocytes. If Bim is required for negative selection or not therefore remains a subject to debate and needs to be further investigated.

Furthermore, the mechanism how Nur77 affects negative selection is still unclear. As a transcription factor, it may induce apoptosis in a transcription-dependent manner or by a direct interaction on Bcl-2 (Rajpal et al. 2003; Thompson and Winoto 2008). On the contrary, one study revealed that Nur77 is negligible for thymic and peripheral T cell death (Lee et al. 1995). Taken together, only one study showed that Nur77 does not affect apoptosis in thymocytes, whereas multiple studies could prove otherwise. Thus, it is a well-accepted assumption that Nur77 plays a role in apoptosis in the context of negative selection in the thymus. Previous work could show that transcriptional levels of Bim and Nur77 remain unaffected in Gadd45β-deficient thymocytes (Keil 2008). Therefore, Gadd45β-MEKK4-p38 interaction possibly constitutes a new signalling pathway in the negative selection process (Fig. 32).

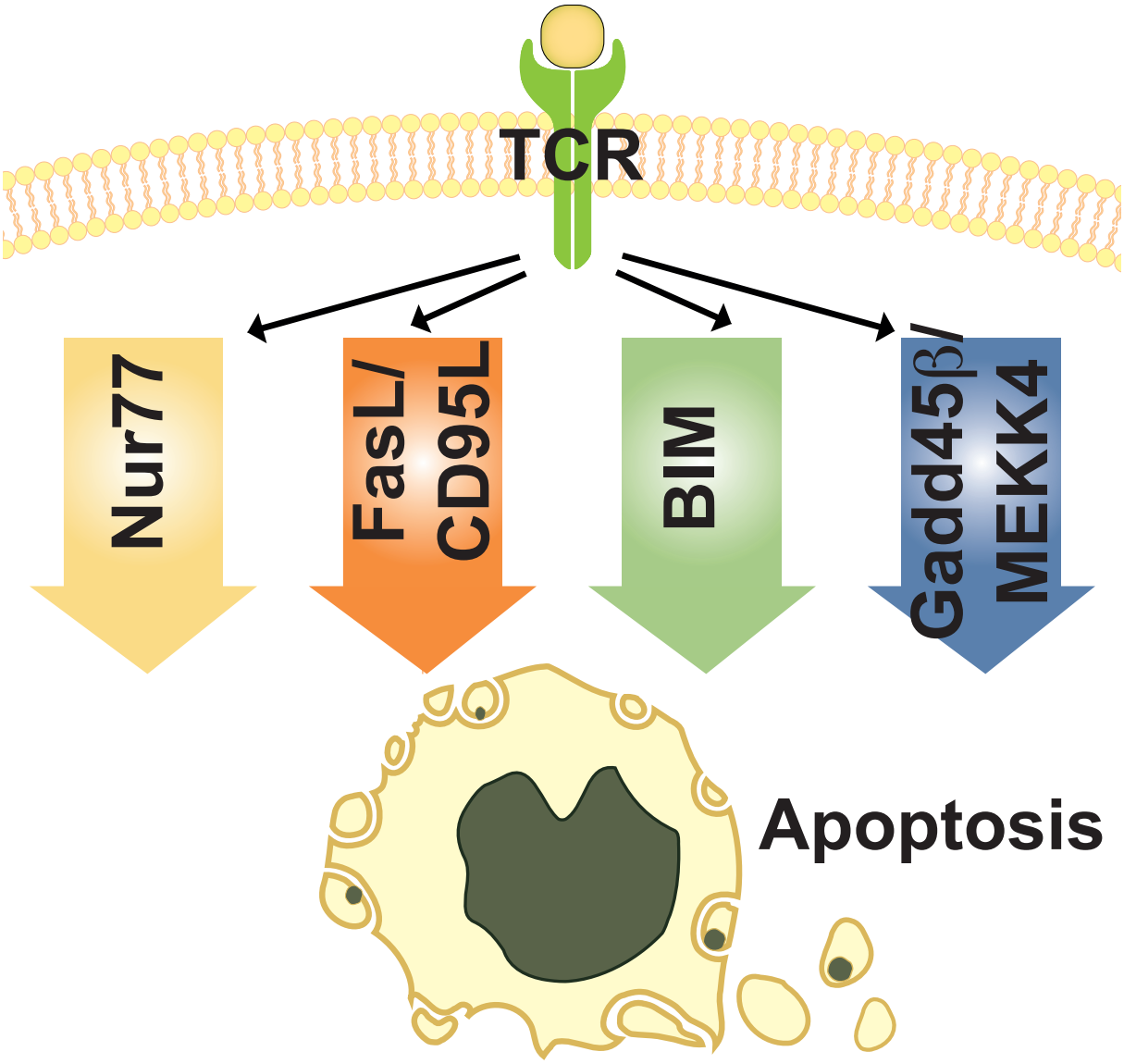


Figure 32: Revised model of negative selection upon TCR stimulation in thymocytes.
Upon a specific TCR stimulation, apoptosis is triggered in negatively selected thymocytes. Depicted are the published pathways, by which this selection process is mediated. Additionally, the new, suggested Gadd45β/MEKK4 pathway is depicted in parallel.

