Schmitz, I.
Gadd45 proteins in immunity
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Ingo Schmitz
Professor of Immunology
Systems-oriented immunology and inflammation research
Helmholtz Center for Infection Research
Inhoffenstrasse 7
38124 Braunschweig
Germany
Email: Ingo.Schmitz@helmholtz-hzi.de
Tel: +49 531 6181 3500 FAX: +49 531 6181 3599

Chapter 4: Gadd45 Proteins in Immunity

Abbreviations

ATG	autophagy-related
Bcl-x_L	B cell lymphoma x large
c-FLIP	cellular FLICE inhibitory protein
Cbl-b	casitas B-lineage lymphoma proto-oncogene b
CD	cluster of differentiation
$CD4^+$	cluster of differentiation 4-positive
$CD8^+$	cluster of differentiation 8-positive
CLR	C-type lectin receptor
CR6	cytokine response gene 6
DISC	death inducing signaling complex
EAE	experimental autoimmune encephalomyelitis
Egr	early growth response
G-CSF	granulocyte colony stimulating factor
Gadd45	growth arrest and DNA damage 45
GM-CSF	granulocyte macrophage colony stimulating factor
GRAIL	gene related to anergy in lymphocytes protein
IFN	interferon
IL	interleukin
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
MAPK	mitogen-activated protein kinase
MEKK4	MAPK/ERK kinase kinase 4
MHC	major histocompatibility complex
MKK	mitogen-activated protein kinase kinase
MOG	myelin oligodendrocyte glycoprotein
Myd118	myeloid differentiation primary response protein 118
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NKT	natural killer T cell
NLR	Nod-like receptor
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription

TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	T helper
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1
vMIA	viral mitochondrial-localized inhibitor of apoptosis
ZAP-70	zeta-chain associated protein of 70 kDa

Abstract

The vertebrate immune system protects the host against invading pathogens such as viruses, bacteria and parasites. It consists of an innate and an adaptive branch that provide immediate and long lasting protection, respectively. As the immune system is composed of different cell types and distributed throughout the whole body, immune cells need to communicate with each other. Intercellular communication in the immune system is mediated by cytokines, which bind to specific receptors on the cell surface and activate intracellular signaling networks. Growth arrest and DNA damage-inducible 45 (Gadd45) proteins are important components of these intracellular signaling networks. They are induced by a number of cytokines and by bacterial lipopolysaccharide. Within the innate immune system, Gadd45 proteins are crucial for the differentiation of myeloid cells as well as for the function of granulocytes and macrophages. Moreover, Gadd45β regulates autophagy, a catabolic pathway that also degrades intracellular pathogens. Regarding adaptive immunity, Gadd45 proteins are especially well characterized in T cells. For instance, Gadd45β and Gadd45γ regulate cytokine expression and Th1 differentiation while Gadd45α inhibits p38 kinase activation downstream of the T cell receptor. Due to their many functions in the immune system, deficiency in Gadd45 proteins causes autoimmune diseases and less efficient tumor immunosurveillance.

4.1 Introduction

The immune system of vertebrates consists of an innate and an adaptive branch. Responses by the innate immune system are immediate and activated by germ-line encoded pattern recognition receptors (PRRs), which recognize conserved pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). The PRRs include toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs) and cytosolic DNA sensors (RIG-I like receptors, RLRs) (Takeuchi and Akira, 2010). Upon triggering, these receptors activate the NF-κB signaling cascade and type I interferons (IFNα and IFNβ) that in turn activate and recruit immune cells. Innate immune cells include granulocytes such as neutrophils, as well as macrophages and dendritic cells. The latter two cell types act as professional antigen presenting cells (APCs) that present peptide-antigens to T cells, which then mount an adaptive immune response. T cells are lymphocytes that develop in the thymus (hence named T cell) and mediate cellular immunity. There are different types of T cells, namely cytotoxic T cells and helper T cells. Cytotoxic T cells express the co-receptor CD8 ($CD8^+$ CTLs) and kill infected target cells via the induction of apoptosis. Helper T cells express the co-receptor CD4 ($CD4^+$ Th cells) and activate macrophages to digest intracellular pathogens. $CD4^+$ Th cells also provide help to another type of lymphocyte called B cells, which develop in the bone marrow (hence named B cell), via co-stimulatory molecules such as CD40. Activated B cells then secrete antibodies (immunoglobulins) and thereby mediate humoral immunity. The

