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Viral induction of Zac1b through TLR3- and IRF3-dependent pathways

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ABSTRACT

Zinc finger protein regulator of apoptosis and cell cycle arrest (Zac1) is a transcription factor able to induce apoptosis or cell cycle arrest through independent pathways. In spite of the important potential functions attributed to Zac1, little is known of its physiological regulation and biological function. We discovered that variant Zac1b was expressed in murine embryonic fibroblasts (MEFs) treated with polyriboinosinic polyribocytidylic acid [poly(I:C)], a synthetic double-stranded RNA. This regulation occurred mainly through Toll-Like Receptor 3 (TLR3)- and Interferon Regulatory Factor 3 (IRF3)-dependent pathways. As TLR3 and IRF3 are central activators of antiviral immunity, we hypothesized that Zac1 may be implicated in antiviral responses. In line with this notion, we observed that Zac1b was expressed in MEFs infected with Encephalomyocarditis virus (EMCV). We also observed that Zac1-deficient MEFs were less sensitive to EMCV-induced cell death than wild-type MEFs. However, Zac1 gene inactivation had no effect on the survival of mice infected with EMCV. In conclusion, this study describes for the first time a transcriptional regulation of Zac1b, induced by synthetic dsRNA and RNA viruses, the functional significance of which remains to be further investigated.

1. Introduction

Zinc finger protein regulator of apoptosis and cell cycle arrest 1 (Zac1), also called Lost on transformation 1 (Lot1) or Pleiomorphic Adenoma Gene Like 1 (Plagl1), is a vertebrate zinc finger protein transcription factor (Abdollahi et al., 1997b; Spengler et al., 1997; Kas et al., 1998; Varrault et al., 1998). Zac1 mRNA is widely expressed in human and murine organs such as the anterior pituitary gland, other brain areas, the kidney and the adrenal gland (Spengler et al., 1997; Kas et al., 1998; Varrault et al., 1998; Piras et al., 2000). Different splice variants for Zac1 have been identified in human (Kas et al., 1998) and mouse (Huang and Stallcup, 2000). In mice, two variants exist: Zac1a and Zac1b. Zac1b is identical to Zac1a except for 11 additional C-terminal amino acids (Huang and Stallcup, 2000).

Zac1 is a paternally expressed imprinted gene (Kamiya et al., 2000; Piras et al., 2000). Consequently, it is suspected that Zac1 may be implicated in embryonic development, as well as in certain disorders such as neonatal diabetes (Kamiya et al., 2000; Arima et al., 2001; Varrault et al., 2001). In support of such a role, inactivation of the Zac1 gene in mice results in intrauterine growth restriction, altered bone formation and neonatal lethality. Only 30% of Zac1-deficient pups reach adulthood (Varrault et al., 2006). Loss of Zac1 expression has also been observed in numerous tumor types, including breast tumors, ovary tumors and pituitary adenomas (Abdollahi et al., 1997a, 1997b; Pagotto et al., 2000; Cvetkovic et al., 2004; Koy et al., 2004; Basyuk et al., 2005). Thereby, Zac1 has been proposed as a candidate tumor suppressor gene. In support of this hypothesis, overexpression of Zac1 inhibits tumor cell growth through induction of both apoptosis and cell cycle arrest (Spengler et al., 1997; Varrault et al., 1998). Furthermore, Zac1 has been proposed as a co-factor for p53 (Huang et al., 2001), synergizing with it to induce certain anti-proliferative genes such as $p21^{WAF1/Cip1}$ or Apoptosis protease-activating factor-1 (Apaf-1) (Huang et al., 2001; Rozenfeld-Granot et al., 2002; Liu et al., 2008). Thus, Zac1 was the first transcription factor beside p53 identified as being able to concurrently induce apoptosis and cell cycle arrest (Spengler et al., 1997).

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In spite of the important potential functions attributed to Zac1, little is known of its physiological regulation. The aims of this study were to identify stimuli that modulate the expression of Zac1, and to investigate the potential functional significance of this regulation. We found that synthetic double-stranded RNA polyriboinosinic polyribocytidylic acid [poly(I:C)] induced expression of Zac1b, mainly through Toll-Like Receptor 3 (TLR3)- and Interferon Regulatory Factor 3 (IRF3)-dependent pathways. As poly(I:C) mimics viral double-stranded (ds)RNA, this result unexpectedly suggested that Zac1b may be implicated in antiviral immunity. We also observed that Zac1b was expressed after infection with Encephalomyocarditis virus (EMCV), and that Zac1-deficient MEFs were modestly but significantly less sensitive to EMCV-induced cell death than wild-type MEFs. However, Zac1-deficient mice did not show any significant changes in their survival rates following EMCV infection. Thus, this study describes for the first time a physiological induction of Zac1b, by synthetic dsRNA and RNA viruses, the functional significance of which remains to be further investigated.

