

# Regulation of the expression and processing of caspase-12

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Phylogenetic analysis clusters caspase-12 with the inflammatory caspases 1 and 11. We analyzed the expression of caspase-12 in mouse embryos, adult organs, and different cell types and tested the effect of interferons (IFNs) and other proinflammatory stimuli. Constitutive expression of the caspase-12 protein was restricted to certain cell types, such as epithelial cells, primary fibroblasts, and L929 fibrosarcoma cells. In fibroblasts and B16/B16 melanoma cells, caspase-12 expression is stimulated by IFN- $\gamma$  but not by IFN- $\alpha$  or - $\beta$ . The effect is increased further when IFN- $\gamma$  is combined with TNF, lipopolysaccharide (LPS), or dsRNA.

These stimuli also induce caspase-1 and -11 but inhibit the expression of caspase-3 and -9. In contrast to caspase-1 and -11, no caspase-12 protein was detected in macrophages in any of these treatments. Transient overexpression of full-length caspase-12 leads to proteolytic processing of the enzyme and apoptosis. Similar processing occurs in TNF-, LPS-, Fas ligand-, and thapsigargin (Tg)-induced apoptosis. However, B16/B16 melanoma cells die when treated with the ER stress-inducing agent Tg whether they express caspase-12 or not.

## Introduction

Caspases are a family of cysteinyl aspartate proteinases central in mediating cellular signals in apoptosis and inflammation (Lamkanfi et al., 2003). The enzymes are produced as zymogens consisting of a NH<sub>2</sub>-terminal prodomain of variable length followed by a large and a small catalytic subunit. The large prodomains of certain members of this protein family, referred to as initiator caspases (e.g., caspase-8, -9, and -10), contain interaction motifs consisting of six or seven antiparallel amphipathic  $\alpha$  helices. These prodomains were shown to allow the recruitment and proximity-induced activation of the enzymes in protein complexes (Stennicke and Salvesen, 2000). Caspases are usually activated by proteolysis that leads to conformational changes of their catalytic parts (Riedl et al., 2001). The mature caspase is a heterotetramer consisting of two large and two small catalytic subunits aligned in a head-to-tail configuration and contains an active site in each of the opposite ends. Caspases typically signal through proteolytic cascades in which initiator caspases cleave and activate executioner caspases that lack a large prodomain (Slee et al., 1999; Van de Craen et al., 1999; Chang

and Yang, 2000). The final outcome of these cascades is the specific proteolysis of a wide variety of substrates, leading either to the loss of a life-saving function or the activation of a proapoptotic or proinflammatory function (Chang and Yang, 2000; Lamkanfi et al., 2003).

So far, 11 human and 10 murine caspases were identified (Chang and Yang, 2000; Lamkanfi et al., 2002). Phylogenetic analysis reveals that caspases segregate into three major groups with different functions and substrate specificities. The three groups are often referred to as inflammatory caspases, apoptotic executioner caspases, and apoptotic initiator caspases (Lamkanfi et al., 2002). Caspase-12 segregates with the inflammatory caspases including caspase-1, -4, -5, and -11 (Chang and Yang, 2000; Lamkanfi et al., 2002). The amino acid sequence identity of murine caspase-12 to the other members of this group is as follows: murine caspase-1 (42%), human caspase-1 (40%), murine caspase-11 (41%), human caspase-4 (48%), and caspase-5 (45%). These caspases seem to be involved more in the maturation and secretion of proinflammatory cytokines such as interleukin (IL)-1 $\beta$  and IL-18 than in the induction of death receptor-mediated apoptosis (Martinon et al., 2002). All of the caspases included in the group have a large prodomain containing a typical caspase

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Abbreviations used in this paper: FasL, Fas ligand; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; Tg, thapsigargin.

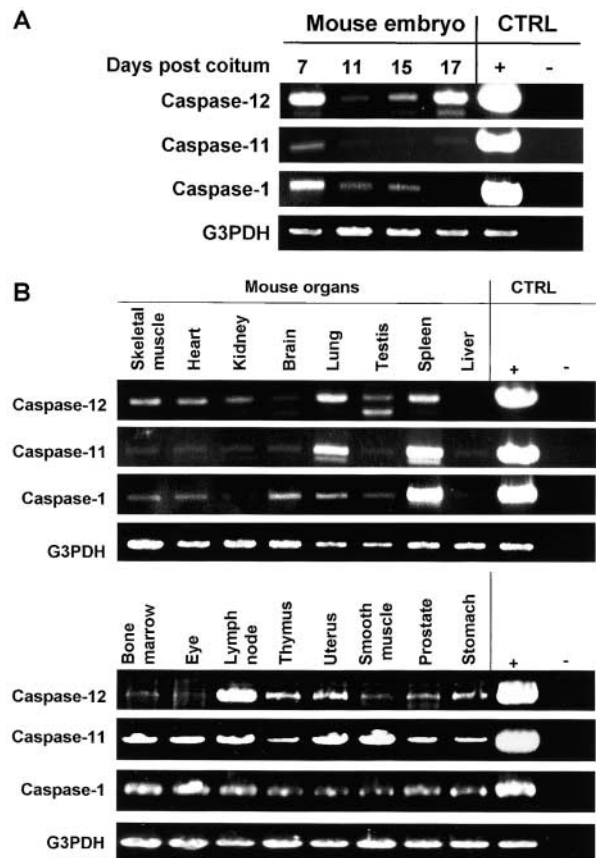
recruitment domain (CARD). The genes encoding the murine members of the group are located on chromosome 9 in the following order: caspase-1, caspase-11, and caspase-12. The genes encoding their human counterparts reside on chromosome 11q22, a region with synteny to murine chromosome 9, and are arranged as follows from telomere to centromere: caspase-1, caspase-5, caspase-4, and finally a gene encoding a pseudo caspase-12 (Fischer et al., 2002). The predicted product of the latter gene is reported to be a caspase-12–like protein with a premature stop and several point mutations preventing the translated protein from having any caspase-like enzymatic activity (Fischer et al., 2002). Studies in caspase-12–deficient mice suggested that the protein plays a major role in ER stress–induced apoptosis and in the development of Alzheimer’s disease (Nakagawa et al., 2000). Since then, several other reports have linked processing of caspase-12 to ER stress–induced apoptosis (Rao et al., 2001, 2002; Diaz-Horta et al., 2002; Morishima et al., 2002). In addition, caspase-12 seems to be involved in apoptosis induced by viral infection (Bitko and Barik, 2001; Jordan et al., 2002) or by serum starvation (Kilic et al., 2002).

Several reports have shown that the expression of certain caspases is induced during inflammation. Treatment with interferon (IFN)- $\gamma$  increases the expression of caspase-8 in many types of cells, thus sensitizing the cells to death domain receptor–mediated apoptosis (Dai and Krantz, 1999; Ruiz-Ruiz et al., 2000; Fulda and Debatin, 2002). Both IFN- $\gamma$  and lipopolysaccharide (LPS) induce the expression of human caspase-1, human caspase-5, and murine caspase-11 but not of human caspase-4 (Tamura et al., 1996; Dai and Krantz, 1999; Lin et al., 2000; Hur et al., 2001; Schauvliege et al., 2002). Although phylogenetic analysis suggests that caspase-12 resembles other inflammatory caspases, it is still unclear whether the regulation and processing of this caspase is modulated in inflammation. Therefore, we analyzed the constitutive expression pattern of caspase-1, -11, and -12 in mouse organs and during embryonic development. We also tested the effect of IFNs and other proinflammatory stimuli on the expression of caspase-12 in several cell lines both at the mRNA and protein levels. We compared these effects with those observed with other caspases. In addition we tested the effect of TNF-, Fas ligand (FasL)-, LPS-, and thapsigargin (Tg)-induced apoptosis on the processing state of caspase-12.