adaptive immune system has two important characteristics, which make it essential for complex organisms such as vertebrates. First, cells of the adaptive immune system, i.e. lymphocytes, bare antigen receptors that are highly specific for a certain antigen. During the development of lymphocytes, gene fragments of the immunoglobulin and T cell receptor (TCR) genes are rearranged in a stochastic manner to generate up to 10^{15} receptor specificities (Davis and Bjorkman, 1988). In contrast to innate immune cells, which react only to conserved pathogen structures such as lipopolysaccharide (LPS), a compound from the cell wall of gram-negative bacteria, lymphocytes are able to react to specific amino acid sequences and, thus, to a particular strain of a pathogen. The second important feature of the adaptive immune system is its ability to form memory, so that re-infection with the same pathogen results in a faster and stronger immune response. This is due to the fact that during the first encounter with a given pathogen some lymphocytes develop into long-lived memory cells that survive in lymphoid organs and other tissues for months and even years and have a lower threshold of activation (Woodland and Kohlmeier, 2009). Therefore, they acquire effector functions much faster than naïve lymphocytes upon repeated antigen encounter. Consequently, they pathogens get cleared much faster and the infected host does not develop any symptoms of disease – the host is immune.

Cytotoxic T cells and B cells function as effector cells of the adaptive immune system by killing target cells and producing antibodies, respectively. Yet, $CD4^+$ Th cells play a leading part during an adaptive immune response by providing co-stimulatory signals and secreting cytokines. Depending on the nature of the invading pathogen they are able to differentiate into various Th cell subsets, namely Th1, Th2, Th17 cells and others, each tailored to drive a type of immune response appropriate for the particular pathogen (Korn et al., 2009; Murphy and Reiner, 2002; Reiner, 2007). For instance, Th1 cells are induced by interleukin-12 (IL-12) and produce interferon-γ (IFN-γ), which activates infected macrophages to degrade microbes that persist in intracellular vesicles such as mycobacteria or Listeria (Murphy and Reiner, 2002). Th2 cells are induced by IL-4 and are important for controlling infections caused by extracellular, multicellular parasites such as helminthes (Murphy and Reiner, 2002). Th2 cells produce IL-4, IL-5 and IL-13 to activate eosinophiles, mast cells and B cells, the latter differentiating into plasma cells to produce high amounts of specific antibodies. Th17 cells were named after the cytokine they secrete, IL-17 (Harrington et al., 2005; Park et al., 2005). They develop upon stimulation of naïve T cells with both the immunosuppressive cytokine transforming growth factor beta (TGF-β) and with the pro-inflammatory cytokine IL-6 and are instrumental in fighting extracellular bacteria and fungi by enhancing neutrophil responses.

Since the immune system is composed of many different cell types distributed over the whole body, it is compulsory that immune cells need to communicate with each other, either via direct cell-cell-contact or via cytokines over a distance. Inside the cells, complex signal transduction networks transmit and integrate these communication signals. Gadd45 proteins are part of the signaling networks in immune cells. Here, I will first discuss signals that induce expression of Gadd45 proteins in cells of the immune system. The next two sections will be on the function of Gadd45 proteins in the innate and adaptive immune system, respectively. Finally, I will discuss the role of Gadd45 proteins in pathological settings, such as autoimmune diseases.

4.2 Expression of Gadd45 Proteins in Immune Cells

The Gadd45 proteins, namely Gadd45α (Gadd45), Gadd45β (Myd118) and Gadd45γ (CR6), are small proteins of 18-20 kDa with no enzymatic activity of their own. They execute their physiological functions by protein-protein-interactions in the nucleus and cytoplasm of cells

and are able to modulate cell proliferation, cell death and cell survival. Due to their high homology (Takekawa and Saito, 1998), Gadd45 family members are expected to have largely overlapping functions. Specificity might be brought about by different signals that drive the transcription of the various *Gadd45* genes. For instance, Gadd45α is a p53 target gene (Kastan et al., 1992), the transcription of Gadd45β is induced by TGF-β, interleukins as well as the T cell receptor (Sanjuan et al., 2007; Selvakumaran et al., 1994; Yang et al., 2001), and Gadd45γ transcription is stimulated by interleukin-2 (Beadling et al., 1993).

