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GADD45γ Mediates the Activation of the p38 and JNK MAP Kinase Pathways and Cytokine Production in Effector T_H1 Cells

Binfeng Lu,[1,2] Hong Yu,[1,2] Chi-wing Chow,[3] Baiyong Li,[1,2,5] Wei-ping Zheng,[1,6] Roger J. Davis,[3] and Richard A. Flavell[1,2,4]

[1] Section of Immunobiology and
[2] Howard Hughes Medical Institute
Yale University School of Medicine
310 Cedar Street
New Haven, Connecticut 06520
[3] Howard Hughes Medical Institute and
Program in Molecular Medicine
University of Massachusetts Medical School
Worcester, Massachusetts 01605

Summary

The p38 and JNK stress-activated MAPK signal transduction pathways are activated by T cell receptor (TCR) signaling and are required for IFN-γ production by T_H1 effector cells. Here, we show that the expression of GADD45γ is induced during T cell activation and that the level of expression is higher in T_H1 cells than in T_H2 cells. T_H1 cells from *GADD45*$\gamma^{-/-}$ mice are severely compromised in their abilities to activate p38 and JNK in response to TCR signaling, produce much less IFN-γ upon restimulation, and are deficient in activation-induced cell death (AICD). Additionally, GADD45γ deficiencies caused reduced contact hypersensitivity in mice. Thus, GADD45γ mediates activation of the p38 and JNK pathways and effector function of T_H1 cells.

Introduction

GADD45 family proteins are involved in a plethora of biological processes, including the maintenance of genome stability (Fornace et al., 1992). Three members of this gene family have been identified (*GADD45/GADD45*α, *MyD118/GADD45*β, and *CR6/OIG37/GADD45*γ), and the expression of these genes has been implicated as regulators of cell cycle progression and differentiation (Abdollahi et al., 1991; Fan et al., 1999; Fornace et al., 1988; Nakayama et al., 1999; Zhang et al., 1999). GADD45 family proteins are able to interact with a wide spectrum of proteins, such as cdc2, the cyclin-dependent kinase inhibitor p21, PCNA, histone, MEKK4/MTK1, and nuclear hormone receptors (Kearsey et al., 1995; Smith et al., 1994; Takekawa and Saito, 1998; Vairapandi et al., 1996; Yi et al., 2000; Zhan et al., 1999). GADD45α is a ubiquitously expressed mammalian gene that is induced by genotoxic stresses (Smith et al., 1994). It is regulated by many factors that are involved in maintaining genome stability, such as p53 and BRCA1 (Harkin et al., 1999; Smith et al., 1994). The analysis of *GADD45*$\alpha^{-/-}$ mice confirmed its role in maintaining genome stability (Hollander et al., 1999). In contrast, MyD118/GADD45β and GADD45γ/CR6/OIG37 appear to be regulated by signals that are involved in cell differentiation. GADD45β expression was induced following stimulation of $M1D^+$ myeloid precursors by IL-6 (Abdollahi et al., 1991), which caused terminal differentiation and growth arrest in these cells. GADD45γ was induced in a hemangioblastic cell line by Oncostatin, which drives the differentiation of this type of cell (Nakayama et al., 1999). Additionally, GADD45γ was induced by IL-2 in a T cell line, which suggested a role in T cell activation (Beadling et al., 1993). These observations imply that GADD45β and GADD45γ may play important roles in cell differentiation.

One major property of GADD45 family proteins is that overexpression of these proteins can cause growth arrest and apoptosis. This was believed to be due to their abilities to interact with components of the cell cycle machinery, such as cdc2, p21, and PCNA. A recent study proposed an alternative mechanism (Takekawa and Saito, 1998). In this case, GADD45α, GADD45β, and GADD45γ were found to physically interact with the MTK1/MEKK4, an upstream activator of the p38 and JNK MAP kinase pathways (Gerwins et al., 1997; Takekawa et al., 1997). In addition, GADD45 protein can activate both the p38 and JNK pathways when overexpressed in COS-7 cells (Takekawa and Saito, 1998). Since activation of the p38/JNK pathways can cause cell growth arrest and apoptosis (reviewed in Davis, 2000), these authors proposed that some of the effects of GADD45 proteins on cell growth and apoptosis are mediated by activation of p38/JNK pathways.

Studies in this laboratory have established that the p38 and JNK pathways are very important for the responses of T_H1 effector cells (Lu et al., 1999; Rincon et al., 1998; Yang et al., 1998). The p38 kinase can be activated efficiently in T_H1 effector cells but not in T_H2 effector cells; blocking the pathway inhibits and agonists of the pathway potentiate T_H1 responses (Rincon et al., 1998). Likewise, mice deficient in MKK3, a direct upstream activator of the p38 kinase pathway, have impaired type I cytokine immune responses (Lu et al., 1999). Similarly, rapid induction of JNK activity was observed in T_H1 effector cells upon ConA or PMA plus ionomycin but not in T_H2 effector cells. IFN-γ production is impaired in T_H1 cells from JNK2-deficient mice (Yang et al., 1998). Recently, Rac2 was found to be specifically expressed in T_H1 cells, and overexpression of Rac2 in T cells seems to drive T_H1 differentiation (Li et al., 2000). This observation provides insight into a mechanism for the selective activation of p38/JNK pathways in T_H1 cells. However, since IFN-γ production in T_H1 cells is not totally blocked in Rac2-deficient mice, there may be other alternative pathways that are also required for the activation of p38/JNK pathways in T_H1 cells (Li et al., 2000).

In this study, we show that GADD45γ is expressed at a higher level in T_H1 than T_H2 effector cells. *GADD45*$\gamma^{-/-}$ T_H1 effector cells fail to activate the p38/JNK pathways

[4] Correspondence: richard.flavell@yale.edu
[5] Present address: Pfizer, Incorporated, Groton, Connecticut 06340.
[6] Present address: University of Rochester, Center for Vaccine Biology and Immunology, Post Office Box 609, Rochester, New York 14623.