## Results

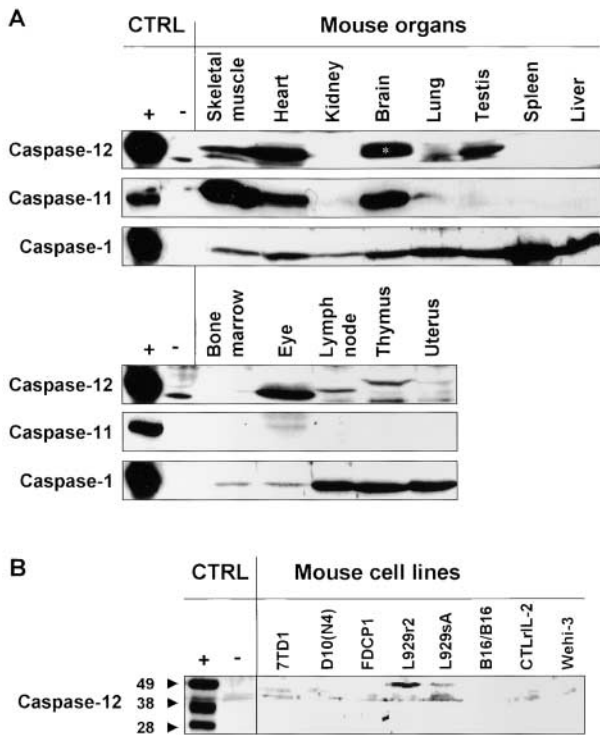
### Analysis of the expression of caspase-12 during mouse embryonic development and in mouse organs and cell lines

A previous analysis of the expression pattern of caspase-12 in newborn mouse organs demonstrated that the enzyme is found in many tissues and suggested that the protein is widely expressed in a variety of cells (Nakagawa and Yuan, 2000). However, the Northern blot analysis presented in our previous report demonstrated that the mRNA of caspase-12 is mainly expressed in the lungs and skeletal muscle (Van de Craen et al., 1997). In an attempt to resolve this apparent discrepancy, we analyzed the expression pattern of



**Figure 1. Differential gene expression patterns of the mouse inflammatory caspase subfamily.** Single-stranded cDNAs from multiple mouse tissues (panel I and II) were amplified by PCR using primers specific for caspase-1, -11, or -12. PCR for glyceraldehyde-3-phosphate dehydrogenase (G3DPH) was performed to ensure that an equal quality and quantity of cDNA was used. Negative (–) and positive (+) PCR controls (CTRL) lacking or containing the specific cDNAs, respectively. (A) Expression of caspase-1, -11, and -12 mRNA during mouse embryonic development. (B) Expression of caspase-1, -11, and -12 mRNA in different mouse organs.

caspase-12 by RT-PCR during embryonic development and in different adult mouse organs and by Western blotting in different cell lines (Figs. 1 and 2). As caspase-12 is most similar to caspase-1 and -11, we compared the constitutive expression pattern of these three caspases. The mRNAs of the three proteins were highly expressed at day 7 of embryonic development. All three caspases showed a drop in expression at day 11, but whereas the mRNA of caspase-1 disappeared by day 17, the expression levels of caspase-11 and -12 mRNAs increased gradually and reached the initial levels again by that day (Fig. 1 A). The highest level of caspase-12 mRNA expression in adult mouse organs was observed in lymph node, lung, and spleen (Fig. 1 B), confirming our previous results (Van de Craen et al., 1997). The lowest caspase-12 mRNA expression levels were detected in brain, bone marrow, eye, and liver (Fig. 1 B). Interestingly, a double band appeared, for example, in the testis, suggesting the existence of a splice variant. Indeed, splice variants of murine caspase-12 and human pseudo caspase-12 were reported before (Van de Craen et al., 1997; Fischer et al.,



**Figure 2. Western blot analysis of the expression of caspase-12 protein in lysates of different mouse organs and cell lines.**

(A) Expression of caspase-1, -11, and -12 in adult mouse organs using anti-caspase-12 antiserum (Ab-1). \*, antiserum G149 failed to detect caspase-12 expression in the brain (not depicted).

(B) Analysis of the expression of caspase-12 protein in mouse cell lines representing different cell lineages. The cell lines used were 7TD1 (mouse/rat B cell hybridoma), D10(N1) T cell lymphoma, FDCP1 (promyelocytoma), L929r2 and L929sA (fibrosarcoma), B16/B16 (melanoma), CTLrIL-2 (cytotoxic T cells), and WEHI-3 (myelomonocytoma). Lysates of HEK293T cells transfected (+) or not (-) with a cDNA encoding for the corresponding caspase were used as positive and negative controls (CTRL), respectively. Arrows indicate bands corresponding to full-length (49 kD) or processed (38 and 28 kD) caspase-12.

2002). Caspase-11 mRNA was observed in all analyzed organs, though the expression levels in the lung, spleen, uterus, and smooth muscle were clearly the highest (Fig. 1 B). Caspase-1 mRNA expression was detected in all of the organs, with the lowest level in the liver and the kidney and the highest in the spleen.

Using Western blot analysis, we checked the expression of caspase-1, -11, and -12 at the protein level in lysates of several organs from 5-wk-old mice (Fig. 2 A). The expression pattern of caspase-1 at the protein level corresponded with the RT-PCR results in Fig. 1 B. However, for caspase-12 and caspase-11, this correlation between protein and mRNA expression levels was not obvious (Fig. 2). Expression of caspase-12 protein was highest in skeletal muscle, heart, brain, testis, and eye (Fig. 2 A). However, no, or hardly any, caspase-12 protein was detected in the other organs tested. Although high caspase-12 mRNA expression levels were detected in the lymph node and the spleen, only very low amounts of the protein could be detected in any of the hematopoietic organs tested. Caspase-11 expression was pre-

dominant in skeletal muscle followed by the heart and the brain (Fig. 2 A).

Next, we analyzed, by Western blotting, extracts from seven murine cell lines representing different cell types (B cell hybridoma, T cells, preB cells, fibrosarcoma, melanoma, and promyelocytes). A control lysate of HEK293T cells transiently transfected with a plasmid leading to overexpression of caspase-12 revealed the 49-kD band expected for full-length pro-caspase-12 and two additional bands running at 38- and 28-kD, respectively (Fig. 2 B). Caspase-12 protein was detected only in cells derived from the murine fibrosarcoma cell line L929 (Fig. 2 B). In agreement with the apparent absence of caspase-12 in lymphoid organs, no caspase-12 protein was detected in cell lines of hematopoietic origin. The strongest expression was detected in L929r2, the cells from which the cDNA of caspase-12 was originally cloned (Van de Craen et al., 1997). Analysis of 13.5-d-post-coitum mouse embryos by immunohistochemistry revealed that the most positively stained organ is the heart (Fig. 3, A and B). In several other organs, including the lung and the nose, the expression of the protein was confined to certain cell types or layers, such as epithelium of the trachea and the nose trail (Fig. 3, C and D). These results suggest that the constitutive expression of caspase-12 is probably restricted to certain cell types.

### IFN- $\gamma$ induces the expression of caspase-12 in mouse fibrosarcoma and melanoma cells: synergy with other proinflammatory stimuli