With respect to innate immune signals, it has been shown that LPS, a cell wall component of gram-negative bacteria, induced Gadd45β *in vivo* (Zhang et al., 2005). Of note, Gadd45β induction could be inhibited by co-administration of the glucocorticoid analogue dexamethasone or by thalidomide. Since both drugs are inhibitors of the pro-inflammatory transcription factor NF-κB (Keifer et al., 2001; Majumdar et al., 2002; Neumann and Naumann, 2007), these data suggested that *Gadd45b* is an NF-κB target gene. Indeed, bortezomib, a proteasome inhibitor that impairs degradation of IκB proteins and thereby NF-κB activation, but not pharmacological inhibitors of mitogen-activated protein kinases (MAPKs), prevented Gadd45β induction by LPS in mice (Zhang et al., 2005). In line with this observation, analysis of the *Gadd45b* gene indicated several functional NF-κB binding sites (Balliet et al., 2001; Jin et al., 2002). It was shown that NF-κB-induced Gadd45β inhibits activation of the MAPK JNK via inhibition of the upstream kinase MKK7 in TNFα-treated T cell hybridomas (De Smaele et al., 2001; Papa et al., 2004). However, it is currently unknown whether this pathway operates in immune cells *in vivo*. Furthermore, so far it has not been addressed so far whether other stimuli of the innate immune systems (i.e. PAMPs) that activate the NF-κB pathway also induce Gadd45β protein expression.

A number of cytokines have been reported to induce the expression of Gadd45β proteins in hematopoietic cells and cells of the immune system. First, hematopoietins such as granulocyte-macrophage colony stimulating factor (GM-CSF), M-CSF, G-CSF and interleukin-3 (IL-3) were shown to induce expression of all *Gadd45* genes at the mRNA level in bone marrow cells (Gupta et al., 2006). Hematopoietic cells from the bone marrow, which are either deficient for Gadd45α or Gadd45β and are more sensitive to apoptosis upon several kinds of cellular stresses, suggesting that these two proteins act in an anti-apoptotic fashion in this cell type (Gupta et al., 2005; Gupta et al., 2006). IL-6, a cytokine that supports differentiation of hematopoietic cells and that has pro-inflammatory activity, induces Gadd45β in the murine myelomonocytic cell line M1 (Zhan et al., 1994). Another pro-inflammatory cytokine that induces Gadd45 expression and belongs to the IL-1 family is IL-18 (Arend et al., 2008). IL-18 induced the expression of *Gadd45b* and *Gadd45g* genes in $CD4^+$ Th cells, which was dramatically enhanced by co-treatment with IL-12 (Yang et al., 2001). Of note, Gadd45α was not induced by IL-12 and IL-18. Similarly to IL-18 in $CD4^+$ Th cells, IL-33, another IL-1 family cytokine, synergized with IL-12 to induce Gadd45β expression in $CD8^+$ cytotoxic T cells (Yang et al., 2011). In sum, pro-inflammatory cytokines appear to be crucial inducers of Gadd45 gene expression in hematopoieitic cells.

Since the action of these pro-inflammatory cytokines on T cells often depends on the activation status of T cells, it is not surprising that the stimulation of the T cell receptor (TCR) also induced *Gadd45* gene expression. Thus, stimulation of naïve $CD4^+$ T cells with anti-CD3 antibodies (triggering the TCR complex) led to Gadd45β expression at early time points (within 4 hours), an effect that was augmented by costimulation via CD28 (Lu et al., 2004). This early induction of Gadd45β was also observed *in vivo* in thymocytes using TCR transgenic mice and injection of cognate peptide antigens (Schmitz et al., 2003). In contrast, induction of Gadd45γ required prolonged stimulation of naïve $CD4^+$ T cells for 48 to 96 hours

(Lu et al., 2001). This may be related to the fact that IL-2 signaling rather than TCR or CD28 signals induces Gadd45γ expression (Hoffmeyer et al., 2001).
Surprisingly, Gadd45β expression is not only induced by the above mentioned immunostimulatory signals, but also by transforming growth factor beta (TGF-β), which is a strong immunosuppressive cytokine (Li and Flavell, 2008). Thus, Gadd45β can be induced by TGF-β in the murine myelomonocytic cell line M1 and in the T cell line EL-4 (Selvakumaran et al., 1994). Accordingly, *Gadd45b* gene expression is regulated by Smad proteins, which are transcription factors that are activated at the TGF-β receptor complex (Takekawa et al., 2002; Ungefroren et al., 2005; Yoo et al., 2003). However, it is currently unknown whether Gadd45β is actually required for the immunosuppressive function of TGF-β on immune cells *in vivo*.