2. Materials and methods

2.1. Mice

The generation of Zac1-deficient mice has been described previously (Varrault et al., 2006). Zac1-deficient mice were kept on a C57Bl/6 background. Zac1-deficient and wild-type (WT) C57Bl/6 mice were bred and housed in filtered cages in ventilated racks in the institutional conventional facility. $Zac1^{+/-pat}$ heterozygous mice were generated by crossing $Zac1^{+/-}$ heterozygous males to wild-type C57Bl/6 females. Age-paired groups of mice were used at 8–10 weeks of age. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Liege. We also followed the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996).

2.2. Murine embryonic fibroblasts

WT, $Zac1^{-/-}$, and $IRF3^{-/-}$ MEFs were isolated from 12.5 to 13.5 days postcoitum mouse embryos. The embryos were dissociated and digested with trypsin/EDTA (GIBCO/Invitrogen) to produce single-cell suspensions. IRF3-deficient mice (Sato et al., 2000) were purchased from RIKEN BioResource Center. Mice were bred and maintained in specific pathogen-free conditions. $TLR3^{-/-}$ and $TLR3^{+/+}$ MEFs were a kind gift from the Professor S. Akira (Osaka University, Osaka, Japan). MEFs were cultured in Dulbecco's Minimal Essential Medium (DMEM) complemented with 10% fetal bovine serum, 2 mM L-glutamin, 0.1 mM non-essential amino acids, 10 μg/ml gentamycin and 10 μg/ml β-mercaptoethanol (GIBCO/Invitrogen).

2.3. Reagents

Actinomycin D, doxorubicin, H_2O_2, recombinant murine Interferon-β1 (IFN-β1), and nutlin-3a were from Sigma–Aldrich. CpG, flagellin, Pam3CSK4, poly(I:C) and R848 were from Invivogen. Lipopolysaccharide (LPS) was from Calbiochem. Recombinant murine Interferon (IFN)-α (hybrid IFN-α1/α4) was from Hycult Biotechnology, recombinant murine Interleukin (IL)-1β from Biosource/Invitrogen, and recombinant murine Tumor Necrosis Factor (TNF)-α from PeproTech.

2.4. Cell irradiation

Cells were irradiated with 10 Gy of gamma irradiation (Caesium-137) using a Gammacell-40 Irradiator (MDS Nordion) or with UV irradiation (50 J/m^2).

2.5. Murine peritoneal macrophages

Seven- to ten-week-old C57BL/6 mice were treated intraperitoneally with poly(I:C) or vehicle (PBS). Mice were sacrificed 6 h after treatment and peritoneal cells were obtained by peritoneal lavages with PBS/EDTA (GIBCO/Invitrogen). Cell counts showed that approximately 85% of isolated cells were macrophages.

2.6. Viruses

Encephalomyocarditis virus (EMCV) was obtained from the American Type Culture Collection (ATCC, chimpanzee, Florida, 1944) and Vesicular stomatitis virus (VSV, strain Indiana) was a kind gift from Professor P. Lebon (Paris University, Paris, France). The viruses were propagated in Vero cells (ATCC). When more than 90% of the infected cells showed signs of cytopathy, the cultures were frozen and thawed twice, and the culture medium was clarified by low-speed centrifugation. Virus titers in culture medium were measured by serial dilution in Vero cells to determine the tissue culture infective dose 50 (TCID50) using the Reed–Muench method. The viral stocks were stored at −80 °C in DMEM medium (GIBCO/Invitrogen).

Sendai virus (SeV) (ATCC, lung material from fatal case of newborn pneumonitis, Japan, 1952) was propagated in 11-day-old chick embryos for 48 h and viral stocks were titrated in LLC-MK2 cells (ATCC), as described previously (Faisca et al., 2005).