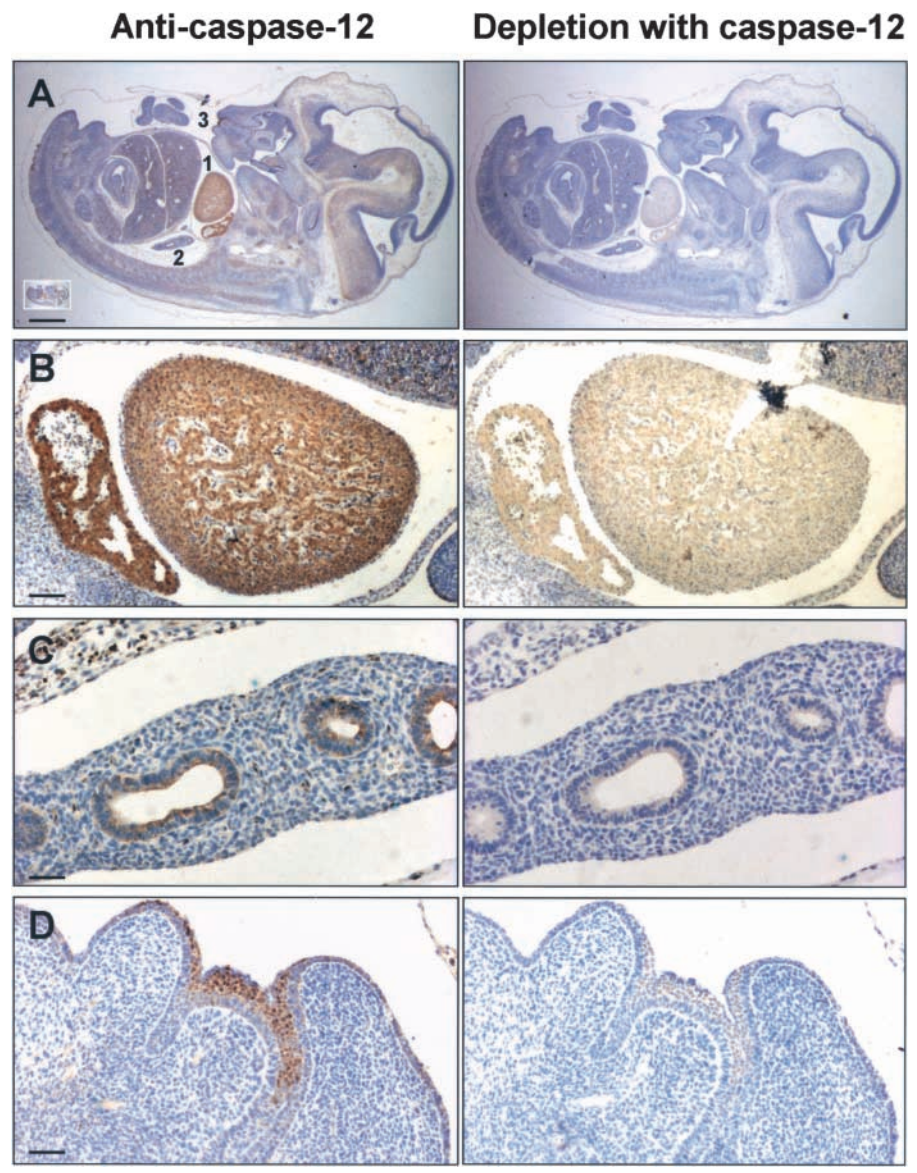
Several reports have demonstrated that the expression of caspase-1 and -11 can be induced by proinflammatory stimuli such as IFN- $\gamma$ , TNF, or LPS (Lin et al., 2000; Schauliege et al., 2002). The L929r2 murine fibrosarcoma and B16/B16 murine melanoma cells respond to TNF only when cotreated with IFN- $\gamma$  (Mareel et al., 1988; Vanhaesebroeck et al., 1991). Therefore, we compared the effect of IFN- $\gamma$  with and without TNF on the expression levels of caspase-1, -11, and -12 in these two cell lines. Northern blot analysis demonstrated that the expression of caspase-12 mRNA is further enhanced in L929r2 and induced in B16/B16 cells by IFN- $\gamma$  (Fig. 4). Synergism between TNF and IFN- $\gamma$  was observed with caspase-12 mRNA in B16/B16 cells and with caspase-11 mRNA in both L929r2 and B16/B16 cells. Such an effect was not observed for caspase-1 and -12 in L929r2 cells. Treatment of L929r2 cells for 24 h with IFN- $\gamma$  and TNF leads to cell death, about half of the cells are propidium iodide positive at 24 h, whereas B16/B16 cells still survive at that time point. This may explain the apparent difference observed in the response of these cell lines. An increase in caspase-1 mRNA was observed only in L929r2 treated with IFN- $\gamma$ , whereas no caspase-1 mRNA expression was observed in B16/B16 cells in any of the treatments.

Using Western blot analysis, we determined if the induction of caspase-12 mRNA expression in B16/B16 cells was also reflected at the protein level and compared the effect with that observed with other caspases (Fig. 5). Treatment with IFN- $\gamma$  increased the expression of caspase-1, -11, and -12. When the treatment with IFN- $\gamma$  was combined with



### Figure 3. Immunohistochemical analysis of the expression of caspase-12 in the mouse embryo.

Caspase-12 expression was detected in sections using either anti-caspase-12 rabbit antiserum Ab-1 or Ab-2. Results obtained with both antisera are similar (not depicted). Only those obtained with Ab-2 are presented. Negative controls of adjacent sections were stained by the anti-caspase-12 antisera depleted with recombinant caspase-12. Sections stained by secondary antibody alone were completely negative (not depicted). (A) Sections of a 13.5-d-postcoitum embryo. Note the strong staining of heart, lung, and nose. White box shows the embryo at its relative real size (6 mm). (B) A close-up of the heart (1) in A. (C) A close-up of the lung (2) in A. Note the positive staining of the epithelial layer of the trachea. (D) A close-up of the nose (3) in A. Note the positive staining of the epithelium in the entrance of the nose. Bars: (A) 455  $\mu\text{m}$ ; (B) 80  $\mu\text{m}$ ; (C) 30  $\mu\text{m}$ ; (D) 45  $\mu\text{m}$ .

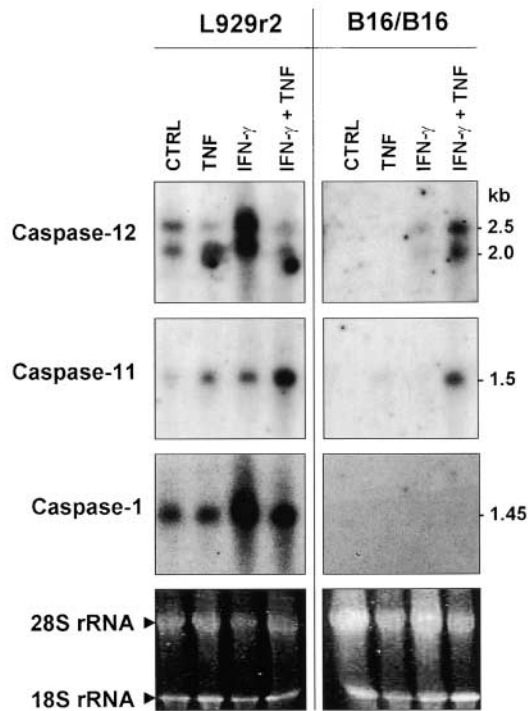


either TNF or LPS, the effect on the expression of caspase-1, -11, and -12 was intensified (Fig. 5; Fig. 6 A). In contrast to the caspases mentioned above, IFN- $\gamma$  treatment led to a decrease in the expression levels of caspase-3 and -9. The disappearance of the full-length form of these caspases (Fig. 5) in B16/B16 cells was not due to proteolytic processing because no proteolytic fragments of caspase-3 and -9 were detected. The antisera used reveal specific proteolytic processing of caspase-3 and -9 during apoptosis (Fig. 8). As expected, IFN- $\gamma$  also increased the expression of the IFN-inducible dsRNA-activated protein kinase (PKR), which we used as a positive control (Baier et al., 1993). No effect was observed on the level of  $\beta$ -actin used as internal negative control. Treatment with either TNF or LPS alone did not affect the expression levels of any of the proteins. We observed a similar effect of IFN- $\gamma$  with and without TNF or LPS on caspase-12 expression in another murine melanoma cell line (PG19; unpublished data). These results suggest that the activation of the gene coding for caspase-12 is tightly regulated. Interestingly, the distinct effects on the

expression of the different caspases intensified in time, suggesting that the expression of caspase-1, -11, and -12 was induced, whereas that of the proapoptotic caspases 3 and 9 was suppressed (Fig. 5).