4.3 The Function of Gadd45 Proteins in Innate Immunity

Within the innate immune system, the function of Gadd45 proteins is best studied in myeloid cells such as granulocytes and macrophages. For instance, in the absence of either Gadd45α or Gadd45β *in vitro* differentiation of bone marrow cells into macrophage or granulocyte lineages resulted in reduced frequencies of these cell types (Gupta et al., 2006). This correlated with increased apoptosis during differentiation and reduced clonogenicity of Gadd45α-deficient and Gadd45β-deficient cells. Reduced myeloid differentiation was also observed *in vivo* when myeloid cells were ablated by intraperitoneal injection of 5-Fluorouracil and recovery was observed 10 days post injection (Gupta et al., 2006). In contrast, Gadd45γ is not required for myeloid differentiation (Hoffmeyer et al., 2001). Of note, Gadd45α- and Gadd45β-deficiency resulted in a higher proliferative capacity of immature myeloid cells (Gupta et al., 2006). Therefore, Gadd45 protein expression may support terminal differentiation of myeloid cells as well as inhibit the proliferation of these terminally differentiated cells.
In addition to myeloid differentiation, Gadd45α and Gadd45β appear to be important for the function of granulocytes and macrophages. In a mouse model of experimental sepsis, Gadd45α-deficient and Gadd45β-deficient mice exhibited impaired recruitment of myeloid cells into the peritoneal cavity upon LPS injection (Salerno et al., 2012). *In vitro*, macrophages and granulocytes of mice lacking either Gadd45α or Gadd45β were less efficient in migration as chemotactic assays using LPS, N-formylated peptides such as N-formyl- methionine-leucine-phenylalanine (fMLP) or the chemokine IL-8 as stimulus revealed. Both types of myeloid cells produced less reactive oxygene species (ROS) and cytokines. Moreover, the phagocytic capacity of Gadd45α-deficient and Gadd45β-deficient macrophages was strongly impaired (Salerno et al., 2012). Mechanistically, this was attributed to the regulation of p38 and JNK mitogen-activated protein kinase signaling by Gadd45 proteins. In summary, Gadd45 proteins play a crucial role in the differentiation, proliferation and function of myeloid cells.
As stated previously, LPS induces Gadd45β via the NF-κB pathway *in vivo* (Zhang et al., 2005). However, what could be the function of Gadd45β downstream of LPS? A recent study provides evidence that Gadd45β regulates macroautophagy (Keil et al., 2013), a process that is an essential catabolic pathway for maintaining protein homeostasis and for energy production within a cell (Mizushima and Komatsu, 2011). Importantly, an additional function of macroautophagy is the degradation of intracellular pathogens (Levine et al., 2011) and, consequently, TLRs are able to induce macroautophagy (Delgado et al., 2008; Sanjuan et al., 2007; Xu et al., 2007). Macroautophagy starts with *de novo* synthesis of a membrane structure

called the phagophore that encloses a part of the cytosol. Upon elongation and closure of the phagophore, a double-membrane enclosed vesicle is formed, which is called the autophagosome. Finally, the autophagosome fuses with a lysosome and its content is degraded by hydrolases. Important mediators of macroautophagy are the autophagy-related (ATG) proteins (Mizushima et al., 2011). Central to the elongation phase of autophagosome formation is a protein called ATG5, without which macroautophagy cannot take place (Mizushima et al., 2001). Consequently, ATG5-deficient mice die postnatally since they are not able to survive the starvation period newborns experience between nutrient provision via the umbilical cord and milk feeding (Kuma et al., 2004). This essential macroautophagy protein is targeted by a Gadd45β-MEKK4-p38 signaling pathway since Gadd45β and MEKK4 direct p38 to the autophagosome (Keil et al., 2013). MEKK4 is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates p38 and JNK mitogen-activated protein kinases and is present in a closed conformation and, therefore, inactive (Gerwins et al., 1997; Takekawa et al., 1997). Upon binding of Gadd45 proteins MEKK4 adopts an open conformation that allows dimerization, autophosphorylation and activation of downstream kinases (Miyake et al., 2007; Takekawa and Saito, 1998). When localized to the autophagosome, p38 phosphorylates ATG5 at threonine residue 75, an event that prevents fusion of autophagosomes with lysosomes (Keil et al., 2013). Thus, Gadd45β-activated p38 inhibits macroautophagy. Accordingly, Gadd45β-deficient fibroblasts and macrophages exhibited enhanced macroautophagy upon LPS treatment (Keil et al., 2013).