2.7. Transfections and plasmids

For the transduction of Zac1a and Zac1b, Zac1a and Zac1b cDNAs were cloned into the pcDNA3.1 expression vector (Invitrogen). Cells were transfected overnight with 3 μg of pcDNA3.1-Zac1a, pcDNA3.1-Zac1b, or empty vector using 6 μl of TransFectin (Bio-Rad), as recommended by the manufacturer.

For the poly(I:C) transfection experiments, cells were transfected for 6 h with 3 μg of poly(I:C) using 6 μl of TransFectin (Bio-Rad), as recommended by the manufacturer.

2.8. Immunoblotting

Cells were lysed in RIPA buffer [1× PBS, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 100 μM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin (Sigma), 40 μg/ml Complete (Roche)]. The lysates were clarified and protein amounts were determined by Lowry protein assay (Bio-Rad). Equivalent amounts of whole cell lysates were subjected to SDS-PAGE and proteins were electrotransferred to polyvinylidene difluoride membranes (Amersham). The membranes were blocked for 1 h at room temperature with 5% milk in 1× TBS with 0.1% Tween 20 and incubated overnight at 4 °C with polyclonal murine anti-Zac1 antibody (rabbit polyclonal IgG, Santa-Cruz), polyclonal murine anti-p53 antibody (rabbit polyclonal IgG, Santa-Cruz), or monoclonal anti-β-actin (mouse monoclonal IgM, EMB Biosciences). The blots were then incubated for 45 min with HRP-conjugated secondary antibody. Immunoreactive bands were revealed using the ECL detection method (Amersham). Band intensities were estimated by densitometry using the Quantity One software (Bio-Rad). The data were normalized to β-actin and are expressed as mean ± SD (Standard Deviation) from three independent experiments. Results were expressed as fold induction compared with untreated cells (values in untreated cells set to 1).

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2.9. RNA extraction and real-time RT-PCR

Total RNA was extracted from cells using NucleoSpin® RNA II extraction kits (Macherey-Nagel) and was reverse transcribed with Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer's instructions. PCR was then performed with the following primers: Zac1b-F 5-tgagtgctgtgaatctggg-3 and Zac1b-R 5-ttatctaaatgcgtgatggaaa-3; GAPDH-F 5-caacagggtggtggacctcat-3 and GAPDH-R 5-tgggatagggcctctcttgct-3. Real-time PCR analysis was performed on cDNA samples using iQ SYBR Green Supermix (Bio-Rad) and the iQ5 real-time PCR detection system (Bio-Rad). The comparative threshold cycle method was used to quantify the relative levels of gene expression and was normalized to the housekeeping gene GAPDH.

2.10. Immunoassay

The concentration of IFN-α and IFN-β in cell supernatants and sera was measured using enzymelinked immunosorbent assays (ELISAs) kits (PBL InterferonSource), as recommended by the manufacturer.

2.11. MTT assay

Cell viability was determined by 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay (Sigma). Culture medium was removed and MEFs were incubated with MTT solution [0.5 mg/ml in DMEM without phenol red (GIBCO/Invitrogen)] at 37 °C for 3 h. Then, the converted dye was solubilized with acidic isopropanol (0.04 M HCl in absolute isopropanol). Absorbance of the converted dye was measured by spectrophotometry at a wavelength of 570 nm with background substraction at 630 nm.

2.12. BrdU assay

Cell proliferation was measured by bromodeoxyuridine (BrdU) assay according to the manufacturer's instructions (BD Biosciences). Briefly, cells were incubated for 3 h with BrdU at a final concentration of 10 μM in cell culture medium. Then, cells were fixed, permeabilized and treated with DNAse to expose incorporated BrdU. Cells were resuspended with fluorescent anti-BrdU antibody. Total DNA was stained for cell cycle analysis with 7-amino-actinomycin D (7-AAD) solution. Analysis was performed by flow cytometry with FACScanto II (Becton Dickinson).

2.13. TUNEL assay

Apoptosis was measured by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions (Roche). Cells were fixed, permeabilized and labeled with TUNEL reaction mixture. Analysis was performed by flow cytometry using a FACScanto II (Becton Dickinson).

2.14. Statistical analyses

All experiments were repeated three times and data are expressed as mean ± SD (standard deviation). Statistical significance of differences was determined by ANOVA followed by Fisher's PLSD (Protected Least Significant Difference) tests. A generalized Wilcoxon test was used to test for differences in survival between wild-type and Zac1-deficient mice after EMCV infection. Differences were considered statistically significant at $p < 0.05$.