#### The expression of caspase-12 is not induced by type I IFNs

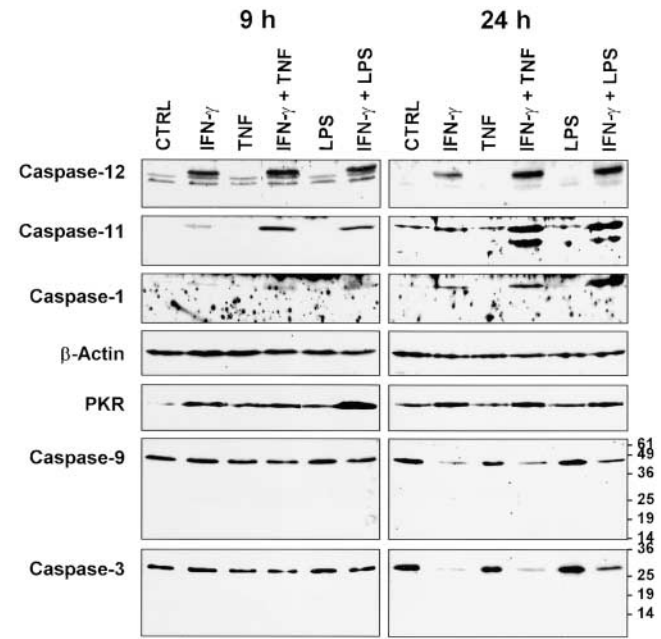
Next, we tested whether the effect on caspase-12 expression is specific for IFN- $\gamma$  or whether it also occurs with IFN- $\alpha$  or - $\beta$ . Both IFN- $\alpha$  and IFN- $\beta$  failed to induce the expression of caspase-12. In contrast with the results obtained with IFN- $\gamma$ , only a very faint caspase-12 signal could be detected in Western blots of lysates of B16/B16 cells treated with the combinations of type I IFNs with either TNF or LPS (Fig. 6 A). However, in cells treated with any one of the IFNs, the expression level of caspase-3 was reduced (Fig. 6 A), confirming the results presented in Fig. 5. All three IFNs induced the expression of PKR, demonstrating that the failure of type I IFNs to induce the expression of caspase-12 did not result from an incapacity of the cells to respond to these cy-



**Figure 4. Effect of IFN- $\gamma$  and TNF on the expression of the mRNAs of the mouse inflammatory caspase subfamily in L929r2 fibrosarcoma and B16/B16 melanoma cells.** Cells were treated or not with IFN- $\gamma$  (1,000 U/ml) or TNF (5,000 U/ml) alone or in combination. The next day, mRNA was extracted and analyzed by Northern blotting using specific probes. The size of the hybridizing bands is indicated in kb. UV imaging of ethidium bromide intercalated in the 18S and 28S rRNAs was used to monitor loading of the gels (bottom).

tokines. IFNs are known inducers of antiviral response. Because caspase-12 was implicated in the response to a viral infection (Bitko and Barik, 2001; Jordan et al., 2002), we wondered if dsRNA, a typical virus by-product, would further modulate caspase-12 expression in the presence of IFN. Interestingly, IFN- $\gamma$ , but not IFN- $\beta$ , which is the antiviral IFN per se, increased the expression of caspase-12 in synergy with dsRNA (Fig. 6 B).

Macrophages play a major role in the response to both viral and bacterial infection and thus present a cellular model that is highly relevant for inflammation (Stoy, 2001). Moreover, macrophages express caspase-1 and -11 and secrete activated IL-1 $\beta$  in response to infection and proinflammatory stimuli (Lin et al., 2000; Schavvliege et al., 2002). Therefore, we determined the effects of the three IFNs alone and of IFN- $\gamma$  in combination with LPS, TNF, or dsRNA on the protein expression levels of caspase-1, -11, and -12 in the Mf4/4 murine macrophage cell line. IFN- $\gamma$ , and to a lesser extent also LPS and IFN- $\beta$ , clearly induced the expression of caspase-11. The expression of caspase-1 remained high and stable (Fig. 7 A). However, no caspase-12-specific bands were detected in Mf4/4 cells in any of the tested conditions (Fig. 7 A). Similar results were obtained with J774 and pU518, two other murine macrophage cell lines (unpublished data) and primary peritoneal macrophages (Fig. 7 B). The nonspecific bands detected in Fig. 7 A appeared after a long exposure of the blot to the film. Similar bands were de-



**Figure 5. Analysis of the expression of caspase-12 and other caspases at the protein level in B16/B16 cells treated with IFN- $\gamma$ , TNF, and LPS.** Western blot analysis of lysates of B16/B16 cells treated for 9 or 24 h with IFN- $\gamma$  (1,000 U/ml), TNF (5,000 U/ml), or LPS (1  $\mu$ g/ml) alone or in combinations, using antisera specific for the indicated murine caspases. Blots were revealed also with an anti- $\beta$ -actin antibody to verify that equal amounts of protein were loaded. The IFN-inducible kinase PKR was used as a positive control, demonstrating responsiveness to IFN. CTRL indicates untreated controls.

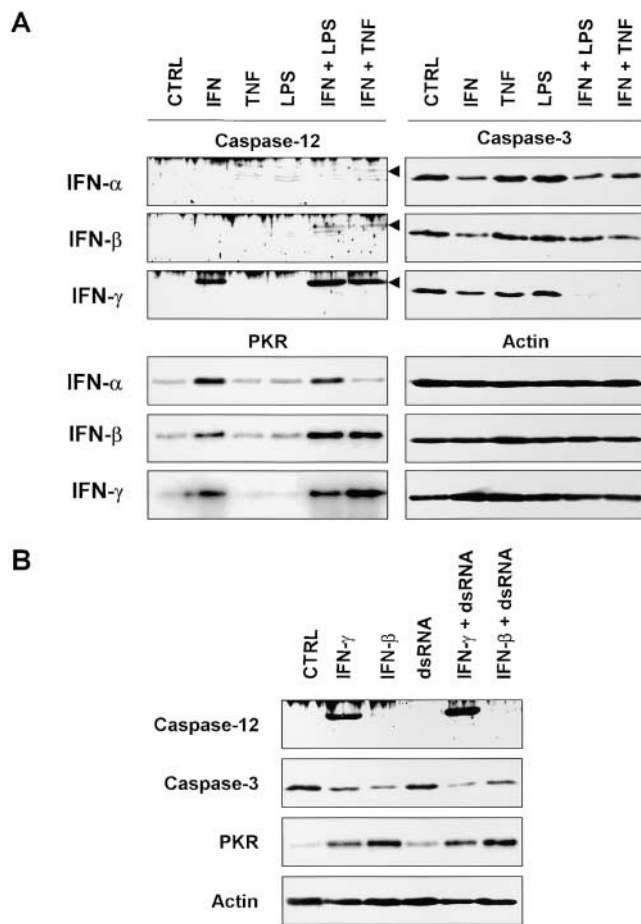
tected by the G149 antiserum in HEK293T cells that do not express murine caspase-12. Moreover, these bands were not detected using two other distinct anti-caspase-12 antisera (unpublished data).

Untreated primary fibroblasts, like the L929 fibrosarcoma cells, constitutively expressed caspase-12 (Fig. 7 B). Treatment of primary fibroblasts with IFN- $\gamma$  strongly enhanced the expression of the protein, and addition of either LPS or TNF even intensified the effect further (Fig. 7 B). These results demonstrate that the expression of caspase-12, like that of the proinflammatory caspases 1 and 11, can be induced in cells exposed to IFN- $\gamma$ . However, the response might be restricted to certain cell types, for example fibroblasts and melanocytes, because macrophages that constitutively express caspase-1 and can be induced to produce caspase-11 apparently fail to express caspase-12.