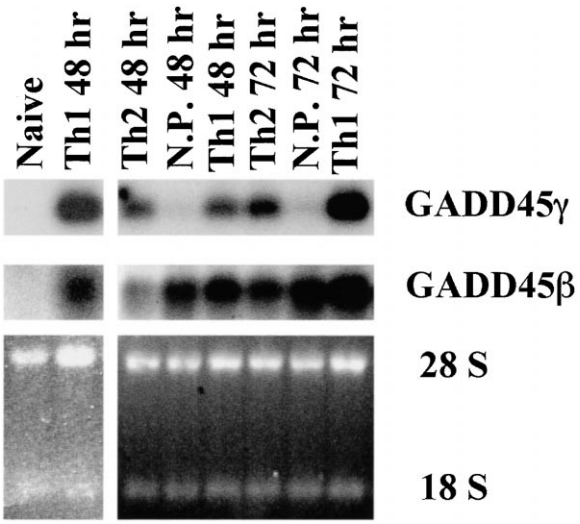

Figure 1. GADD45γ Is Highly Induced in T_H1 Cells

Naive ($CD62L^+CD44^-$) $CD4^+$ T cells (lane 1) were isolated from 8-week-old C57/B6 mice. These cells were then cultured on α-CD3 (10 μg/ml) and α-CD28 (5 μg/ml) coated plates. Cells were cultured under T_H1, T_H2, or no polarizing conditions for 48 and 72 hr.

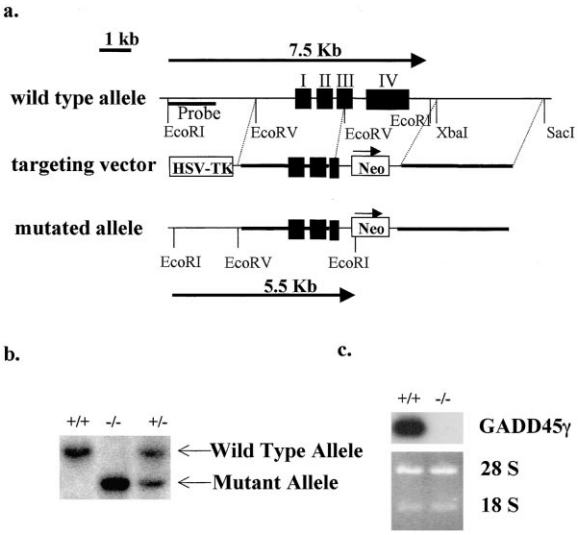

Figure 2. Generation of *GADD45*$\gamma^{-/-}$ Mice

(A) The targeting strategy is illustrated schematically.

(B) Southern analysis of genomic DNA prepared from tails of *GADD45*$\gamma^{+/+}$, *GADD45*$\gamma^{-/-}$, and *GADD45*$\gamma^{+/-}$ mice.

(C) Northern analysis of total RNA prepared from $CD4^+$ T cells that were isolated from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice and cultured under T_H1 conditions for 3 days. The probe was prepared using a full-length GADD45γ cDNA.

in response to TCR stimulation, and this results in compromised IFN-γ production in these cells. Our study provides direct in vivo evidence that GADD45γ mediates activation of the p38 and JNK MAP kinase pathways and the function of T_H1 effector cells.

Results

T_H1 and T_H2 cells can be differentiated in vitro from common naive precursor T cells during the course of a few days. To discover genes that are responsible for the functional differences between T_H1 and T_H2 cells, we performed representational display analysis (RDA) (Hubank and Schatz, 1994) using in vitro-differentiated T_H1 and T_H2 cells (Li et al., 2000; Zheng and Flavell, 1997). GADD45γ was highly represented in the T_H1 cDNA population. Northern blot analysis was performed to examine the kinetics of GADD45γ expression during the early phase of T_H1/T_H2 differentiation. We found that GADD45γ was not expressed in naive $CD4^+$ T cells (Figure 1) but that GADD45γ mRNA was induced during T cell activation (Figure 1). GADD45γ mRNA increased to a similar level in $CD4^+$ T cells cultured in both T_H1 and T_H2 polarizing conditions up to 48 hr after T cell activation was initiated. However, on day 3, GADD45γ message in T_H1 cells was strongly induced and was at least 3-fold higher than that in T_H2 cells (Figure 1). GADD45β expression was also examined. Similarly, GADD45β mRNA was highly induced during T cell activation (Figure 1). GADD45β was also highly expressed in T_H1 versus T_H2 cells (Figure 1). In contrast, GADD45α was expressed at a low level and is not induced during T cell activation (data not shown). Naive $CD4^+$ T cells were also cultured under nonpolarizing conditions for 2 or 3 days, and the expression of GADD45β and GADD45γ in these cells was examined. GADD45β was expressed at a similar level on day 2. On day 3, its level of expression was significantly higher in nonpolarized cells than in T_H2 cells but lower than in T_H1 cells. Thus, the expression of this gene is both upregulated by TCR signaling and IL-12 but repressed by IL-4. Interestingly, GADD45γ was not expressed in cells cultured under nonpolarizing conditions. These data suggest that polarizing cytokines such as IL-4 and IL-12 are required for the expression of GADD45γ in activated T cells. The difference in regulation of GADD45β and GADD45γ during T cell activation suggests that these molecules could play different roles in activated T cells. It is well established that the activity of p38 and JNK MAP kinases can be stimulated to much higher levels in T_H1 effector cells than T_H2 effector cells (Rincon et al., 1998; Yang et al., 1998). Moreover, both p38 and JNK MAP kinases are required for IFN-γ production by T_H1 effector cells (Lu et al., 1999; Rincon et al., 1998; Yang et al., 1998). Interestingly, a recent study of COS-7 cells demonstrated that the expression of the GADD45 family of proteins resulted in strong induction of p38 and JNK activity, mediated by direct interaction between GADD45 proteins and MEKK4/MTK1 (Takekawa and Saito, 1998). We considered, therefore, that the high expression level of GADD45γ mRNA could be an attractive mechanism that might potentiate p38/JNK activation in T_H1 cells.

In order to understand the in vivo role of GADD45γ in $CD4^+$ T cells, GADD45γ-deficient mice were generated. The *GADD45*γ gene is composed of four coding exons (Figure 2A). The targeting construct was designed to delete exon 4 and part of exon 3. Homologous recombination between the targeting construct and the endogenous *GADD45*γ locus results in deletion of sequences that encode amino acids 92–159. This region of GADD45γ has

been shown to be crucial for interaction with MTK-1/MEKK4 (Takekawa and Saito, 1998). Chimeric mice that transmitted the disrupted allele through the germline were generated from three independent embryonic stem cell clones. Southern blot analysis at weaning revealed that offspring of heterozygous parents included the three expected genotypes (Figure 2B) in approximately Mendelian proportions. Both male and female *GADD45*$\gamma^{-/-}$ mice were fertile. We differentiated both wild-type (wt) CD4$^+$ T cells and *GADD45*$\gamma^{-/-}$ CD4$^+$ T cells under T_H1 polarizing conditions with IL-12 for 3 days. RNA was made from these cells, and the GADD45γ mRNA level was examined by Northern blot analysis using the full-length GADD45γ cDNA as a probe. GADD45γ was highly expressed in wild-type CD4$^+$ T cells but was absent in *GADD45*$\gamma^{-/-}$ CD4$^+$ T cells (Figure 2C). These data suggest that the targeting strategy utilized in this study resulted in a null mutation of the *GADD45*γ gene.