4.4 The Function of Gadd45 Proteins in Adaptive Immunity

With respect to adaptive immunity, most of the work on Gadd45 proteins has concentrated on T cells. However, there are also a few reports on other cell types of the adaptive immune system. For instance, it was shown that B cells strongly induce Gadd45β along with known anti-apoptotic proteins such as Bcl-x_L and c-FLIP upon ligation of CD40, a TNF receptor superfamily member that provides co-stimulatory signals to B cells (Zazzeroni et al., 2003). Generation of cell lines stably overexpressing Gadd45β demonstrated that this protein inhibits CD95/Fas-induced (i.e. extrinsic) apoptosis, but has no effect on early events in the CD95 signaling cascade such as the formation of the death-inducing signaling complex (DISC). Instead, Gadd45β impaired the activation of the mitochondrial amplification loop although direct triggering of the mitochondrial (i.e. intrinsic) pathway was not affected (Zazzeroni et al., 2003). The latter discovery seems to contradict the finding that Gadd45 proteins are able to bind to cellular Bcl-x_L as well as to the anti-apoptotic protein vMIA from the cytomegalovirus and thereby enhances the cell's resistance towards CD95-induced apoptosis (Smith and Mocarski, 2005). Therefore, the exact mechanism of the influence of Gadd45β on apoptosis is unknown and it remains to be dissected whether Gadd45β targets the CD95 signaling cascade similar to the TNFR1 pathway (De Smaele et al., 2001), or whether it targets mitochondria (Smith and Mocarski, 2005). Nevertheless, as in myeloid precursor cells (Gupta et al., 2005), Gadd45β appears to be an anti-apoptotic protein in B cells since it protected them from activation-induced cell death.

NKT cells are a type of lymphocyte, possessing characteristics of NK cells and memory T cells. They express an invariant TCR with a TCRα chain containing a variable region encoded by the Vα14 gene and a joining region encoded by the Jα18 gene that restricts these cells to CD1d molecules (Kronenberg and Gapin, 2002; Taniguchi et al., 2003). The latter are MHC-like molecules that present glycolipids as antigens such as α-galactosylceramide (Porcelli and Modlin, 1999). NKT cells possess immunoregulatory functions and secrete large quantities of cytokines such as IFN-γ and IL-4 (Kronenberg and Gapin, 2002; Taniguchi et

al., 2003). Interestingly, NKT cells are more resistant to TCR-induced apoptosis than conventional T cells, which correlated with a higher induction of anti-apoptotic genes such as *Gadd45b* (Harada et al., 2004). However, no data has been provided so far that supports a functional role for Gadd45β in NKT cell survival. Therefore, the importance of Gadd45 proteins for NKT cell biology awaits further studies.

Dendritic cells are the most potent professional antigen presenting cells (Shortman and Liu, 2002). They capture antigens from the environment and present them via MHC class II to $CD4^+$ Th cells. Depending on the nature of antigen and the route of antigen uptake, dendritic cells express cytokines that drive immune responses into a given direction, e.g. they secrete IL-12 and IFN-γ to promote a Th1 response (Murphy and Reiner, 2002). Bone marrow-derived dendritic cells express all three Gadd45 proteins (Jirmanova et al., 2007). Interestingly, dendritic cells from Gadd45α-deficient mice exhibited less activation of the classical MKK3/6-p38 mitogen-activated protein kinase (MAPK) cascade, less production of the Th1 cytokines IL-12 and IFN-γ and reduced expression of the co-stimulatory molecule CD40 upon stimulation with soluble antigens from *Toxoplasma gondii* (Jirmanova et al., 2007). Similarly, Gadd45β-deficient dendritic cells produce less IFN-γ upon stimulation with LPS (Lu et al., 2004). Therefore, the activation of classical MAPK signaling by Gadd45 proteins is crucial for mounting a Th1 response via activation of dendritic cells. Currently, it is not known whether the Gadd45-p38-axis regulates cytokine expression in dendritic cells via transcriptional or post-transcriptional mechanisms. However, since cytokine expression is often regulated by mRNA stability, it is tempting to speculate that p38 activation by Gadd45 proteins is required for stabilization of cytokine mRNAs in dendritic cells, as has been shown for TNFα mRNA (Ronkina et al., 2010).