### Caspase-12 is processed in apoptotic cell death induced by FasL, TNF, or LPS

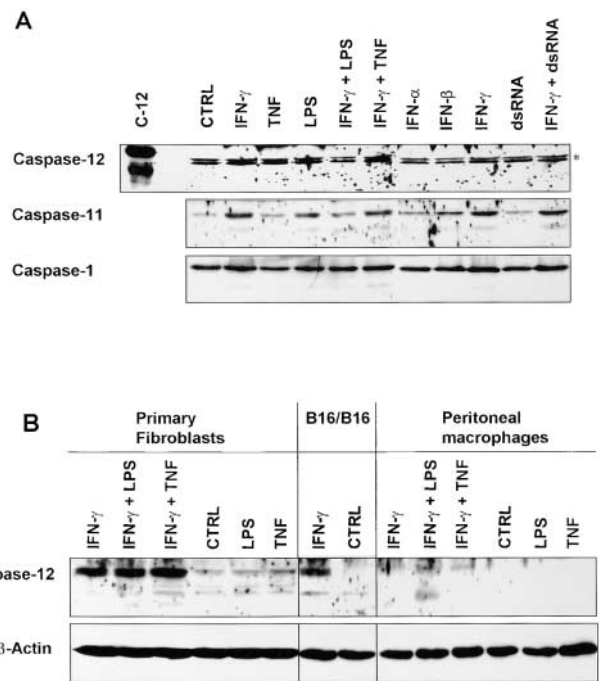
Transient overexpression of full-length wild-type caspase-12, but not of its inactive C298A mutant, in HEK293T cells led to processing of the protein and to apoptosis (Fig. 2 B; Fig. 8 A; Fig. 9 B). Processing of caspase-12 in these conditions resulted in fragments of  $\sim$ 38 and 28 kD. These results suggest that a link exists between the processing of caspase-12 and the induction of an apoptotic cell death. In rapid apoptosis occurring in L929sAhFas cells treated with FasL, caspase-12 was fully processed, and only the 28-kD fragment





**Figure 6. Comparison of the effect of type I and type II IFNs on the expression of caspase-12 and -3 by B16/B16 cells.** (A) Western blot analysis of cells treated for 24 h with IFN- $\alpha$ , - $\beta$ , or - $\gamma$  (1,000 IU/ml) in the presence or absence of TNF (5,000 IU/ml) or LPS (1  $\mu$ g/ml). (B) Western blot analysis of cells treated for 24 h with IFN- $\beta$  or - $\gamma$  (1,000 IU/ml) in the presence or absence of dsRNA (100  $\mu$ g/ml). The IFN-inducible kinase PKR was used as a positive control for responsiveness to the different IFNs. An anti- $\beta$ -actin antibody was used to verify that equal amounts of protein were loaded.

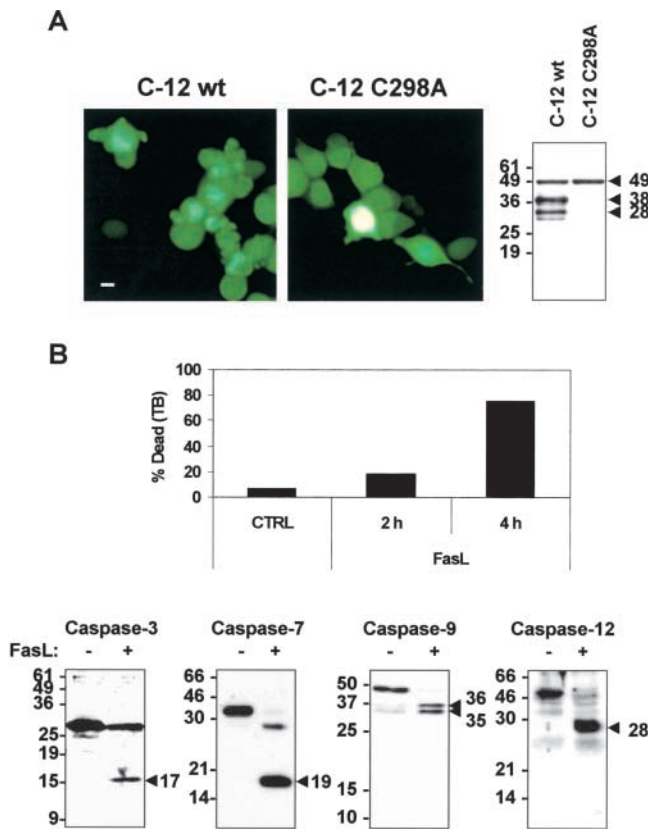
was observed (Fig. 8 B). The processing of caspase-12 in the latter cells coincided with the activation of caspase-3, -7, and -9 (Fig. 8 B). Treatment of B16/B16 cells with TNF alone had no effect on their growth, whereas treatment with either IFN- $\gamma$  or LPS alone led to a decrease in the proliferation rate without any apparent signs of cell death (Fig. 9 A). However, light microscopy analysis demonstrated that cells treated with a combination of IFN- $\gamma$  with LPS or TNF rounded up and started floating and eventually died and disintegrated (Fig. 9 A; unpublished data). We tested whether caspase-12 is also processed in this kind of cell death. As seen before (Figs. 5 and 6), the protein was highly expressed in all the conditions that included IFN- $\gamma$ . However, processing of caspase-12 was detected only when the cells were treated with the combination of IFN- $\gamma$  with either LPS or TNF (Fig. 9 B). These results show that caspase-12 is processed in different proapoptotic conditions, including those induced by TNF, LPS, and FasL, and suggest that it may actively contribute to the cell death process.



**Figure 7. Analysis of the expression of caspase-12 in macrophages and primary fibroblasts.** (A) Western blot analysis of Mf4/4 macrophages treated for 24 h with IFN- $\alpha$ , - $\beta$  or, - $\gamma$  (1,000 IU/ml) in the presence or absence of TNF (5,000 IU/ml), LPS (1  $\mu$ g/ml), or dsRNA (50  $\mu$ g/ml). C-12 indicates a lysate of HEK293T cells overexpressing caspase-12, used as a positive control. \*, nonspecific bands. (B) Western blot analysis of primary fibroblasts and peritoneal macrophages treated for 24 h with IFN- $\gamma$  (1,000 IU/ml) in the presence or absence of TNF (5,000 IU/ml) or LPS (1  $\mu$ g/ml). B16/B16 cells treated or not with IFN- $\gamma$  (1,000 IU/ml) were used as a positive control. An anti- $\beta$ -actin antibody was used to verify that equal amounts of protein were loaded.

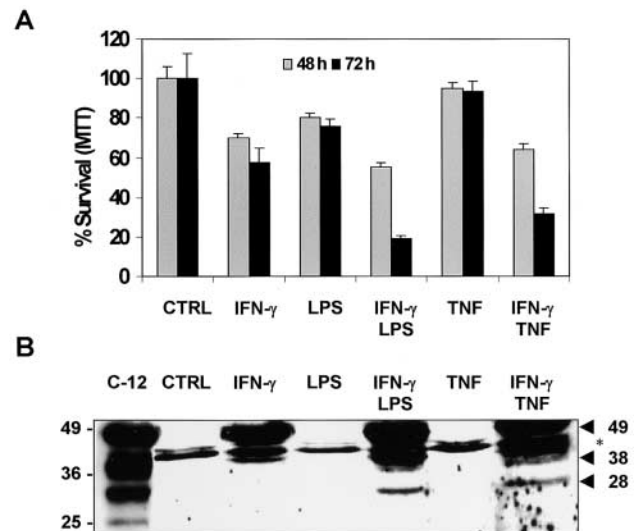
### Caspase-12 is dispensable for Tg-induced ER stress-mediated apoptosis

Several reports have proposed that caspase-12 plays a major role in ER stress-induced apoptosis (Rao et al., 2001, 2002; Diaz-Horta et al., 2002; Morishima et al., 2002). As B16/B16 cells express caspase-12 only when they are treated with IFN- $\gamma$ , we used these cells to test if caspase-12 is required for ER stress-mediated apoptosis induced by Tg. B16/B16 cells died in response to Tg in the presence or absence IFN- $\gamma$  (Fig. 10 A). Light microscopy analysis revealed that in both cases, cells dying in response to Tg were blebbing and had the typical apoptotic morphology similar to that seen with HEK293T overexpressing caspase-12 (Fig. 8 A). Although IFN- $\gamma$  treatment clearly sensitized the cells to TNF-induced cytotoxicity (Fig. 10 A), the effect of IFN- $\gamma$  on Tg-induced cell death was minor. To analyze the expression and processing pattern of caspase-12 in response to Tg and compare it with that observed in IFN- $\gamma$  + TNF-treated cells, we used two different anti-caspase-12 antisera. The first antiserum, Ab-2, was raised against a peptide spanning residues 2–17 in the amino acid sequence of caspase-12 and thus recognized fragments containing the prodomain of the protein, and the second antiserum, G149, was raised against the catalytic parts of the enzyme (Fig. 10 B). Both antisera detected the



