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Letter to the Editor

Bcl-2 transgene expression fails to prevent fatal hepatocyte apoptosis induced by endogenous TNFα in mice lacking *RelA*

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Dear Editor,

Mammals have two distinct, but ultimately converging apoptosis signalling pathways, one initiated by so-called 'death receptors' and the other regulated by the Bcl-2 protein family.[1] Death receptors, such as TNF-R1 and Fas (APO-1/CD95) constitute a subgroup of the tumour necrosis factor receptor (TNF-R) superfamily, which contain an intracellular death domain essential for FADD adaptor protein-mediated activation of caspase-8 and initiation of apoptosis. However, under most circumstances TNF-R1 engagement fails to induce apoptosis, instead promoting the differentiation and proliferation of cells. Under these circumstances, survival is guaranteed by triggering the recruitment of TRAF2, TRAF5 and the kinase RIP, leading to Rel/NF-κB activation. In many cell types, Rel/NF-κB transcription factors induce expression of antiapoptotic Bcl-2 family members and other apoptosis inhibitors, notably c-FLIP and IAP proteins.[2] The physiological importance of Rel/NF-κB antiapoptotic function is highlighted by the observation that mice lacking RelA or its activator IKKβ, die from excessive hepatocyte apoptosis at around E14.5 and E13 of development, respectively.[3,4] The generation of viable *rela*$^{-/-}$ mice on either a *tnf*$\alpha^{-/-}$ or *tnfr1*$^{-/-}$ background demonstrates that this hepatocyte death is mediated by TNFα > TNFR1-signalling.[5] In healthy mice, TNF-R1 is expressed at high levels on hepatocytes and Kupffer cells, which are normally resistant to TNFα-induced apoptosis. However, certain bacterial products such as LPS, septic shock, or ischaemia-reperfusion injury can render hepatocytes sensitive to TNFα-induced apoptosis, possibly through inhibition of the Rel/NF-κB pathway.

The essential role of pro-and antiapoptotic Bcl-2 family members in the regulation of developmentally programmed cell death and stress-induced apoptosis is well established, yet their role in death-receptor-mediated apoptosis remains controversial, possibly due to the use in some instances of nonphysiological experimental systems. Certain whole animal and cell culture studies indicate that TNF-R1 ligation triggers apoptosis through a Bcl-2-inhibitable mechanism involving caspase-8-mediated activation of the proapoptotic BH3-only protein Bid.[6] In contrast, other studies found no effect of Bcl-2 or related prosurvival homologues on death receptor-induced apoptosis.

To investigate the ability of Bcl-2 to block cell death *in vivo* mediated by endogenous TNFα, mice lacking RelA[3] were intercrossed with ATT-*bcl*-2 transgenic mice, which express a human *bcl*-2 cDNA under control of the hepatocyte-specific α-1-anti-trypsin promoter.[7] Consistent with previous reports,[7] livers of embryonic and adult (E15) ATT-*bcl*-2 transgenic mice were indistinguishable from livers of wild-type counterparts (data not shown). Western blot analysis of foetal liver extracts revealed readily detectable levels of transgenic (human) Bcl-2 on days 12.5, 13.5 and 14.5 of gestation in ATT-*bcl*-2 transgenic embryos (Figure 1a). Immunohistochemical staining demonstrated that in the liver of transgenic mice human Bcl-2 was only expressed in hepatocytes (Figure 1b). To determine whether Bcl-2 overexpression could prevent the embryonic death of *rela*$^{-/-}$ mice, *rela*$^{+/-}$ ATT-*bcl*-2 transgenic mice were intercrossed with *rela*$^{+/-}$ mice. Out of a total of 124 offspring, no *rela*$^{-/-}$ homozygotes (expected 31), with (expected 15) or without the *bcl*-2 transgene (expected 15), were detected at weaning, indicating that Bcl-2 was unable to prevent TNFα-mediated hepatocyte destruction. To confirm this conclusion, timed matings between *rela*$^{+/-}$ and *ATT-bcl*-2 transgenic *rela*$^{+/-}$ mice were established. Wild-type, *rela*$^{+/-}$ and *rela*$^{-/-}$ embryos, with or without the *bcl*-2 transgene, were present at the expected Mendelian frequency up to day 14 of gestation, a time preceding the TNFα-induced death of *rela*$^{-/-}$ embryos. Consistent with previous reports,[3] all *rela*$^{-/-}$ animals were dead by E15.5 (from 10 litters; expected 8). Notably, all 10 *bcl*-2 transgenic *rela*$^{-/-}$ embryos (from 10 litters; expected 9) were also dead by day 15.5 of gestation presenting with extensive haemorrhageing in the abdominal cavity (Figure 1c). Histological examination revealed severely abnormal liver architecture with extensive hepatocyte apoptosis (Figure 1c). By contrast and as shown previously,[5] matings of *rela*$^{+/-}$ *tnf*$\alpha^{-/-}$ mice generated viable *rela*$^{-/-}$ *tnf*$\alpha^{-/-}$ offspring at the expected Mendelian frequency, with liver sections from these mice appearing normal (Figure 1c).