To examine if GADD45γ deficiency affected lymphoid development in general, thymocytes, splenocytes, and bone marrow cells from 6- to 8-week-old mice of both wild-type and *GADD45*$\gamma^{-/-}$ genotypes were examined by FACS analysis. Thymocytes were examined for the expression of surface markers such as CD4, CD8, CD44, CD25, and CD3. The expression of surface markers such as CD4, CD8, CD44, CD62L, CD25, B220, IgM, Mac-1, and Gr-1 was assessed on splenocytes. Furthermore, bone marrow cells were also assayed for the surface expression of B220, IgM, CD43, Mac-1, and Gr-1. No significant changes were observed in any of these parameters (data not shown). These data suggest that *GADD45*$\gamma^{-/-}$ is not essential for lymphoid development.

It has been well documented that GADD45 family members can cause growth arrest when overexpressed. In fact, T_H1 cells proliferate at a much lower rate than T_H2 cells. These prior observations prompted us to examine the effect of loss of GADD45γ on the proliferation of T_H1 cells in response to TCR signaling. Wild-type and *GADD45*$\gamma^{-/-}$ CD4$^+$ T cells were polarized under T_H1 conditions for 4 days. The cells were then stimulated with different concentrations of plate-bound α-CD3 antibodies for 24 hr. Proliferation was then measured using [^{3}H]thymidine incorporation. However, *GADD45*$\gamma^{-/-}$ T_H1 effector cells proliferated at a similar, if not a slightly reduced, rate as the wild-type T_H1 effector cells (Figure 3). These data suggest that GADD45γ does not significantly affect the proliferation of T_H1 effector cells.

An earlier study suggested a role for GADD45 family members in the activation of the p38/JNK MAP kinase pathway (Takekawa and Saito, 1998). However, the in vivo significance of this discovery is controversial because p38/JNK activation stimulated by several stress signals precedes the induced expression of the GADD45 family of genes (Shaulian and Karin, 1999; Wang et al., 1999). Since GADD45γ is highly expressed in effector T_H1 cells, we decided to study whether GADD45γ plays any role in the activation of the p38 MAP kinase pathway in these cells. To detect activated p38, we used α-phospho-p38 MAP kinase antibodies. Wild-type and *GADD45*$\gamma^{-/-}$ CD4$^+$ T cells were polarized under T_H1 conditions for 5 days. Loss of GADD45γ expression did not affect the basal level of phosphorylation of p38 MAP kinase in effector T_H1 cells (Figure 3A). These data suggest that high-level expression of the *GADD45*γ gene

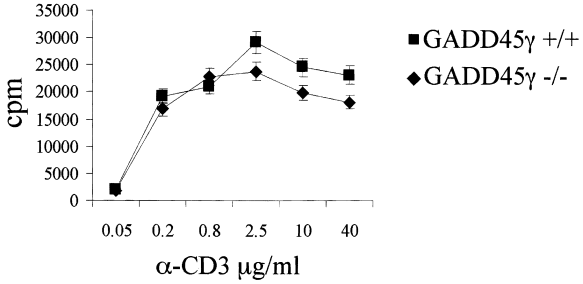

Figure 3. Proliferation of T_H1 Effector Cells Is Not Affected by Mutation of GADD45γ

Naive (CD62L$^+$CD44$^-$) CD4$^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice are cultured under T_H1 conditions for 4 days. These cells were harvested and washed three times. Cells were stimulated with plate-bound α-CD3 at the indicated concentration for 24 hr. Then, cells were pulsed with [^{3}H]thymidine for another 16 hr. Proliferation was assayed by determining the incorporation of [^{3}H]thymidine.

alone does not result in p38 activation. The cells were then restimulated with α-CD3 antibody or α-CD3 plus α-CD28 antibodies for 1 hr. Phosphorylation of the activation sites of p38 MAP kinase was induced in wild-type T_H1 effector cells when these cells were restimulated with α-CD3 or α-CD3 plus α-CD28 antibodies. However, the phosphorylation of these sites was not induced when *GADD45*$\gamma^{-/-}$ T_H1 effector cells were stimulated with α-CD3 or α-CD3 plus α-CD28 antibodies (Figure 4A). In contrast, both ERK isoforms were similarly activated in wild-type and GADD45γ-deficient T cells (Figure 4A). In vitro kinase assays were performed using extracts prepared from similarly cultured T_H1 cells. Consistent with the immunoblot data, p38 kinase activity was induced when T_H1 cells were activated by α-CD3 antibody. However, it was not induced in *GADD45*$\gamma^{-/-}$ T_H1 effector cells (Figure 4B). GADD45γ protein therefore mediates TCR-stimulated p38 MAP kinase activation in T_H1 effector cells. JNK has also been shown to be activated by the overexpression of GADD45 family member proteins (Takekawa and Saito, 1998). To examine whether the JNK pathway was affected by GADD45γ deficiency, in vitro kinase assays were carried out using the same extracts used for p38 kinase assay. While JNK activity was induced in wild-type T_H1 effector cells upon α-CD3 antibody treatment, it was not induced in *GADD45*$\gamma^{-/-}$ T_H1 effector cells (Figure 4B). Therefore, GADD45γ is required for both p38 and JNK MAP kinase activation through TCR stimulation in T_H1 effector cells. A dose-dependent study of p38 activation was also carried out (Figure 4C). p38 is activated in wild-type T_H1 effector cells, even when a small dose of α-CD3 antibody was used. Activation of p38 was increased in proportion to increasing amounts of α-CD3 antibody. In contrast, p38 was activated in *GADD45*$\gamma^{-/-}$ T_H1 only when large amounts of α-CD3 antibody was used. These data suggest that GADD45γ controls the threshold of p38 activation in effector T_H1 cells. Since GADD45γ is not expressed in cells cultured under nonpolarizing conditions, we examined whether GADD45γ deficiency affected p38 activation in these cells. Interestingly, TCR stimulation by using α-CD3 antibody was still able to activate p38 in these