**Figure 8. Processing of caspase-12 in apoptosis induced by overexpression of the full-length protein in HEK293T cells and in L929sAhFas cells treated with FasL.** (A) HEK293T cells were transiently cotransfected with a plasmid encoding for a nuclear localization signal containing GFP and a plasmid encoding for either the wild type (C-12 wt) or an inactive caspase-12 mutant (C-12 C298A). Fluorescent microscopy photographs and Western blot analysis demonstrate that although both of the caspase-12 variants are expressed in equal levels, only the wild-type protein is processed and leads to apoptotic cell death. Bar, 5  $\mu$ m. Arrows indicate fragments corresponding to caspase-12 and their size in kD. (B) L929sAhFas cells were treated with recombinant soluble FasL for the indicated time. Cytotoxicity was measured by trypan blue (TB) exclusion. CTRL indicates untreated controls. Processing of caspase-3, -7, -9, and -12 after a 2-h treatment with FasL was determined by Western blotting. Arrows indicate the proteolytically processed caspase fragments and their size in kD.

full-length 49-kD protein in cells treated with IFN- $\gamma$  but failed to detect the expression of caspase-12 in cells treated with Tg alone (Fig. 10 C). The level of caspase-12 expression seen in cells cotreated with IFN- $\gamma$  and Tg was lower than in cells treated with IFN- $\gamma$  in the absence of Tg. This may be explained by a decrease in translation due to Tg treatment (Wong et al., 1993). Caspase-12 processing clearly occurred in cells treated with the combination of IFN- $\gamma$  and TNF or Tg (Fig. 10 C). Ab-2 detected the 38-kD fragment of caspase-12 in cells treated with IFN- $\gamma$  + TNF but failed to detect this fragment in cells treated with IFN- $\gamma$  + Tg, whereas G149 detected the 28-kD fragment in both types of treatments (Fig. 10 C). These results suggest that the 38-kD fragment lacks only the COOH-terminal small catalytic subunit (SCSU), and that the 28-kD fragment is missing both the SCSU and the prodomain and,

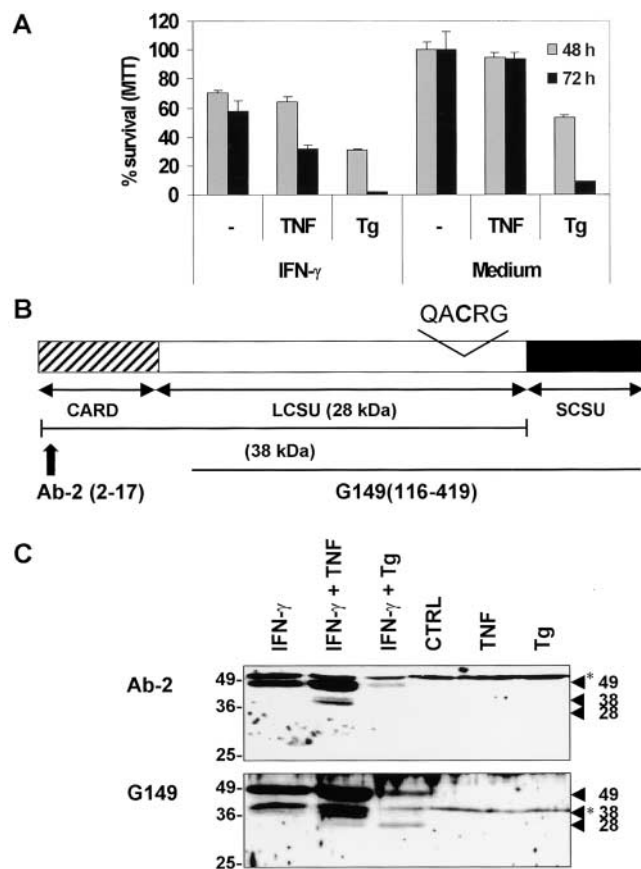


**Figure 9. Caspase-12 is processed in B16/B16 cells dying from the combination of IFN- $\gamma$  with either LPS or TNF.** (A) Analysis of the cytotoxic potential of IFN- $\gamma$ , TNF, or LPS. Cells were treated with IFN- $\gamma$  (1,000 U/ml), TNF (5,000 U/ml), or LPS (1  $\mu$ g/ml) alone or in combinations. Cell viability was measured at the indicated time using MTT. CTRL indicates untreated controls. Error bars, standard deviation of six replicates of a representative experiment. (B) Western blot analysis of cells treated for 48 h with IFN- $\gamma$  (1,000 U/ml), TNF (5,000 U/ml), or LPS (1  $\mu$ g/ml) alone or in combinations. C-12 indicates lysate of HEK293T cells overexpressing caspase-12, used as a positive control. Arrows indicate bands corresponding to the 49-kD full-length pro-caspase-12 and to the 38- and 28-kD fragments of the processed caspase. \*, nonspecific bands.

thus, probably corresponds to the mature large catalytic subunit (LCSU) (Fig. 10, B and C). Taken together, these results demonstrate that caspase-12 is not required for Tg-induced cell death, although treatment with Tg can induce the proteolytic cleavage of the protease.

## Discussion

In the current report, we investigated the expression of caspase-12 during mouse embryonic development and in adult mouse organs. We compared the regulation of the expression of caspase-12 by proinflammatory stimuli with that of other caspases. Although the mRNAs of caspase-1, -11, and -12 are expressed in most of the organs tested, the level of expression varies substantially. For example, all three caspases were highly expressed in the spleen, whereas only caspase-11 and -12 were highly expressed in the lungs. Similar results were obtained before for murine caspase-1, -11, and -12 using Northern blots (Van de Craen et al., 1997) and for human caspase-1, -4, and -5 by RT-PCR (Lin et al., 2000). By analyzing a battery of mouse cell lines of various lineages, we show that the expression of caspase-12 at the protein level is more restricted than expected. Indeed, the only cells we observed to constitutively express the protein are primary fibroblasts and L929 fibrosarcoma cells. This suggests that although caspase-12 can be detected in most organs, only some of the cells in these organs are expressing the protease. This suggestion was clearly supported by the immunohistochemical analysis that demonstrated that in



**Figure 10. Caspase-12 is dispensable for Tg-induced ER stress-mediated apoptosis in B16/B16 cells.** (A) Analysis of the cytotoxic effect of Tg (2  $\mu$ M) alone or in combination with IFN- $\gamma$  (1,000 IU/ml) compared with TNF (5,000 IU/ml). Cell viability was measured at the indicated time using MTT. CTRL is untreated control. Error bars, standard deviation of six replicates of a representative experiment. (B) Schematic representation of pro-caspase-12 indicating the relative location of the peptides used to produce the anti-caspase-12 antisera Ab-2 and G149. Ab-2 was raised against a peptide spanning amino acids 2–17 of caspase-12 and is specific for the NH<sub>2</sub>-terminal prodomain. G149 was raised against a recombinant protein spanning amino acids 116–419 of caspase-12 and recognizes the catalytic part of the protein. LCSU and SCSU indicate the large and the small catalytic subunits of the caspase, respectively. (C) Western blot analysis of cells treated for 72 h with IFN- $\gamma$  (1,000 IU/ml), TNF (5,000 IU/ml), or Tg (2  $\mu$ M) alone or in combinations. C-12 indicates lysate of HEK293T cells overexpressing caspase-12, used as a positive control. Arrows indicate bands corresponding to the 49-kD full-length pro-caspase-12 and to the 38- and 28-kD fragments of the processed caspase. \*, nonspecific bands.

most positive organs, only certain cell layers, such as epithelium of the trachea in the lung and the nose trail, express the caspase.