Thus, it can be concluded that Gadd45β-deficient thymocytes, which should have been deleted in the context of negative selection in the thymus, instead mature and migrate into the periphery. Therefore, this defect in central tolerance possibly leads to the accumulation of autoreactive T cells in the periphery and subsequently to autoimmunity. This finding would also be in line with the reported increased autoimmunity of Gadd45β-deficient mice in contrast to wildtype animals (Liu et al. 2005).

6. List of abbreviations

°C	Degree Celsius
7AAD	7-aminoactinomycin
A	Absorbance
AIRE	Autoimmune regulator
Amp	Ampicillin
Atg	Autophagy related gene
Bak	Bcl-2 antagonist killer
Bax	Bcl-2 associated protein X
BCL-10	B cell lymphoma protein 10
BCL-2	B cell lymphoma protein 2
Bcl-xL	Bcl-2-like 1 large
Beclin 1	Coiled-coil, myosin like Bcl-2 interacting protein
Bid	BH3-interacting death agonist
Bim	Bcl-2-interacting mediator of cell death
bp	Base pair
BSA	Bovine serum albumin
c-Flip	Cellular *FLICE*-inhibitory protein
CARD	Caspase-recruitment domain
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
DC	Dendritic cell
DD	Death domain
DED	Death effector domain
dH_2O	Distilled water
DISC	Death inducing complex

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside-5´-triphosphate
DP	Double positive ($CD4^+CD8^+$)
dsRNA	Double stranded ribonucleic acid
EAE	Experimental induced encepahlomyelitis
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunoabsorbant assay
ER	Endoplasmatic reticulum
ERK	Extracellular signal related kinase
FACS	Fluorescence activated cell sorter
FADD	FAS-associated death domain protein
FITC	Fluorescein-5-isothiocyanat
Flip	Flice-inhibitory protein
Foxp3	Forkhead box protein 3
g	Grams
Gadd	Growth Arrest and DNA-Damage inducible gene
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GST	Glutathione-S-transferase
h	Hours
Ig	Immunoglobulin
JNK	Jun-kinase
Kan	Kanamycin
kb	Kilobase pairs
kDa	Kilodaltons
l	Litre

LB	Luria-Bertani médium
LC3	Microtubule-associated protein 1 light chain 3
LPS	Lipopolysaccharide
M	Molar
mA	Milliamps
MAPK	Mitogen-activated protein kinase
MAPK	Mitogen-activated protein kinase
MEKK	Mapk kinase kinase
mg	Milligrams
MHC	Major histocompatibility complex
min	Minutes
MKK	Mapk kinase
ml	Millilitres
mM	Millimolar
mm	Millimeter
mol	Moles
mRNA	Messenger RNA
mTEC	Medularry thymic epithelial cell
mTOR	Mammalian target of rapamycin
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear factor kappa-B
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCC	Pigeon cytochrome C
PCR	Polymerase chain reaction
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2

RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
s	Seconds
SDS	Sodium dodecyl sulphate
t	Time
TAE	Tris-acetate EDTA
Taq	Thermostable DNA polymerase derived from *Thermus aquaticus*
tBid	truncated Bid
TBS	Tris-buffered saline
TCR	T cell receptor
TEMED	N,N,N´N´-tetramethyl-ethylenediamine
Tet	Tetracycline
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand receptor
Treg	Regulatory T cell
Tris	Tris(hydroxymethyl) aminomethane
tRNA	Transfer RNA
V	Volt
v/v	Volume for volume
vol	Volume
VSV8	Vesicular stomatitis virus (octapeptide)
W	Watt
w/v	Weight for volume
wt	Wildtype
µg	Micrograms
µl	Microlitres

μM	Micromolar

7. References

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9. Declaration of originality

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Hiermit erkläre ich, dass die eingereichte Dissertation mit dem Thema

The role of Gadd45β in apoptosis and autophagy

von mir selbständig verfasst wurde, nicht schon als Dissertation verwendet wurde und benutze Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. Nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 27.11.2012

Ralf Höcker

10. Curriculum vitae

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Originalarbeiten:

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