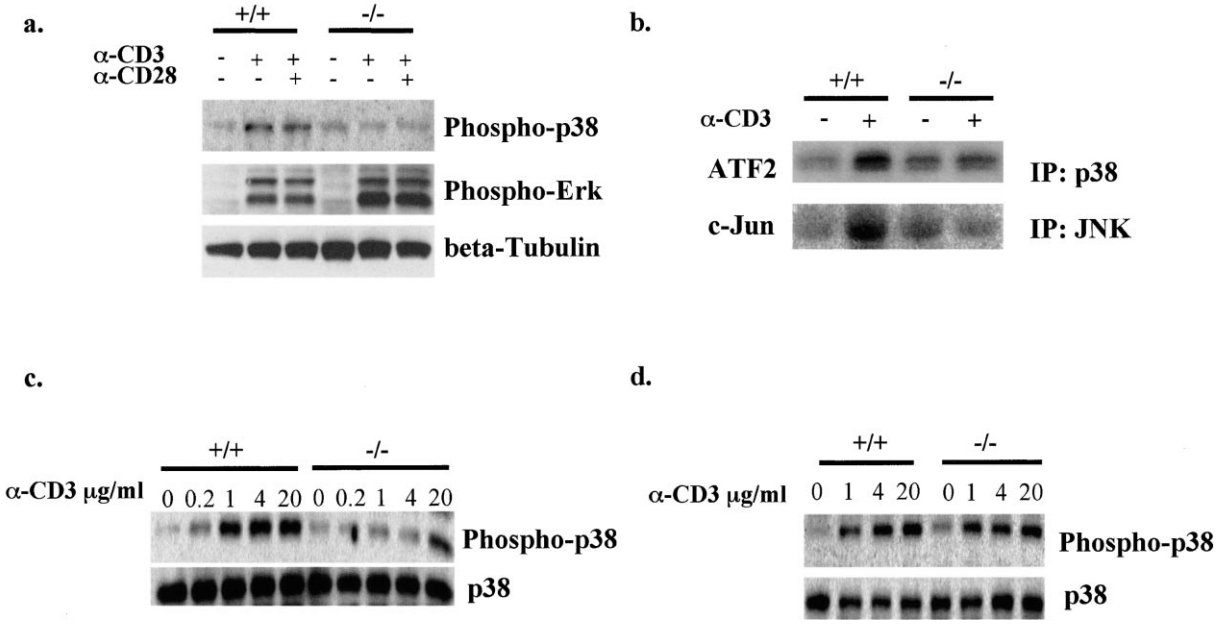

Figure 4. JNK and p38 MAP Kinase Activation in T_H1 Effector Cells Is Dependent on GADD45γ

(A) Naive ($CD62L^+CD44^-$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under T_H1 conditions for 5 days. These cells were harvested and washed three times. Cells were stimulated with plate-bound α-CD3 (10 μg/ml) or α-CD3 (10 μg/ml) and α-CD28 (5 μg/ml) for 1 hr. The activated forms of p38 and ERK were detected by immunoblot analysis using phospho-specific antibodies.

(B) Naive ($CD62L^+CD44^-$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under T_H1 conditions for 5 days. These cells were harvested and washed three times and were stimulated with plate-bound α-CD3 (10 μg/ml) for 1 hr. JNK and p38 MAP kinase activity was measured using the substrates c-Jun and ATF2, respectively.

(C) Naive ($CD62L^+CD44^-$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under T_H1 conditions for 4 days. These cells were harvested and washed three times. The cells were stimulated with plate-bound α-CD3 for 15 min. The activated form of p38 was detected by immunoblot analysis using phospho-specific antibodies.

(D) Naive ($CD62L^+CD44^-$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under the nonpolarizing condition for 4 days. These cells were harvested and washed three times. The cells were stimulated with plate-bound α-CD3 for 15 min. The activated form of p38 was detected by immunoblot analysis using phospho-specific antibodies.

nonpolarized cells independent of GADD45γ (Figure 4D). These data suggest that there is a GADD45γ-independent pathway that mediates TCR signaling to the p38 MAP kinase pathway in cells cultured under nonpolarizing conditions.

GADD45 family proteins are known to cause apoptosis in cells when highly expressed. Interestingly, T_H1 effector cells are more susceptible to activation-induced cell death (AICD) (Zhang et al., 1997). We suspected that GADD45γ might play a role in AICD of T_H1 effector cells. To test this hypothesis, we generated T_H1 effector cells by priming naive $CD4^+$ T cells for 4 days with α-CD3 and α-CD28 antibodies and restimulated these effectors with plate-bound α-CD3 antibodies for 8 hr and 24 hr. Apoptosis was measured by staining cells with Annexin V and propidium iodide (PI). A significant reduction in AICD was observed upon treatment with low levels of α-CD3 at both time points (Figures 5A and 5B). Therefore, GADD45γ is at least partially responsible for AICD in T_H1 effector cells. Apoptosis at 8 hr was also examined using the TUNEL assay. A significantly lower number of apoptotic cells was detected on two doses of α-CD3-stimulated GADD45γ-deficient cells than in wild-type cells (Figure 5C). Similar results were obtained with T_H1 effector cells derived from ConA-primed naive $CD4^+$ T cells (data not shown).

Since GADD45γ was highly expressed in T_H1 cells and the p38 and JNK pathways are key to T_H1 function, we examined if GADD45γ deficiency affected T_H1 effector function. Naive $CD4^+$ T cells were isolated from both wild-type and *GADD45*$\gamma^{-/-}$ mice. These cells were cultured with α-CD3 and α-CD28 antibodies under T_H1 and T_H2 conditions for 5 days. Cells were then restimulated with plate-bound α-CD3 antibody. Culture supernatant obtained following overnight stimulation was harvested and assayed for cytokine production. Wild-type $CD4^+$ T cells differentiated under T_H2 polarizing conditions produced a large amount of IL-4 upon restimulation (Figure 6A). Under T_H2 conditions, *GADD45*$\gamma^{-/-}$ $CD4^+$ T cells produced slightly reduced amounts of IL-4 upon restimulation. In contrast, when compared with wild-type $CD4^+$ T cells cultured under T_H1 polarizing conditions, IFN-γ secreted by *GADD45*$\gamma^{-/-}$ $CD4^+$ T cells cultured under the same conditions was significantly reduced (Figure 6B). The reduction in IFN-γ secretion was the result of reduced expression of IFN-γ mRNA by *GADD45*$\gamma^{-/-}$ $CD4^+$ T cells (Figure 6C). In contrast, levels of TNF-α and TNF-β (Figure 6C) and IL-2 (data not shown) were not significantly changed. We also obtained Th1 effector cells using a second condition in which naive $CD4^+$ T cells were primed under T_H1 conditions with ConA. Four days later, these cells were stimulated with α-CD3 antibody