Regarding Th1-mediated immunity, Gadd45 proteins were also described to affect Th1 cells in an intrinsic manner. For instance, Gadd45γ was shown to be important for the IFN-γ production by Th1 cells (Lu et al., 2001). In contrast, Th2 polarized Gadd45γ-deficient cells showed similar IL-4 levels when compared to wildtype Th2 cells. Gadd45γ-deficient T cells also exhibited less p38 and JNK MAPK activity and were less prone to activation induced cell death (Lu et al., 2001). However, Gadd45γ was not required for hematopoiesis, T cell proliferation or T cell responsiveness to IL-2 (Hoffmeyer et al., 2001; Lu et al., 2001). Importantly, Gadd45γ-deficient mice showed reduced contact hypersensitivity demonstrating that Th1 responses were also impaired *in vivo* (Lu et al., 2001).

Similar to Gadd45γ, Gadd45β also supports Th1 responses. Using retroviral overexpression and Gadd45β-deficient T cells, it was shown that Gadd45β promotes IFN-γ secretion upon TCR triggering or upon stimulation with IL-12 and IL-18, which drive Th1 differentiation (Lu et al., 2004; Yang et al., 2001). This was mediated via prolonged p38 MAPK activation (Lu et al., 2004) and inhibited via a dominant-negative version of MEKK4 (Yang et al., 2001), which is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates p38 and JNK (Gerwins et al., 1997; Takekawa et al., 1997). In line with the data obtained with dominant negative MEKK4, $CD4^+$ T cells from MEKK4-deficient mice exhibited less IFN-γ secretion during Th1 differentiation and reduced p38 activation upon stimulation of the TCR or with IL-12 and IL-18 (Chi et al., 2004). Importantly, overexpression of Gadd45β or Gadd45γ in MEKK4-deficient T cells did not increase IFN-γ production while it did so in wildtype T cells demonstrating that the Gadd45 proteins together with MEKK4 comprise a common pathway that potentiates IFN-γ production and thereby Th1-mediated immunity (Chi et al., 2004). However, while one study described the expression of IFN-γ to be independent of STAT4 and its phosphorylation state (Chi et al., 2004), another study found that Gadd45β and Gadd45γ induced phosphorylation of STAT4 at serine residue 721 and that Ser721-phosphorylated STAT4 was crucial for IFN-γ expression and Th1 differentiation (Morinobu

et al., 2002). Despite this controversy, it is clear that Gadd45 proteins are important regulators of IFN-γ expression in T cells and of Th1 differentiation.

Next to IFN-γ production, Gadd45β was shown to be important for IL-2 secretion in differentiated Th1 cells as well as in naïve T cells (Lu et al., 2004). As in differentiated Th1 cells, Gadd45β-deficient naïve T cells showed reduced p38 MAPK activity suggesting reduced T cell activation (Lu et al., 2004). Surprisingly, Gadd45β was also identified by DNA array technology as a gene induced during induction of T cell anergy, a state of unresponsiveness that is induced in T cells when they receive TCR stimulation in the absence of a co-stimulatory signal (Safford et al., 2005). Accordingly, anergy is a mechanism to ensure immunological tolerance along with clonal deletion by apoptosis or suppression of immune responses by regulatory T cells (Schwartz, 2003). Besides other mechanisms, anergy is mediated on the molecular level by nuclear factor of activated T cells (NFAT) as well as early growth response 2 (Egr2) and Egr3 that induce expression of E3 ubiquitin ligases such as Cbl-b and GRAIL (Fathman and Lineberry, 2007). Recently, a connection between T cell anergy and Gadd45β was reported. When analyzing the effect of the Notch target gene Deltex1 on T cell physiology, it was found that Deltex1 induced anergy of $CD4^+$ Th cells (Hsiao et al., 2009). Next to induction of the E3 ubiquitin ligase Cbl-b, Deltex1 induced transcriptional activation of the *Gadd45b* gene (Hsiao et al., 2009). It was suggested that the inhibitory effect of Gadd45β on JNK mitogen-activated protein kinase activity mediates T cell anergy in an E3 ubiquitin ligase independent manner. However, a functional role for Gadd45β in T cells anergy has not been directly tested.