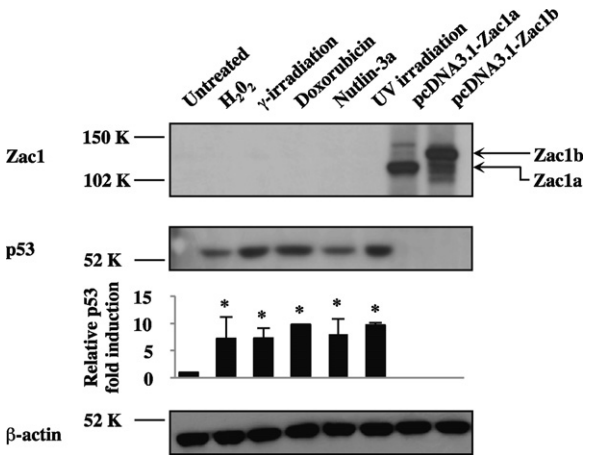

Fig. 1. DNA damage inducers do not induce Zac1 expression. WT MEFs were treated for 6 h with H_2O_2 (200 μM), γ-irradiation (10 Gy), doxorubicin (0.2 μg/ml), nutlin-3a (5 μM) or UV irradiation (50 J/m²). For reference, WT MEFs were transfected overnight with Zac1a or Zac1b expression vectors. Total protein extracts were analyzed for Zac1 and p53 expression by Western blotting. β-Actin was used as loading control. Comparable results were obtained in three independent experiments. (*) Significantly different from untreated control cells.

3. Results

3.1. DNA damage inducers do not induce Zac1 expression

Zac1 has been proposed to act as a tumor suppressor, as it notably shares the ability to induce concurrently apoptosis and cell cycle arrest with p53 (Spengler et al., 1997). We thus initially studied the effect of DNA damage inducers on Zac1 expression in mouse embryonic fibroblasts (MEFs). Surprisingly, H_2O_2, γ-irradiation, doxorubicin, nutlin-3a and UV irradiation did not induce Zac1 protein expression, although, as expected, they induced the accumulation of p53 (Fig. 1).

3.2. Poly(I:C) induces Zac1b expression mainly through TLR3- and IRF3-dependent mechanisms

Next, we sought to determine whether pro-inflammatory stimuli, which may act as potent apoptosis inducers (Rath and Aggarwal, 1999; Salaun et al., 2006; Ma et al., 2007; Grunnet et al., 2009; Taura et al., 2010), could regulate Zac1 expression. MEFs were stimulated with various TLR ligands [Pam3CSK4 (TLR1/2), poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), R848 (TLR7/8), CpG (TLR9)] and pro-inflammatory cytokines (TNF-α, IL-1β), and assessed for their expression of Zac1. As shown in Fig. 2A, the TLR3 ligand poly(I:C) uniquely induced Zac1 protein expression. The induction of Zac1 by poly(I:C) was time-dependent, starting 3 h, peaking 6 h and going back to baseline 12 h post-treatment (Fig. 2B). None of the other treatments induced Zac1 at any of the time points tested (data not shown).

To determine which of the two murine variant transcripts of Zac1 was induced by poly(I:C), we transfected MEFs with expression vectors coding for Zac1a or Zac1b. Western blot analysis showed that Zac1a and Zac1b had molecular weights of approximately 105 kDa and 125 kDa (Fig. 2C). Comparison with exogenously expressed Zac1 isoforms indicated that poly(I:C) induced predominantly the Zac1b isoform in MEFs. The anti-Zac1 antibody also recognized a non-specific band of approximately 140 kDa, which we suspect could be the enzyme Myosin light chain kinase (MYLK) based on molecular weight and sequence homology with the immunogen used for anti-Zac1 antibody production (Fig. 2C).