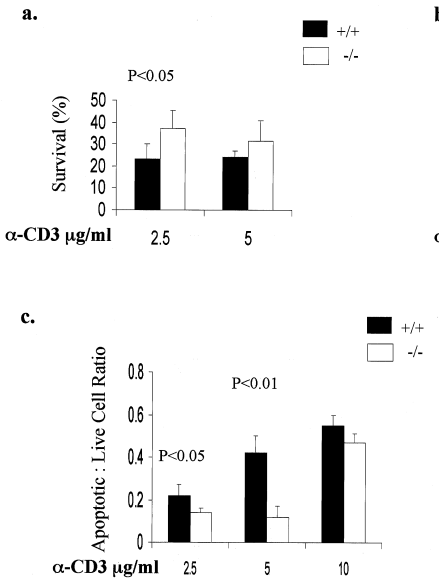

Figure 5. GADD45γ Deficiency Causes Reduction of Activation-Induced Cell Death

Naive ($CD62L^+CD44^-$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under the T_H1 condition for 5 days. These cells were harvested and washed three times and were stimulated with plate-bound α-CD3 for 8 hr (A) and 24 hr (B). Then, cells were stained with Annexin V and propidium iodide and were analyzed by FACS. Live cells are Annexin V and propidium iodide double-negative cells. The results were the average of three independent experiments, and each was from a different pair of *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice. Error bars are based on the standard deviation of the data set. TUNEL assay was also carried out, and live cells and TUNEL-positive cells were counted, and the ratio was an average from three fields. The result is representative of three independent experiments. Error bars are based on the standard deviation of the data set.

for 24 hr. Again, knockout T_H1 effector cells produced significantly less IFN-γ than wild-type T_H1 cells (Figure 6D). Together, these data demonstrate that GADD45γ is critical for the function of T_H1 cells in general and IFN-γ expression specifically.

Since in vitro T_H1 responses were dependent on GADD45γ, we wished to examine whether this requirement was reflected in the in vivo T_H1 immune response. Contact hypersensitivity (CHS) represents the prototype of delayed-type hypersensitivity and is a T_H1 cell-mediated cutaneous immune/inflammatory reaction to haptens (Eisen et al., 1952). Mice were sensitized with dinitrofluorobenzene (DNFB) and challenged 5 days later. Ear-swelling responses to DNFB were significantly depressed in *GADD45*$\gamma^{-/-}$ mice as compared with wt mice (Figure 7). Therefore, GADD45γ is important for this type of cell-mediated immune response in vivo.

Discussion

Previous studies established that the p38 and JNK MAP kinase pathways are selectively induced in T_H1 effector cells and are required for T_H1 immune responses in general and IFN-γ production specifically in $CD4^+$ T cells (Rincon et al., 2000; Yang et al., 1998). Another study demonstrated that GADD45 family proteins are sufficient for the activation of the p38 and JNK MAP kinase pathways

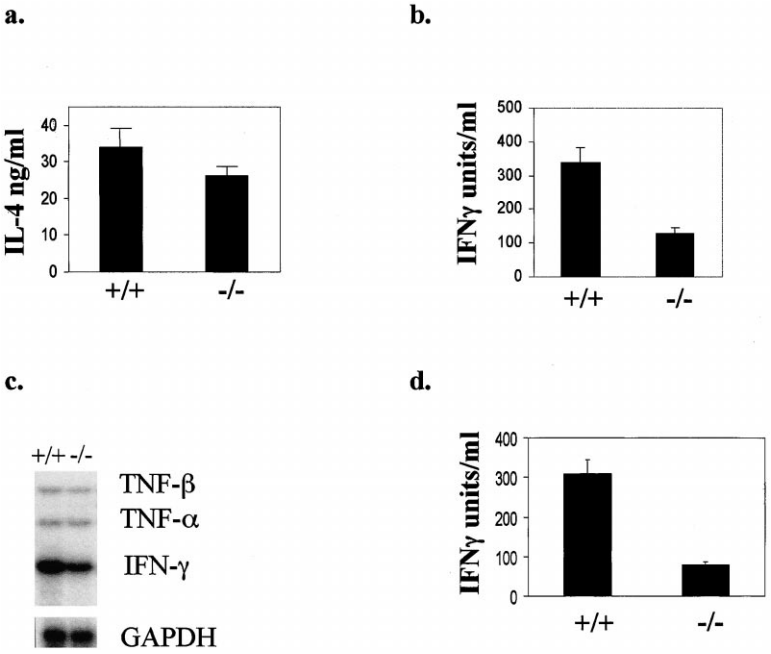

Figure 6. GADD45γ Deficiency Compromises IFN-γ Production by T_H1 Cells

Naive ($CD62L^+CD44^-$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under T_H1 and T_H2 conditions with α-CD3 and α-CD28 antibodies for 5 days. These cells were harvested and washed three times and were stimulated with plate-bound α-CD3 (5 μg/ml) overnight. (A) Enzyme-linked immunosorbent assay (ELISA) analysis of IL-4 production by *GADD45*$\gamma^{+/+}$ and *GADD45*$\gamma^{-/-}$ T_H2 cells. The data are representative of four independent experiments. (B) IFN-γ production by *GADD45*$\gamma^{+/+}$ and *GADD45*$\gamma^{-/-}$ T_H1 cells was measured by ELISA. The data are representative of four independent experiments. (C) RNase protection assay (RPA) of IFN-γ mRNA in *GADD45*$\gamma^{+/+}$ and *GADD45*$\gamma^{-/-}$ T_H1 cells. (D) Naive ($CD62L^+CD44^+$) $CD4^+$ T cells from either *GADD45*$\gamma^{+/+}$ or *GADD45*$\gamma^{-/-}$ mice were cultured under T_H1 conditions with APC and ConA for 4 days. These cells were harvested, washed three times, and were stimulated with plate-bound α-CD3 (5 μg/ml) overnight. IFN-γ production by *GADD45*$\gamma^{+/+}$ and *GADD45*$\gamma^{-/-}$ T_H1 cells was measured by ELISA. The data are representative of three independent experiments.