As TNFα was shown to induce endothelial and haemopoietic cell apoptosis under certain circumstances, it was conceivable that the failure of the *ATT-bcl*-2 transgene to protect *rela*$^{-/-}$ hepatocytes may indicate that the death of these cells was an indirect consequence of disruption to the foetal liver architecture resulting from the death of nonhepatic cells that precedes hepatocyte cell death. To address this issue, endothelial and haemopoietic cells in *rela*$^{+/-}$ and *rela*$^{-/-}$ foetal livers were compared on day 14 of gestation when *rela*$^{-/-}$ mice are still viable. Although liver sections from *rela*$^{-/-}$ embryos, with or without the *ATT-bcl*-2 transgene, exhibited apoptotic bodies (Figure 2a and b), vascular endothelial cells in the *rela*$^{-/-}$ foetal liver displayed no sign of nuclear

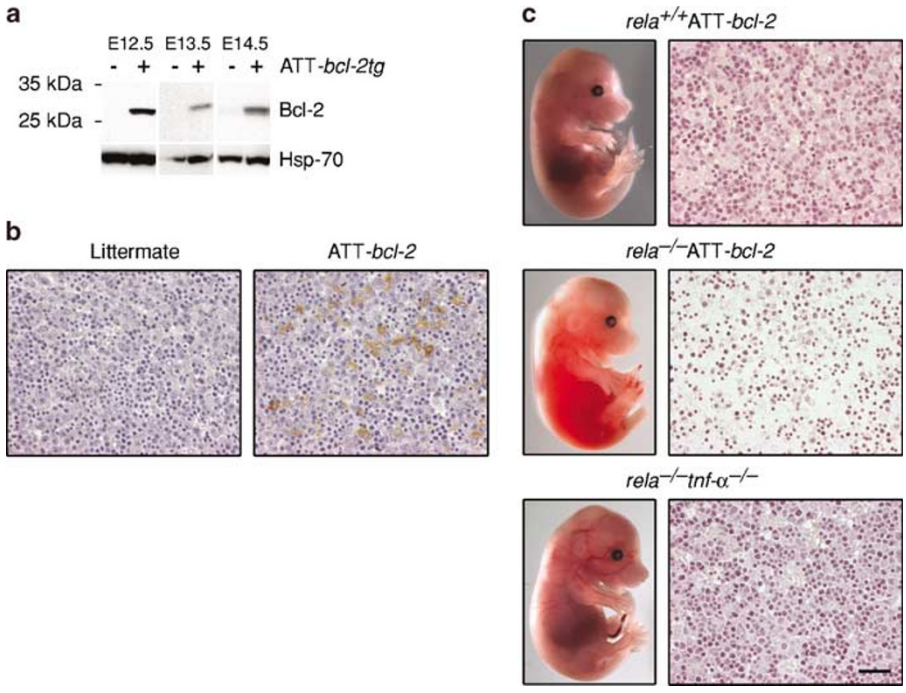

Figure 1 Enforced Bcl-2 expression does not prevent hepatocyte apoptosis in *rela*$^{-/-}$ embryos. (**a**) Bcl-2 expression in foetal hepatocytes of ATT-*bcl-2* transgenic mice. Protein lysates from foetal livers (E12.5, 13.5, 14.5) of ATT-*bcl-2* transgenic mice and littermate controls were subjected to Western blot analysis using mouse anti-human Bcl-2 mAb (Bcl-2-100) and mouse anti-HSP70 mAb N6. (**b**) Immunohistochemical staining of foetal liver sections from E13.5 ATT-*bcl-2* transgenic or littermate control embryos using mouse anti-human Bcl-2 mAb (Bcl-2-100). Similar staining was observed in foetal liver sections of E12.5 transgenic mice (**c**) Embryos of the indicated genotypes were isolated at E15.5 and images taken with a digital camera (Zeiss). Embryos were processed for histology, stained with H&E and foetal livers analysed with a compound microscope. Bar, 225 μm. For timed matings, day 0 was defined by the appearance of a vaginal plug. Genotyping of knockout and transgenic mice was performed by PCR of genomic DNA