Protein sequence comparison shows that caspase-12 most resembles caspase-1 and -11. Caspase-1 and its cytokine substrates IL-1 $\beta$  and IL-18 play an important role in immune and inflammatory responses to endotoxins and bacterial infection such as *Shigella flexneri* and *Escherichia coli* (Dinarello, 1998; Sansonetti et al., 2000; Joshi et al., 2002). Mice deficient in caspase-1 are resistant to endotoxin shock and are defective in LPS-induced secretion of IL-1 $\alpha$ , IL-1 $\beta$ , IL-18, and IFN- $\gamma$  (Li et al., 1995; Fantuzzi et al., 1997,

1998; Burgess et al., 1998). Caspase-11-deficient mice present a similar phenotype in addition to the lack of the ability to respond to LPS by caspase-1 processing, a function that apparently requires caspase-11 (Wang et al., 1998). In humans, caspase-1 and IL-1 $\beta$  activation was shown to involve the formation of a complex including caspase-1 and caspase-5 (Martinon et al., 2002). Murine caspase-11 can also form a complex with, and is required for the activation of, caspase-1 (Wang et al., 1998) and therefore may have a function similar to that of human caspase-5. The phylogenetic clustering of caspase-1, -4, -5, -11, and -12 suggests a role for the latter caspase in inflammatory responses. As seen with caspase-1 and -11, the expression of caspase-12 is stimulated by IFN- $\gamma$  in fibrosarcoma and melanoma cells. In contrast to IFN- $\alpha$  and - $\beta$ , which are produced by most cell types in response to viruses or dsRNA, production of IFN- $\gamma$  is restricted to cells of the immune system (Katze et al., 2002). These include natural killer (NK) cells, CD4<sup>+</sup> T helper 1 (T<sub>H</sub>1) cells, and CD8<sup>+</sup> cytotoxic T cells stimulated by IL-12 and IL-18 secreted from activated macrophage or dendritic cells (Murphy and Reiner, 2002). IFN- $\alpha$  and - $\beta$  are mainly involved in the response to viral infection (Katze et al., 2002; Taniguchi and Takaoka, 2002). Although IFN- $\gamma$  has an antiviral function, the cytokine acts mainly as an effector cytokine required for cell-mediated immunity (Murphy and Reiner, 2002; Gordon, 2003). Moreover, IFN- $\gamma$  is a strong activator of proinflammatory and microbicidal functions of macrophages (Gordon, 2003). Remarkably, expression of caspase-12 is induced exclusively by IFN- $\gamma$  and not by IFN- $\alpha$  or - $\beta$ . Does this suggest that caspase-12 plays a role in antibacterial or proinflammatory activities? The answer is far from clear, as the cellular substrates of the protease are still unknown. In contrast to caspase-1 and -11, caspase-12 is not expressed in macrophages, a typical example of inflammatory cells. Moreover, overexpression of caspase-12 in several cell lines together with pro-IL-1 $\beta$  does not lead to secretion of active IL-1 $\beta$  (Van de Craen et al., 1997; unpublished data), excluding a direct role for the protease in IL-1 $\beta$  maturation. In addition, although caspase-12-deficient mice were made (Nakagawa et al., 2000), nothing has been published yet on the response of these deficient mice to proinflammatory or endotoxic stimuli or to bacterial infection.

Activation of caspase-1 or -11 due to exposure to LPS or bacterial infection can also lead to apoptosis (Chen et al., 1996; Hilbi et al., 1998; Kang et al., 2000, 2002; Hisahara et al., 2001). Caspase-12 may exert a similar function. Our results demonstrate that overexpression of caspase-12 in HEK293T cells leads to processing of the enzyme and to apoptosis. We observed similar processing of caspase-12 in B16/B16 cells dying in response to IFN- $\gamma$  combined with either TNF or LPS and in L929sAhFas cells dying by Fas-mediated apoptosis. Interestingly, in B16/B16 cells treated with IFN- $\gamma$ , expression of the typical apoptotic caspases 3 and 9 decreased, whereas that of caspases 1, 11, and 12 went up. This down-regulation of the apoptotic caspases versus the up-regulation of the inflammatory caspases may suggest that IFN- $\gamma$  prepares the cells for an alternative caspase cascade, avoiding the intrinsic apoptotic pathway.

The inducible expression of caspase-12 in B16/B16 cells allowed us to study the involvement of the protein in Tg-



induced ER stress-mediated apoptosis. Our results clearly show that the cells die in response to Tg whether caspase-12 is present or not. They also demonstrate that in cells expressing caspase-12, the enzyme is processed and probably activated in a variety of apoptotic conditions. In view of the restricted expression pattern of caspase-12 to certain cell types and the fact that its closest homologue encoded in the human genome is not a functional protease, we suggest that although the protein may have a function in apoptotic cell death, it is not likely to be the initiator of ER stress-mediated apoptosis. Despite the absence of caspase-12 in stimulated macrophages, the induction of the expression and processing of the protease by proinflammatory stimuli in fibroblasts may still suggest a role in the immune or inflammatory response in the mouse.

## Materials and methods

### Antibodies, cytokines, and reagents

Recombinant murine TNF (specific biological activity of  $2.2 \times 10^8$  IU/ml) was produced in *E. coli* and purified in our laboratory. Recombinant murine IFN- $\alpha$  was from BioSource International. Recombinant murine IFN- $\gamma$  and IFN- $\beta$  were produced in *E. coli* and purified in our laboratory. Flag-tagged human FasL was expressed in HEK293T cells and purified in house. Poly(I)-poly(C) (synthetic dsRNA) was dissolved at 3.5 mg/ml in water (Amersham Biosciences). LPS (*Salmonella abortus equi*) was from Sigma-Aldrich. Anti-murine caspase-9 antibody was from New England Biolabs, Inc. Rabbit polyclonal antiserum against recombinant murine caspase-1, -3, -7, and -11 and caspase-12 (G149) were prepared at the Centre d'Economie Rurale (Laboratoire d'Hormonologie Animale). Purified anti-caspase-12 antisera, Ab-1 and Ab-2, were from Oncogene Research Products. The anti-caspase-12 antiserum used in all Western blot analyses, unless otherwise indicated, is G149. Antiserum specific for mPKR was from Santa Cruz Biotechnology, Inc., and antibody to  $\beta$ -actin was from ICN Biomedicals. Determination of protein concentration in cell lysates was performed using a Bio-Rad Laboratories protein assay.

### RT-PCR analysis

Mouse multiple tissue cDNA (MTC<sup>TM</sup>) panels I and II were from CLONTECH Laboratories, Inc. PCR was performed in a total volume of 50  $\mu$ l PCR buffer containing 2 mM MgSO<sub>4</sub>, 0.4 mM deoxynucleoside triphosphates, 0.5  $\mu$ M each primer, 2.5 U of Pfx Platinum DNA polymerase (Invitrogen), and 5  $\mu$ l of first strand cDNA of tissue or embryo cDNA. The PCR cycle started at 94°C for 2 min, followed by a three-step cycling: denaturation at 94°C for 15 s, annealing at either 58°C or 62°C (depending on the primers used) for 30 s, and extension at 72°C for 90 s. This was followed by a final extension step at 72°C for 5 min. In each experiment, PCR for glyceraldehyde-3-phosphate dehydrogenase (G3DPH) was performed according to the manufacturer's instructions using primers provided by CLONTECH Laboratories, Inc., to ensure that an equal quality and quantity of cDNA was used. To avoid PCR products reaching saturation, 30 PCR cycles were used for caspase-1, -11, and -12 and 22 cycles for G3DPH. The primers for mouse caspase-12 were derived from the 5' end of the full-length cDNA and from the 3' end of the predicted large catalytic subunit (Van de Craen et al., 1997). The primers for caspase-1 and -11 were derived from the 5' and 3' ends of the respective open reading frames.