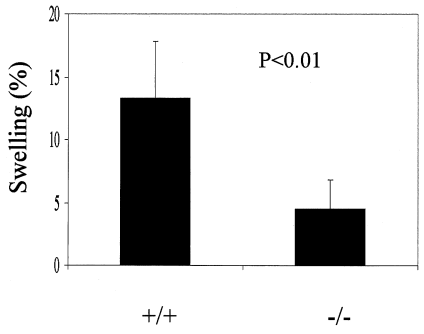

Figure 7. GADD45γ Deficiency Alters Contact Hypersensitivity in Mice

Four 8-week-old female wild-type and four 8-week-old female *GADD45*$\gamma^{-/-}$ mice were used in the CHS, as described in Experimental Procedures. The statistical significance of differences between the means was determined by applying a Student's t test. Error bars are based on the standard deviation of the data set.

through a novel direct interaction between GADD45 family proteins and MEKK4/MTK1 in COS-7 cells (Takekawa and Saito, 1998). Our observation that GADD45β and GADD45γ are highly expressed in T_H1 effector cells merges these two findings and provides an attractive model that high-level expression of GADD45 proteins may be responsible for selective activation of the p38 and JNK MAP kinase pathways in T_H1 effector cells. Indeed, deletion of GADD45γ in mice drastically reduced the TCR-induced activation of the p38 and JNK MAP kinase pathways in T_H1 effector cells. Additionally, deletion of GADD45γ also affects IFN-γ production in T_H1 effector cells. This work provides a mechanism for selective activation of the p38 and JNK MAP kinase pathways in T_H1 effector cells. Moreover, it has resolved a highly disputed issue in GADD45 biology, which is whether GADD45 is required for activation of the p38 and JNK MAP kinase pathways in vivo (Shaulian and Karin, 1999; Wang et al., 1999).

It is interesting that, although both GADD45β and GADD45γ are highly expressed in T_H1 cells compared to T_H2 cells, GADD45γ deficiency was able to significantly reduce TCR-induced activation of the p38 and JNK MAP kinase pathways. This suggests that GADD45β and GADD45γ proteins are either involved in different signaling pathways that lead to p38/JNK activation or that they act in a complex in which both proteins are essential. Because GADD45 family proteins are very similar in their primary sequences and they are functionally interchangeable in many in vitro assays, it is also possible that certain threshold levels of GADD45 family protein must be achieved for efficient activation of the p38 and JNK MAP kinase pathways by TCR activation. Deletion of GADD45γ might reduce the total level of GADD45 family proteins below this threshold. Since their expression seems to be regulated by different stimuli, they are also expected to play distinct roles in immune responses. This notion is consistent with our data that under nonpolarizing conditions, where GADD45β but not GADD45γ is expressed, TCR-stimulated p38 MAP kinase activation is normal. It is likely GADD45β plays an important role in activating p38 MAP kinase pathway in these cells.

An important function of GADD45 family proteins suggested by many studies is cell cycle control, especially the control of G2 cell cycle progression (Fan et al., 1999; Zhan et al., 1999). GADD45α deficiency in mice causes defects in G2/M checkpoint activation in response to UV irradiation (Hollander et al., 1999). Mouse embryonic fibroblasts (MEF) isolated from these mice demonstrated genome instability during long-term culture (Hollander et al., 1999). In addition, *GADD45*$\alpha^{-/-}$ mice also develop thymus hyperplasia. These phenotypes of *GADD45*$\alpha^{-/-}$ mice are likely the result of defects in G2/M checkpoint control in response to genotoxic stimuli. The phenotypes of *GADD45*$\alpha^{-/-}$ mice are consistent with the activation of GADD45α by p53 and BRCA1 (Harkin et al., 1999; Smith et al., 1994) and the ability of GADD45α to affect the cell cycle by interacting with cdc2, p21, and PCNA (Smith et al., 1994; Vairapandi et al., 1996). Gadd45γ was also shown to interact with p21 and PCNA and negatively regulate cell growth (Nakayama et al., 1999). However, we did not observe significant differences in the cell cycle between wild-type and *GADD45*$\gamma^{-/-}$ T_H1 cells. No thymic abnormality was observed in *GADD45*$\gamma^{-/-}$ mice. It was recently reported that *p21*$^{-/-}$ adult mice accumulate abnormal numbers of memory $CD4^+$ T cells (Balomenos et al., 2000). However, we did not detect a significant difference in memory $CD4^+$ T cells between adult wild-type and *GADD45*$\gamma^{-/-}$ mice. The differences in the phenotype of *GADD45*$\alpha^{-/-}$ and *GADD45*$\gamma^{-/-}$ mice are consistent with the fact that their expression is differentially regulated (i.e., GADD45α is induced in response to genotoxic stimuli, while GADD45γ is induced during T cell activation or by cytokines).

One property of T_H1 cells that differs dramatically from T_H2 cells is that they are more susceptible to AICD (Zhang et al., 1997). It has been shown in many cell contexts that overexpression of a GADD45 family member is able to induced apoptosis. Indeed, we observed reduced AICD in *GADD45*$\gamma^{-/-}$ T_H1 cells. Therefore, GADD45γ plays a role in AICD in T_H1 cells.

During the revision of this manuscript, an interesting report was published, which demonstrated that GADD45β is required for cytokine-induced but not TCR-induced IFN-γ production in T_H1 cells (Yang et al., 2001). In our present study, however, we show that GADD45γ does mediate TCR-induced IFN-γ production. The report of Yang et al. (Yang et al., 2001) focused on the study of the effect of overexpression of GADD45β or blocking this pathway using dominant-negative MEKK4 in resting T_H1 cells, a developmental stage at which GADD45β and GADD45γ have decreased from the high levels of T_H1 effector cells to negligible levels. Using this particular stage of T_H1 cells, Yang et al. also showed that p38 inhibitor (SB203580) blocked IL-12- and IL-18-induced IFN-γ production but was not able to block TCR-induced IFN-γ production in T_H1 cells (Yang et al., 2001). These results apparently contradict our previous studies which demonstrated that p38 inhibitors did block TCR-induced IFN-γ production in effector T_H1 cells (Rincon et al., 1998). However, we think this discrepancy is due to differences in the T_H1 cells used in these studies. Since Yang et al. examined the effect of p38 inhibitor on IFN-γ production by resting T_H1 cells, where neither GADD45β nor GADD45γ was expressed, whereas our study examined T_H1 effectors, which ex-