As described above, Gadd45β and Gadd45γ are involved in activation of the p38 mitogen-activated protein kinase via a classical kinase cascade. Thus, these two Gadd45 proteins activate MEKK4 (a mitogen-activated protein kinase kinase kinase) in T cells, which activates MKK3 or MKK6 (mitogen-activated protein kinase kinases) leading to the activation of p38. In contrast to Gadd45β and Gadd45γ, Gadd45α has a different role in the regulation of p38 MAPK activity in T cells. The TCR activates p38 by an alternative mechanism that does not involve a classical three-tire kinase cascade. Instead, TCR triggering activates the tyrosine kinase ZAP70 that phosphorylates p38 on tyrosine residue 323 leading to full p38 activation (Salvador et al., 2005b). The importance of this alternative pathway was demonstrated by the generation of knock-in mice harboring a Tyr323Phe mutation, in which activation of p38α MAPK upon TCR stimulation with or without costimulation was abrogated (Jirmanova et al., 2009). Furthermore, T cells with mutated p38α MAPK exhibited reduced RNA synthesis upon T cell activation and secreted less IFN-γ, indicating impaired Th1 responses (Jirmanova et al., 2009). Importantly, Gadd45α inhibited this T cell-specific, alternative p38 activation pathway. Recombinant Gadd45α inhibited the activity of p38 in an *in vitro* kinase assay and this was specific for ZAP70-mediated but not MKK6-mediated p38 activation suggesting that Gadd45α prevents binding of ZAP-70 to p38 (Salvador et al., 2005a). In line with this notion, Gadd45α-deficient T cells displayed constitutive p38 activation (Salvador et al., 2005a). Therefore, the function of Gadd45α seems to be opposing that of Gadd45β and Gadd45γ regarding p38 MAPK activation in T cells.

4.5 Gadd45 Proteins in Autoimmunity and Tumor Immunosurveillance

Gadd45 proteins have been linked to diseases, in which the immune system plays a pivotal role. For instance, Gadd45 proteins have been associated with autoimmunity. As stated in the previous section, Gadd45α-deficient T cells exhibit constitutive p38 MAPK activity (Salvador et al., 2005a), which could indicate an aberrant activation of T cells in these mice.

In line with this hypothesis, Gadd45α-deficient mice spontaneously develop an autoimmune disease that is characterized by the presence of autoantibodies against double-stranded and single-stranded DNA, as well as against histones (Salvador et al., 2002). At nine months of age Gadd45α-deficient mice showed signs of proteinuria and glomerulonephritits. Furthermore, these mice had reduced numbers of leukocytes and lymphocytes in their peripheral blood (Salvador et al., 2002). Interestingly, female rather than male mice were affected, which is similar to systemic lupus erythematosus (SLE) in humans (Tsokos, 2011). Of note, this phenotype was reverted when Gadd45α-deficient mice were crossed to mice harboring a Tyr323Phe mutation in both p38α and p38β, the two isoforms of p38 MAPK expressed in T cells (Jirmanova et al., 2011). This strongly supports the notion that the alternative p38 activation pathway in T cells is regulated by Gadd45α and accounts for the development of autoimmune disease in Gadd45α-deficient mice. In addition, p38 Tyr323Phe double knock-in mice were less susceptible towards the induction of collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE), which are mouse models for rheumatoid arthritis and multiple sclerosis, respectively (Jirmanova et al., 2011). Therefore, the Gadd45α-regulated alternative p38 activation pathway in T cells might contribute to several autoimmune disorders.

Not only Gadd45α, but also Gadd45β and Gadd45γ have been connected to autoimmunity. For instance, Gadd45β-deficient mice showed exacerbated and prolonged clinical symptoms in myelin oligodendrocyte glycoprotein (MOG) peptide-induced EAE (Liu et al., 2005). The differences to wildtype T cells became even more obvious in a transfer EAE model, in which naïve T cells of either wildtype or the knock-out genotype were transferred into immunodeficient recipients. At later time points, Gadd45β-deficient animals showed severe signs of inflammation as shown by IFN-γ expression of $CD4^+$ Th cells and the activation status of microglia cells (Liu et al., 2005). *In vitro*, Gadd45β-deficient T cells proliferated more than wildtype cells and were more resistant towards the induction of apoptosis, which may provide a mechanistic basis for the observed autoimmune phenotype (Liu et al., 2005). In addition, reduced expression of Gadd45β was found in synovial fibroblasts of rheumatoid arthritis patients (Svensson et al., 2009). Overexpression of Gadd45β in these cells resulted in reduced JNK mitogen-activated protein kinase activation and expression of matrix metalloprotease 3, which plays an important role in joint destruction during rheumatoid arthritis. Of note, Gadd45β-deficient mice exhibited increased JNK activity, expression of matrix metalloprotease 3 and 13 as well as joint inflammation, which resulted in a higher clinical scores in this murine model of serum-induced arthritis (Svensson et al., 2009). Further supporting the notion that Gadd45 proteins regulate autoimmunity, is the fact that Gadd45β and Gadd45γ double-deficient mice develop a spontaneous lymphoproliferative disease and splenomegaly (Liu et al., 2005). This was also associated with increased immunoglobulin levels in the serum and deposition of immunoglobulins in glomeruli suggesting a lupus-like autoimmune phenotype.