condensation and their morphology was comparable to that of endothelial cells in healthy *rela*$^{+/-}$ embryos (Figure 2c and d). Combined TUNEL and Hoechst staining of these tissue sections revealed that in contrast to the *rela*$^{-/-}$ hepatocytes, many of which were TUNEL-positive (Figure 2e–h), *rela*$^{-/-}$ endothelial cells did not exhibit features characteristic of programmed cell death (Figure 2i and j). As early as E14, the frequency of TUNEL-positive hepatocytes in *rela*$^{-/-}$ embryos was significantly higher than that found in developmentally matched controls, with the *bcl-2* transgene failing to reduce the number of apoptotic hepatocytes on the *rela*$^{-/-}$ background (Figure 2h). This finding indicated that the TNFα-mediated hepatocyte death in *rela*$^{-/-}$ embryos was not a secondary consequence of endothelial cell death. Similarly, it was also important to determine whether the death of *rela*$^{-/-}$ hepatocytes coincided with and, hence, may be influenced by the death of neighbouring haemopoietic cells, which constitute a major cellular population in the foetal liver. Two-colour FACS staining was performed to ascertain whether *rela*$^{-/-}$ CD45^{+} haemopoietic cells or CD45^{-}Ter-119^{+} erythroid precursors were undergoing apoptosis. While there were increased numbers of TUNEL-positive cells in the *rela*$^{-/-}$ foetal liver, these cells were neither CD45^{+} nor Ter-119^{+} and most likely comprised hepatocytes that contaminated the foetal liver haemopoietic cell preparations (data not shown). Collectively, these findings demonstrate that in *rela*$^{-/-}$ foetal liver, hepatocytes but not endothelial or haemopoietic cells are undergoing TNFα-mediated apoptosis.

These observations demonstrate that Bcl-2 overexpression in *rela*$^{-/-}$ hepatocytes does not protect against apoptosis triggered by physiological levels of TNFα. It therefore appears that during liver development, RelA protects hepatocytes from TNFα-induced death through activation of antiapoptotic genes other than those encoding Bcl-2-like prosurvival proteins (unless one of them can fulfil a function that Bcl-2 itself cannot). It is currently unclear if the death of *rela*$^{-/-}$ hepatocytes is mediated by soluble or membrane-bound TNFα. Interestingly, soluble TNFα alone does not induce apoptosis in adult mice, but lethal hepatitis does result from coinjection of soluble TNFα plus D-(+)-galactosamine (GalN)[8] and this is mediated primarily through TNF-R1. In apparent contrast to our findings, the ATT-*bcl-2* transgenic mice were reported to resist a lethal challenge of soluble TNFα plus GalN.[8] This may indicate that an adult liver responds differently to TNFα than an embryonic liver. Alternatively, these observations might indicate that Bcl-2 overexpression can only protect hepatocytes against soluble TNFα but not against membrane-bound TNFα, which according to our hypothesis would have to be the trigger of hepatocyte destruction in *rela*$^{-/-}$ foetuses. Bid was found to be cleaved and integrated into the outer mitochondrial membrane of hepatocytes from mice injected with TNFα plus GalN.[6] After such treatment Bid-deficient mice displayed a significant delay in hepatocyte destruction compared to wild-type mice,[6] indicating that it plays a critical role in this cell death pathway. Therefore, it will be of interest to generate *bid*$^{-/-}$*rela*$^{-/-}$ mice

to investigate whether this BH3-only protein is critical for embryonic hepatocyte killing induced by endogenous TNFα in this setting.

It had also been reported that hepatocyte destruction induced by injection of anti-Fas antibody occurs after the death of endothelial cells. However, since the loss of caspase-8 in hepatocytes alone protects mice from anti-Fas-induced liver destruction,[9] this death appears to occur by a hepatocyte-intrinsic process and not as a consequence of endothelial cell apoptosis. Similarly, our histological and FACS analysis of *rela*$^{-/-}$ foetal livers indicates that TNFα-mediated hepatocyte death does not follow the death of endothelial or haemopoietic cells.

In conclusion, our results indicate that TNFα-mediated hepatocyte killing due to loss of RelA relies on a signalling pathway that is insensitive to inhibition by Bcl-2. This work coupled with previous findings[10] may indicate that soluble and membrane-bound TNFα trigger apoptosis through distinct pathways. This in turn may have implications for the aetiology of disease states involving hepatocyte destruction and for the development of therapies to treat various liver diseases.