press high levels of GADD45β and GADD45γ, the interpretation of the result obtained by Yang et al. and its generalized application to T_H1 cells should be qualified. Our present report, like our prior study, focused on the role of p38 MAP kinase pathway in IFN-γ production by effector T_H1 cells where both GADD45β and GADD45γ are highly expressed (Lu et al., 1999; Rincon et al., 1998). Our data show that, when GADD45 family proteins are present, the TCR-stimulated p38 MAP kinase pathway has a significant effect on the production of IFN-γ. However, when GADD45 family proteins are absent, we believe that the p38 MAP kinase pathway is activated in an inefficient fashion and therefore has a less potent effect on IFN-γ production. This is a possible explanation why Yang et al. did not observe inhibition of IFN-γ production by resting T_H1 cells using a p38 inhibitor, while we observed significant inhibition of effector T_H1 cells by both p38 inhibitor and a dominant-negative p38 transgene (Rincon et al., 1998). Like GADD45β (Yang et al., 2001), GADD45γ appears to mediate cytokine-driven IFN-γ production, since wild-type T_H1 cells stimulated with IL-12 and IL-18 make significantly more (3- to 4-fold) IFN-γ than GADD45γ-deficient T_H1 cells (data not shown). It appears that GADD45γ mutation caused a similar reduction of IFN-γ production in T_H1 cells stimulated by TCR ligation or by stimulation with the cytokines IL-12 plus IL-18.

We have demonstrated that deletion of GADD45γ compromises the ability of T_H1 cells to activate p38/JNK in response to TCR signaling. Therefore, GADD45γ and possibly other stress cytokine-induced molecules are required to generate fully functional effector T_H1 cells. These molecules are therefore potential targets of therapeutic intervention.

Experimental Procedures

Targeting Strategy

The *GADD45γ* gene was isolated from a 129/SvE mouse genomic library in Lambda FIX II, using a full-length cDNA probe. The targeting vector was constructed using a thymidine kinase cassette, a 3.5 kb EcoRV gene fragment, a Neo^R cassette, and a 4.5 kb XbaI and SacI gene fragment (Figure 2A). This vector was designed to replace two coding exons of the *GADD45γ* gene with the Neo^R cassette. The vector was electroporated into SW9.5 mouse embryonic stem (ES) cells, and drug-resistant clones were screened by Southern blot analysis of EcoR1-restricted genomic DNA using a 1 kb EcoRI/XhoI genomic fragment as a probe. Three independent correctly targeted ES cell clones were injected into C57BL/6 blastocysts, and germline transmission was obtained from each clone.

T Cell Culture

$CD4^+$ T cells were prepared as described (Zheng and Flavell, 1997). Naive ($CD62l^+CD44^{low}$) cells were sorted on a Becton Dickinson FACStar plus. Two ways of generating T_H1 effector cells were used. First, naive $CD4^+$ T cells were cultured on 24-well plates precoated with α-CD3 (10 μg/ml) and α-CD28 (5 μg/ml), IL-12 (1.5 ng/ml), human IL-2 (20 units/ml), and α-IL4 (clone 11B11) for 4–5 days (Lu et al., 1998). These cells were then washed and restimulated with plate-bound α-CD3 antibodies (5 μg/ml) for various amounts of time. Second, naive $CD4^+$ T cells (5×10^5/ml) and APC (5×10^5/ml) were cultured on 24-well plates with ConA (2 μg/ml), IL-12 (1.5 ng/ml), human IL-2 (20 units/ml), and α-IL4 (clone 11B11) for 4 days to generate effector T_H1 cells. These cells were then washed and restimulated with plate-bound α-CD3 antibodies (5 μg/ml) for various amounts of time. To prepare APCs, spleen cells were treated with mAb Y19, TIB105, GK1.5, and HB191 plus rabbit complement to remove T cells and NK cells, and then were irradiated with 3000 rad. To culture $CD4^+$ T cells under nonpolarizing conditions, naive $CD4^+$ cells were cultured on 24-well plates precoated with α-CD3 (10 μg/ml), α-CD28 (5 μg/ml), and human IL-2 (20 units/ml) for 4–5 days.

Protein Kinase Assays

JNK and p38 MAP kinase activity was measured using an in vitro protein kinase assay using the substrates c-Jun and ATF2, respectively (Diehl et al., 2000; Dong et al., 2000).

Western Blot

For each condition, 10 μg of extracts was used. α-phospho-p38 and α-phospho-ERK antibodies were from New England Biolabs (catalog number 9211S and 9101S, respectively). Western blot was carried out following the manufacturer's instructions.

TUNEL Assay

The ApopTag kit from Intergen was used with a protocol provided by the manufacturer (www.intergenco.com/body_apoptag_index.html).

RNase Protection Assay

Total RNA was isolated from T_H1 cells using TRIzol reagent (GIBCO-BRL). RPA was performed using 5 μg of total RNA under conditions suggested by the manufacturer. The RiboQuant RPA kit was purchased from PharMingen. Probe set mCK-3b was used to detect cytokine expression.

Assay for CHS

Induction of CHS was conducted using the methods described previously (Simon et al., 1990). The shaved mouse abdomen skin was painted with 25 μl of 0.5% DNFB in acetone/olive oil (4:1). Five days later, mice were challenged with 10 μl of 0.2% DNFB on each side of the ear. The ear thickness was measured at 24 hr after challenge. Results were expressed as percentage of ear swelling, which was calculated by subtracting the thickness of ear before treatment from the thickness of the hapten-challenged ear and dividing by the thickness of ear before treatment.

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References

Abdollahi, A., Lord, K.A., Hoffman-Liebermann, B., and Liebermann, D.A. (1991). Sequence and expression of a cDNA encoding MyD118: a novel myeloid differentiation primary response gene induced by multiple cytokines. Oncogene *6*, 165–167.

Balomenos, D., Martin-Caballero, J., Garcia, M.I., Prieto, I., Flores, J.M., Serrano, M., and Martinez, A.C. (2000). The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development. Nat. Med. *6*, 171–176.

Beadling, C., Johnson, K.W., and Smith, K.A. (1993). Isolation of interleukin 2-induced immediate-early genes. Proc. Natl. Acad. Sci. USA *90*, 2719–2723.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases (Review). Cell *103*, 239–252.

Diehl, N.L., Enslen, H., Fortner, K.A., Merritt, C., Stetson, N., Charland, C., Flavell, R.A., Davis, R.J., and Rincon, M. (2000). Activation of the p38 mitogen-activated protein kinase pathway arrests cell cycle progression and differentiation of immature thymocytes in vivo. J. Exp. Med. *191*, 321–334.

Dong, C., Yang, D.D., Tournier, C., Whitmarsh, A.J., Xu, J., Davis, R.J., and Flavell, R.A. (2000). JNK is required for effector T-cell function but not for T-cell activation. Nature *405*, 91–94.