### Plasmids and transfection of HEK293T cells

The cloning of the cDNA encoding for full-length caspase-12 from a cDNA library derived from L929r2 cells and its transfer to the pCAGGS eukaryotic expression vector were described previously (Van de Craen et al., 1997). In pCAGGS-caspase-12 C298A, the codon for the catalytic cysteine was substituted by that coding for alanine, leading to the expression of an inactive form of the enzyme. pNLS-EGFP is a modified pEGFP-N1 plasmid (CLONTECH Laboratories, Inc.) encoding GFP with a nuclear localization signal. HEK293T cells were transiently cotransfected using calcium phosphate precipitation in six-well culture dishes with 50 ng of pNLS-EGFP and 600 ng of either pCAGGS-caspase-12 or pCAGGS-caspase-12 C298A. The next day, cells were examined and photographed using a fluorescent microscope. Cells were harvested and lysed for Western blot analysis as described below.

### Preparation of protein extracts from mouse organs

Mouse organs were isolated, and samples from three to six mice were pooled and frozen in liquid nitrogen. Tissues were thawed and homogenized on ice with an RZR homogenizer (Heidolph-Instruments) in PBS-A supplemented with 1 mM leupeptin, 1 mM aprotinin, 0.1 mM PMSF, and 1 mM oxidized glutathione. The homogenates were diluted in an equal volume of caspase lysis buffer containing 0.05% Nonidet-P40, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Hepes, pH 7.4, 1 mM leupeptin, 1 mM aprotinin, 0.1 mM PMSF, and 1 mM oxidized glutathione and incubated on ice for 10 min. Cell debris was removed by centrifugation, protein concentrations were measured using a Bio-Rad Laboratories protein assay, and 50  $\mu$ g from each sample was taken for Western blot analysis.

### Immunohistochemistry

BALB/c mouse embryos, aged 13.5 d postcoitum, were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced (4–6  $\mu$ m). Endogenous peroxidase was blocked with peroxidase blocking reagent (DakoCytomation). Caspase-12 expression was detected in sections using either anti-caspase-12 rabbit antiserum, Ab-1 or Ab-2. Binding of primary antibody was revealed by 45 min incubation with a rat anti-rabbit antibody conjugated to peroxidase (DakoCytomation) followed by incubation with the 3,3'-diaminobenzidine chromogen (DakoCytomation). Negative controls were stained by the secondary antibody alone or by the anti-caspase-12 rabbit antiserum depleted with a His<sub>6</sub>-tagged recombinant caspase-12 produced in *E. coli* and captured on Ni-NTA (QIAGEN). The specificity of the depletion procedure was controlled by passing anti-caspase-12 antiserum over a Ni-NTA column. Furthermore, the specificity of the antisera to caspase-12 was verified by immunostaining of HEK293T transfected or not with pCAGGS-caspase-12 C298A using a similar staining technique.

### Northern blot analysis

B16/B16 or L929r2 cells were seeded at  $1.2 \times 10^3$  cells per cm<sup>2</sup>. The next day, cells were left untreated or treated with either IFN- $\gamma$  (1,000 U/ml) or TNF (5,000 U/ml) alone or in combination. Cells were harvested 20 h later, and total RNA was extracted from  $4 \times 10^6$  cells using RNAzol<sup>TM</sup> (WAK-Chemie Medical GmbH). After electrophoretic separation on a 1% formaldehyde agarose gel, RNA was transferred to a Hybond-N<sup>+</sup> membrane and fixed by UV light. Membranes were incubated in prehybridization solution (50 mM Tris-HCl, pH 7.4, 40% formamide, 4 $\times$  SSC, 10 $\times$  Denhardt's solution, 0.1% Na<sub>3</sub>P<sub>2</sub>O<sub>7</sub>, 1% SDS, and 20  $\mu$ g/ml herring sperm DNA) for 2 h at 42°C. DNA probes for caspase-1, -11, and -12 were labeled with [<sup>32</sup>P]dCTP using random priming (Boehringer) and incubated overnight at 42°C with the prehybridized membrane. Filters were washed with 2 $\times$  SSC, 0.1% SDS at room temperature and then exposed to an X-ray film at -80°C.

### Cell culture and treatment

Murine Mf4/4 macrophages (Desmedt et al., 1998) and B16/B16 cells were maintained in LPS-free RPMI 1640 medium (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL), 0.03% L-Gln (Merck), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M  $\beta$ -mercaptoethanol at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Before analysis, cells were seeded at  $3 \times 10^5$  cells/2 ml/well in six-well tissue culture plates (Falcon; Becton Dickinson) in fresh growth medium. The next day, cells were left untreated or were treated as indicated.

Mouse embryonic fibroblasts were isolated from BALB/c mouse embryos by collagenase treatment and incubated in DMEM.NUT.MIX.F-12 medium supplemented with GLUTAMAX-1, pyridoxine, and 15% FCS. For analysis, cells were seeded at  $3 \times 10^5$  cells/2 ml/well in six-well tissue culture plates in fresh growth medium. The next day, cells were treated as indicated.

Peritoneal macrophages were obtained by washing the peritoneal cavity of three adult C57/BL6 mice with PBS-A. May-Grünwald/Giemsa (Sigma-Aldrich) staining performed after the removal of erythrocytes by a hypotonic treatment demonstrated that ~90% of the recovered cells had a monocyte/macrophage-like morphology. The remaining cells were seeded at  $10^6$  cells/1 ml/well in six-well tissue culture plates in LPS-free RPMI 1640 medium supplemented with 10% FCS, 0.03% L-Gln, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M  $\beta$ -mercaptoethanol and allowed to adhere for 1.5 h. After the removal of suspension cells and refreshment of the medium (2 ml/well), adherent cells were treated as indicated.

The human Fas-expressing L929sA cells (L929sAhFas) were described previously (Vercammen et al., 1998). L929sAhFas cells were seeded in 9-cm plates at  $3.10^6$ /plate. The next day, cells were treated with medium or recombinant Flag-tagged FasL (1/500 dilution), and cell death was determined by trypan blue staining in 2-h intervals. Cells were lysed 2 h after

FasL treatment in 20 mM Hepes, pH 7.0, 0.1% CHAPS, 5 mM DTT, 1 mM leupeptin, 1 mM aprotinin, and 0.1 mM PMSF. Cell debris was removed by centrifugation, protein concentrations were measured, and 30  $\mu$ g from each sample was taken for Western blot analysis.

### Western blot analysis

With the exception of L929sAhFas, all cells were harvested, washed with ice-cold PBS-A, and lysed on ice with caspase lysis buffer. Cell debris was removed by centrifugation, and protein concentrations were measured. From each sample, 10  $\mu$ g of HEK293T cell lysate, 50  $\mu$ g of melanoma cell or macrophage cell lysate, or 30  $\mu$ g of primary fibroblast cell lysate was taken for Western blot analysis. A lysate of HEK293T cells expressing the wild-type protein was used as a positive control for caspase-12 expression. Samples were boiled for 10 min in SDS-PAGE sample buffer, separated in 12.5 or 15% SDS-PAGE gels, as indicated, and transferred to nitrocellulose. Blocking, antibody incubation steps, and washing of the membrane were performed in PBS supplemented with 0.05% Tween-20 and 3% skim milk. After the incubation with the primary antibody of choice and washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies to mouse and rabbit immunoglobulin (Amersham Biosciences). Proteins were visualized using a chemiluminescence substrate (PerkinElmer), and blots were exposed to film (Amersham Biosciences).

### Cell survival assay

B16/B16 cells were seeded at  $2 \times 10^3$  cells per well, respectively, in 96-well plates. The next day, cells were left untreated or were treated with IFN- $\gamma$  (1,000 IU/ml), TNF (5,000 IU/ml), LPS (1  $\mu$ g/ml), or Tg (1  $\mu$ M) alone or with combinations of IFN- $\gamma$  with TNF, LPS, or Tg. Cell survival was assessed at the indicated time using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) (Tada et al., 1986). The percentage of cell death was calculated using the following equation:  $100\% \times (1 - (A_{595/655}\text{-treated cells} - A_{595/655}\text{ medium}) / (A_{595/655}\text{-untreated cells} - A_{595/655}\text{ medium}))$ .

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