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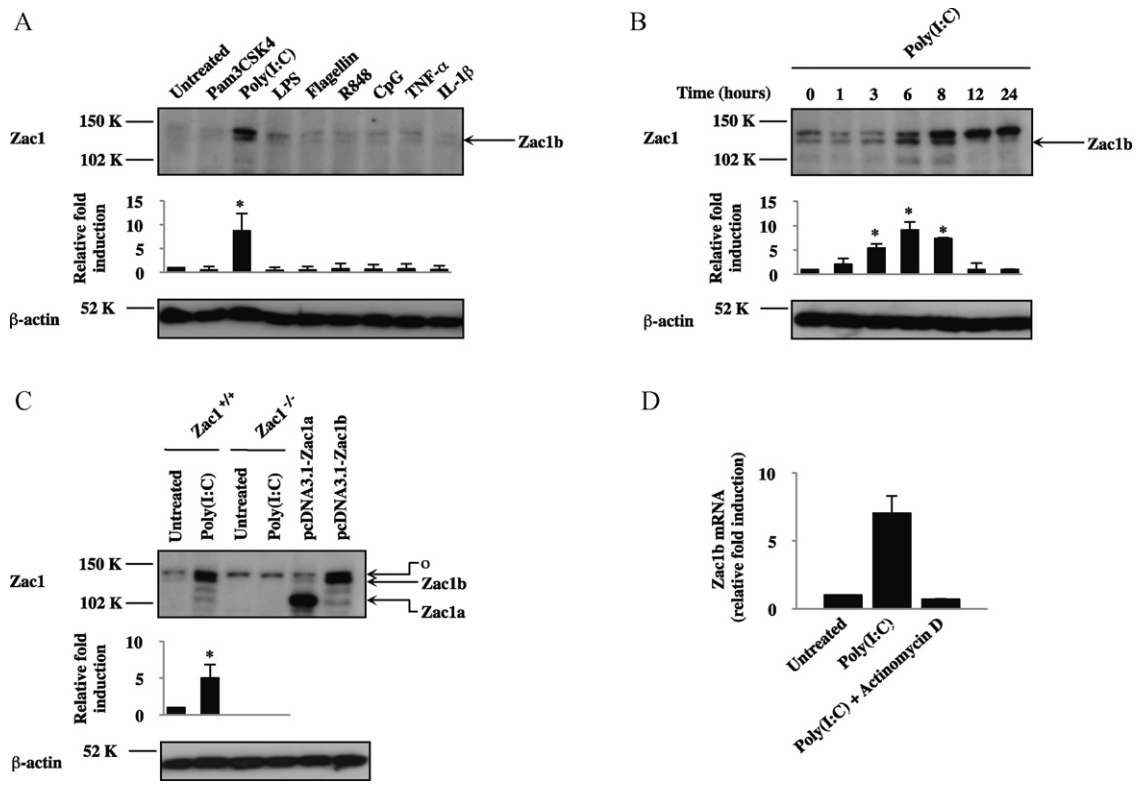

Fig. 2. Poly(I:C) induces Zac1b expression. (A) WT MEFs were stimulated for 6 h with Pam3CSK4 (1 μg/ml, TLR1/2 ligand), poly(I:C) (20 μg/ml, TLR3 ligand), LPS (10 μg/ml, TLR4 ligand), flagellin (10 μg/ml, TLR5 ligand), R848 (5 μg/ml, TLR7/8 ligand), CpG (5 μM, TLR9 ligand), TNF-α (20 ng/ml) or IL-1β (10 ng/ml). Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. (B) WT MEFs were stimulated for 1, 3, 6, 8, 12 and 24 h with poly(I:C) (20 μg/ml). Vehicle-treated cells were used as control. Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. (C) WT MEFs were transfected overnight with Zac1a or Zac1b expression vectors. For comparison, $Zac1^{+/+}$ and $Zac1^{-/-}$ MEFs were treated for 6 h with poly(I:C) (20 μg/ml). Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. (°) Non-specific band. (*) Significantly different from untreated control. (D) WT MEFs were stimulated for 6 h with poly(I:C), with or without actinomycin D (1 μg/ml). Zac1b mRNA expression was estimated by real-time RT-PCR. Results were expressed as fold induction compared with untreated WT MEFs (values in untreated WT MEFs set as 1). The data were normalized to GAPDH and are expressed as mean ± SD. All experiments were repeated three times.

Poly(I:C) induced the expression of Zac1b also at the mRNA level (Fig. 2D). This appeared to depend on transcriptional mechanisms, as the upregulation of Zac1b mRNA expression was abolished by concomitant treatment with actinomycin D (Fig. 2D).

We next investigated whether poly(I:C) also induced Zac1b expression in primary adult cell types. In this regard, we observed that intraperitoneal injection of mice with poly(I:C) led to Zac1b expression in peritoneal macrophages (Fig. 3A and B).