Next to the suppression of autoimmunity, Gadd45 proteins seem to promote anti-tumor responses. In a study that aimed to increase such responses by immunization with inactivated autoreactive T cells that are thought to promote depletion of such autoreactive cells, it was shown that T cells from immunized mice were more resistant to activation-induced cell death and that this correlated with Gadd45β expression (Wang et al., 2006). Importantly, the growth of the tumor T cell line was inhibited in immunized mice compared to non-immunized mice. Supporting the idea of a Gadd45 protein-aided anti-tumor immune response, tumor growth was enhanced in Gadd45β-deficient mice using a mouse B16 melanoma model (Ju et al., 2009). $CD8^+$ T cells from Gadd45β-deficient mice produced less IFN-γ *in vivo* upon tumor challenge and upon stimulation with IL-12 and IL-18 or via TCR triggering *in vitro*.

Moreover, Gadd45β-deficient $CD8^+$ T cells expressed less T-bet and Eomes upon activation, two transcription factors that are crucial for the development of $CD8^+$ memory T cells (Intlekofer et al., 2005). Most importantly, tumor vaccination failed in mice double deficient for Gadd45β and Gadd45γ (Ju et al., 2009). Taken together, Gadd45 proteins have important functions in tumor immunosurveillance.

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Figure legends

Figure 4.1 Gadd45 proteins in hematopoiesis and function of immune cells. Immune cells develop from pluripotent hematopoietic stem cells (HSC) that give rise to myeloid – granulocytes (yellow) and monocytes/macrophages (light green) - and lymphoid - B cells, NK cells and T cells (blue) - lineages. Dendritic cells (dark green) can develop from both, the myeloid and lymphoid lineage. Progenitor cells are shown in purple. Gadd45 proteins are shown in red next to the cell type, in which they play a crucial role in development or function. CMP, common myeloid progenitor; GMP, granulocyte monocyte progenitor; CLP, common lymphoid progenitor. Certain progenitor cell types and red blood cells are not shown for clarity reasons.

Figure 4.2 Gadd45β and autophagy. Upon starvation or infection with intracellular pathogens, the cell mounts an autophagic response. The autophagosomal membrane (light blue) forms *de novo* and elongates in an LC3-II- (light green) and ATG5-dependent (yellow) manner to engulf protein aggregates, organelles or intracellular pathogens. Subsequently, the autophagosome (light blue) fuses with a lysosome (light red) leading to vesicle acidification and subsequent cargo degradation. Gadd45β (red) and MEKK4 (grey) together direct the p38 mitogen-activated protein kinase (dark green) to the autophagosomal membrane, where it phosphorylates ATG5. This event inhibits maturation of the autophagosome and, thus, blocks autophagy. Gadd45b expression is induced by TCR triggering, by binding of lipopolysaccharide (LPS) to toll-like receptor 4 (TLR4) or by certain cytokines.

Figure 4.3 Gadd45 proteins in T cells. Triggering of the T cell receptor (TCR) activates Src family kinases (SFK) and the zeta chain-associated protein of 70 kDa (ZAP-70). These tyrosine kinases lead to the activation of the transcription factor NF-κB and the p38 mitogen-activated protein kinase. NF-κB induces transcription of the *Gadd45b* gene. The same applies to the cytokines IL-12 and IL-18 as well as stimulation of the Notch receptor and its cytoplasmic effector Deltex. The cytokine IL-2 activates transcription of the *Gadd45g* gene. Both, Gadd45β and Gadd45γ proteins interact with the kinase MEKK4, which leads to sustained p38 activation and, subsequently to interferon-γ (IFN-γ) production and Th1 differentiation. Gadd45α is constitutively expressed in T cells and can inhibit the alternative activation of p38 by ZAP70-mediated tyrosine phosphorylation. Activation of *Gadd45b* by Notch and Deltex may lead to T cell anergy by a yet unknown mechanism.