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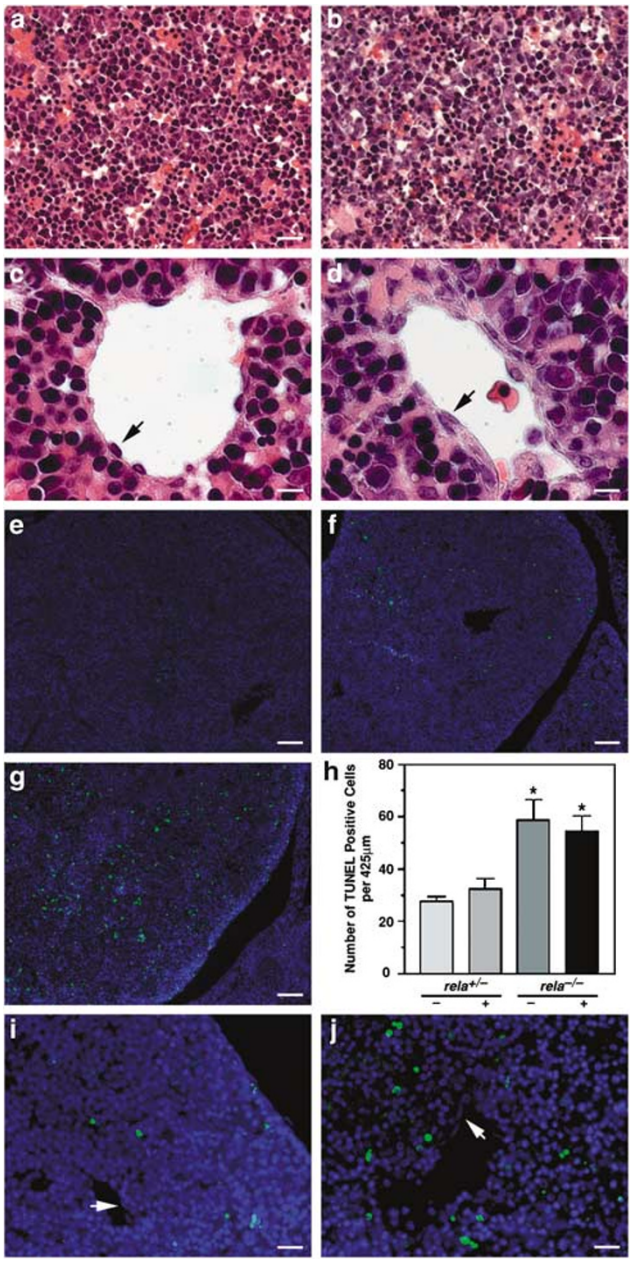

Figure 2 The early phase of apoptosis in *rela*$^{-/-}$ foetal livers coincides with the death of hepatocytes but not endothelial cell killing. (**a**–**d**) H&E stained embryonic sections of E14 littermate *rela*$^{+/-}$ control (**a**) and *rela*$^{-/-}$ mutant mice (**b**) were examined by light microscopy. *rela*$^{-/-}$ but not *rela*$^{+/-}$ foetal livers showed signs of disorganisation and the presence of apoptotic bodies. Bar, 225 μm. Higher power analysis reveals that the vascular endothelial cells (black arrow) in the littermate *rela*$^{+/-}$ control (**c**) and *rela*$^{-/-}$ foetal liver (**d**) appears to be intact ($\times$1000; oil emersion). (**e**–**j**) (**e**) Combined TUNEL and Hoechst counter staining in the absence of terminal deoxynucleotidyltransferase revealed minimal background staining of E14 sections. TUNEL staining of *rela*$^{+/-}$ (**f**) and *rela*$^{-/-}$ (**g**) embryonic sections revealed increased hepatocyte apoptosis in the *rela*$^{-/-}$ foetal livers (Bar, 875 μm). Like those of *rela*$^{+/-}$ controls (**i**) *rela*$^{-/-}$ endothelial cells (**j**) were not TUNEL-positive; the white arrows show representative endothelial cells for both genotypes. Bar, 225 μm. (**h**) Quantitation of TUNEL-positive cells was determined from at least five images of sections for each genotype with or without the ATT-*bcl-2* transgene (+/−). The data shown represents the mean $\pm$ S.E.M. of three independent experiments and statistical significance was observed between *rela*$^{+/-}$ and *rela*$^{-/-}$ mice (* denotes $P<0.01$; Mann–Whitney *U*-test). TUNEL staining of paraffin-embedded embryonic sections was performed according to the manufacturer's instructions (ApopTag TUNEL staining kit; Chemicon) and counter stained with the Hoechst dye. Images were taken with a compound microscope and a digital camera (Zeiss)