Poly(I:C) is a synthetic double-strand RNA that, if deposited extracellularly, activates TLR3 (Alexopoulou et al., 2001) while, if transfected, activates RIG-I (retinoic acid-inducible gene 1 protein)-like helicases (RLHs) (Yoneyama et al., 2004; Gitlin et al., 2006; Kato et al., 2006). Through subsequent activation of the transcription factor IRF3 (O'Neill et al., 2003; Yamamoto et al., 2004), poly(I:C) notably induces type I IFN expression (Yoneyama et al., 1998). To examine the pathway used by poly(I:C) to induce Zac1b, we treated $TLR3^{-/-}$ and WT MEFs with poly(I:C), either deposited in the medium or transfected into the cells. Zac1b was induced by both deposited and transfected poly(I:C) in WT MEFs. However, the induction was much less pronounced in transfected cells (Fig. 4A and B). The induction by poly(I:C) was abolished in $TLR3^{-/-}$ MEFs when poly(I:C) was deposited, but not when it was transfected. These results thus indicate that the expression of Zac1b is most potently activated by TLR3-dependent mechanisms, although intracellular recognition mechanisms may also contribute to its induction.

We next investigated the contribution of IRF3 to TLR3-induced expression of Zac1b. We observed that the expression of Zac1b was strongly decreased in poly(I:C)-treated $IRF3^{-/-}$ MEFs, indicating that Zac1b induction by deposited poly(I:C) depended on classical TLR3/IRF3 signaling (Fig. 5A and B). However, treatment of MEFs with type I IFNs (IFN-α and IFN-β) did not induce

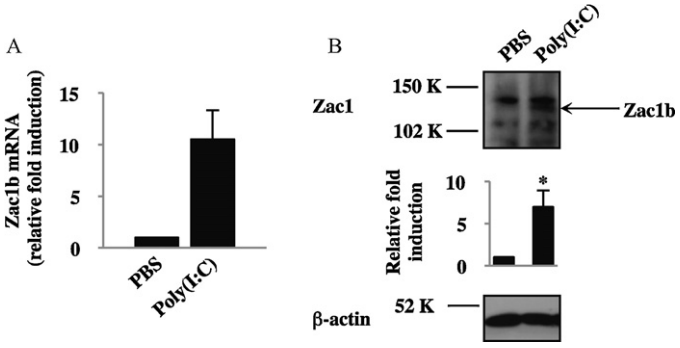

Fig. 3. Poly(I:C) induces Zac1b expression in peritoneal macrophages. Three WT mice were treated intraperitoneally with poly(I:C) (200 μg) or vehicle (PBS). Six hours after treatment, peritoneal macrophages were obtained by peritoneal lavage. (A) Zac1b mRNA expression was estimated by real-time RT-PCR. Results were expressed as fold induction compared with peritoneal macrophages of vehicle-treated mice (values in peritoneal macrophages of vehicle-treated mice set as 1). The data were normalized to GAPDH and are expressed as mean ± SD from three independent experiments. (B) Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. Comparable results were obtained in three independent experiments. (*) Significantly different from untreated control.

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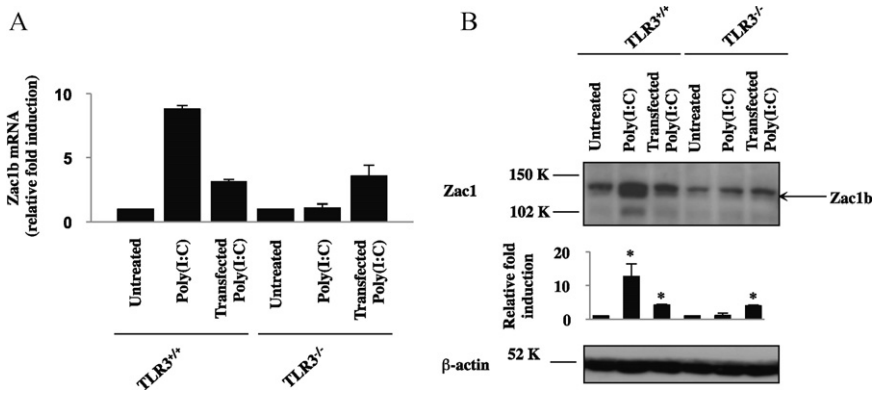

Fig. 4. Poly(I:C) induces Zac1b expression mostly through TLR3-dependent mechanisms. TLR3$^{+/+}$ and TLR3$^{-/-}$ MEFs were stimulated for 6 h with deposited (20 μg/ml) or transfected poly(I:C). (A) Zac1b mRNA expression was estimated by real-time RT-PCR. Results were expressed as fold induction compared with untreated MEFs (values in untreated MEFs set as 1). The data were normalized to GAPDH and are expressed as mean ± SD from three independent experiments. (B) Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. Comparable results were obtained in three independent experiments. (*) Significantly different from untreated control.