Eisen, H.N., Orris, L., and Belman, S. (1952). Elicitation of delayed allergic skin reactions with haptens: the dependence of elicitation on hapten combination with protein. J. Exp. Med. *95*, 473.

Fan, W., Richter, G., Cereseto, A., Beadling, C., and Smith, K.A. (1999). Cytokine response gene 6 induces p21 and regulates both cell growth and arrest. Oncogene *18*, 6573–6582.

Fornace, A.J., Jr., Alamo, I., Jr., and Hollander, M.C. (1988). DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. USA *85*, 8800–8804.

Fornace, A.J., Jr., Jackman, J., Hollander, M.C., Hoffman-Liebermann, B., and Liebermann, D.A. (1992). Genotoxic-stress-response genes and growth-arrest genes. gadd, MyD, and other genes induced by treatments eliciting growth arrest. Ann. NY Acad. Sci. *663*, 139–153.

Gerwins, P., Blank, J.L., and Johnson, G.L. (1997). Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-Jun amino terminal kinase pathway. J. Biol. Chem. *272*, 8288–8295.

Harkin, D.P., Bean, J.M., Miklos, D., Song, Y.H., Truong, V.B., Englert, C., Christians, F.C., Ellisen, L.W., Maheswaran, S., Oliner, J.D., et al. (1999). Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell *97*, 575–586.

Hollander, M.C., Sheikh, M.S., Bulavin, D.V., Lundgren, K., Augeri-Henmueller, L., Shehee, R., Molinaro, T.A., Kim, K.E., Tolosa, E., Ashwell, J.D., et al. (1999). Genomic instability in Gadd45a-deficient mice. Nat. Genet. *23*, 176–184.

Hubank, M., and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. *22*, 5640–5648.

Kearsey, J.M., Coates, P.J., Prescott, A.R., Warbrick, E., and Hall, P.A. (1995). Gadd45 is a nuclear cell cycle regulated protein which interacts with p21Cip1. Oncogene *11*, 1675–1683.

Li, B., Yu, H., Zheng, W., Voll, R., Na, S., Roberts, A.W., Williams, D.A., Davis, R.J., Ghosh, S., and Flavell, R.A. (2000). Role of the guanosine triphosphatase rac2 in T helper 1 cell differentiation. Science *288*, 2219–2222.

Lu, B., Ebensperger, C., Dembic, Z., Wang, Y., Kvatyuk, M., Lu, T., Coffman, R.L., Pestka, S., and Rothman, P.B. (1998). Targeted disruption of the interferon-gamma receptor 2 gene results in severe immune defects in mice. Proc. Natl. Acad. Sci. USA *95*, 8233–8238.

Lu, H.T., Yang, D.D., Wysk, M., Gatti, E., Mellman, I., Davis, R.J., and Flavell, R.A. (1999). Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. EMBO J. *18*, 1845–1857.

Nakayama, K., Hara, T., Hibi, M., Hirano, T., and Miyajima, A. (1999). A novel oncostatin M-inducible gene OIG37 forms a gene family with MyD118 and GADD45 and negatively regulates cell growth. J. Biol. Chem. *274*, 24766–24772.

Rincon, M., Enslen, H., Raingeaud, J., Recht, M., Zapton, T., Su, M.S., Penix, L.A., Davis, R.J., and Flavell, R.A. (1998). Interferon-gamma expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. EMBO J. *17*, 2817–2829.

Rincon, M., Conze, D., Weiss, L., Diehl, N.L., Fortner, K.A., Yang, D., Flavell, R.A., Enslen, H., Whitmarsh, A., and Davis, R.J. (2000). Conference highlight: do T cells care about the mitogen-activated protein kinase signalling pathways? Immunol. Cell. Biol. *78*, 166–175.

Shaulian, E., and Karin, M. (1999). Stress-induced JNK activation is independent of Gadd45 induction. J. Biol. Chem. *274*, 29595–29598.

Simon, J.C., Cruz, P.D., Bergstresser, P.R., and Tigelaar, R.E. (1990). Low dose ultraviolet B-irradiated Langerhans cells preferentially activate CD4+ cells of the T helper 2 subset. J. Immunol. *145*, 2087–2091.

Smith, M.L., Chen, I.T., Zhan, Q., Bae, I., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M., and Fornace, A.J., Jr. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science *266*, 1376–1380.

Takekawa, M., and Saito, H. (1998). A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell *95*, 521–530.

Takekawa, M., Posas, F., and Saito, H. (1997). A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. EMBO J. *16*, 4973–4982.

Vairapandi, M., Balliet, A.G., Fornace, A.J., Jr., Hoffman, B., and Liebermann, D.A. (1996). The differentiation primary response gene MyD118, related to GADD45, encodes for a nuclear protein which interacts with PCNA and p21WAF1/CIP1. Oncogene *12*, 2579–2594.

Wang, X., Gorospe, M., and Holbrook, N.J. (1999). gadd45 is not required for activation of c-Jun N-terminal kinase or p38 during acute stress. J. Biol. Chem. *274*, 29599–29602.

Yang, D.D., Conze, D., Whitmarsh, A.J., Barrett, T., Davis, R.J., Rincon, M., and Flavell, R.A. (1998). Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity *9*, 575–585.

Yang, J., Zhu, H., Murphy, T.L., Ouyang, W., and Murphy, K.M. (2001). IL-18-stimulated GADD45beta required in cytokine-induced, but not TCR-induced, IFN-gamma production. Nat. Immunol. *2*, 157–164.

Yi, Y.W., Kim, D., Jung, N., Hong, S.S., Lee, H.S., and Bae, I. (2000). Gadd45 family proteins are coactivators of nuclear hormone receptors. Biochem. Biophys. Res. Commun. *272*, 193–198.

Zhan, Q., Antinore, M.J., Wang, X.W., Carrier, F., Smith, M.L., Harris, C.C., and Fornace, A.J., Jr. (1999). Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene *18*, 2892–2900.

Zhang, X., Brunner, T., Carter, L., Dutton, R.W., Rogers, P., Bradley, L., Sato, T., Reed, J.C., Green, D., and Swain, S.L. (1997). Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. J. Exp. Med. *185*, 1837–1849.

Zhang, W., Bae, I., Krishnaraju, K., Azam, N., Fan, W., Smith, K., Hoffman, B., and Liebermann, D.A. (1999). CR6: a third member in the MyD118 and Gadd45 gene family which functions in negative growth control. Oncogene *18*, 4899–4907.

Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587–596.