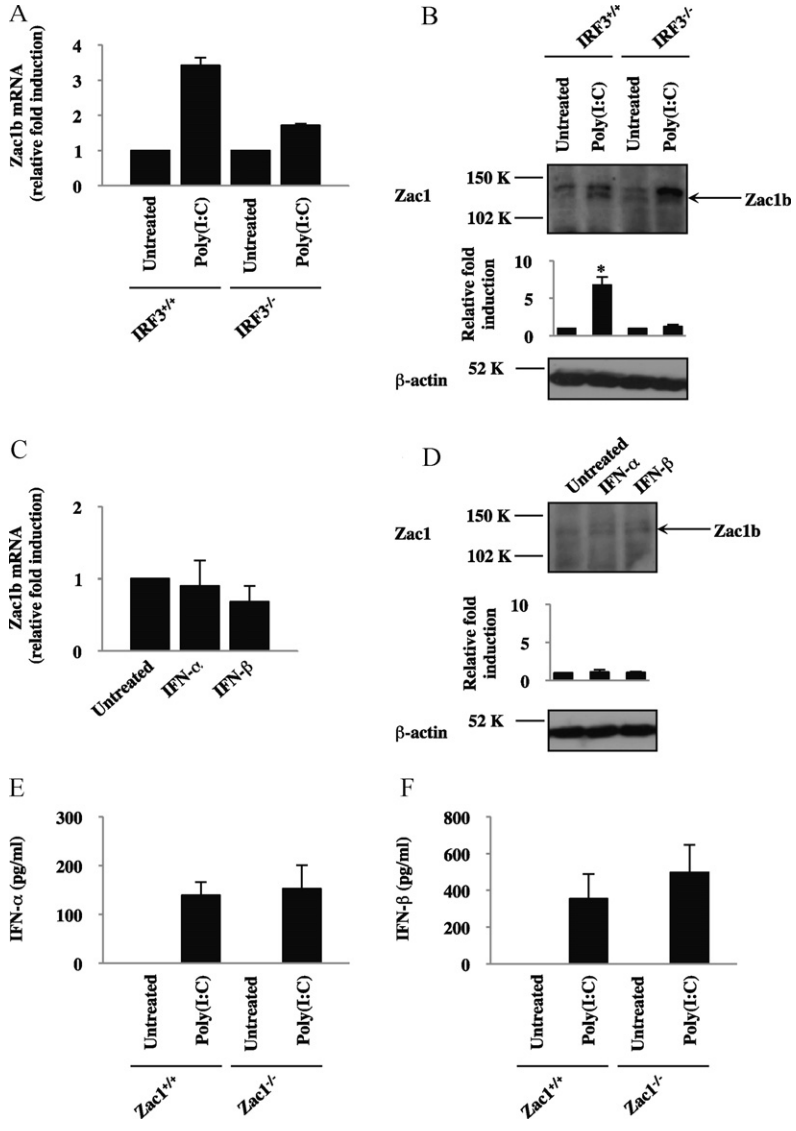

Fig. 5. Poly(I:C) induces Zac1b expression through IRF3-dependent, type I IFN-independent mechanisms. IRF3$^{+/+}$ and IRF3$^{-/-}$ MEFs were stimulated for 6 h with poly(I:C) (20 μg/ml). WT MEFs were stimulated for 6 h with IFN-α or IFN-β (100 U/ml). (A and C) Zac1b mRNA expression was estimated by real-time RT-PCR. Results were expressed as fold induction compared with untreated MEFs (values in untreated MEFs set as 1). The data were normalized to GAPDH and are expressed as mean ± SD from three independent experiments. (B and D) Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. Comparable results were obtained in three independent experiments. (*) Significantly different from untreated control. (E and F) Zac1$^{+/+}$ and Zac1$^{-/-}$ MEFs were stimulated for 24 h with poly(I:C) (20 μg/ml). (E) IFN-α and (F) IFN-β concentrations in cell supernatants were measured by ELISAs. Data are expressed as means ± SD of three independent experiments.

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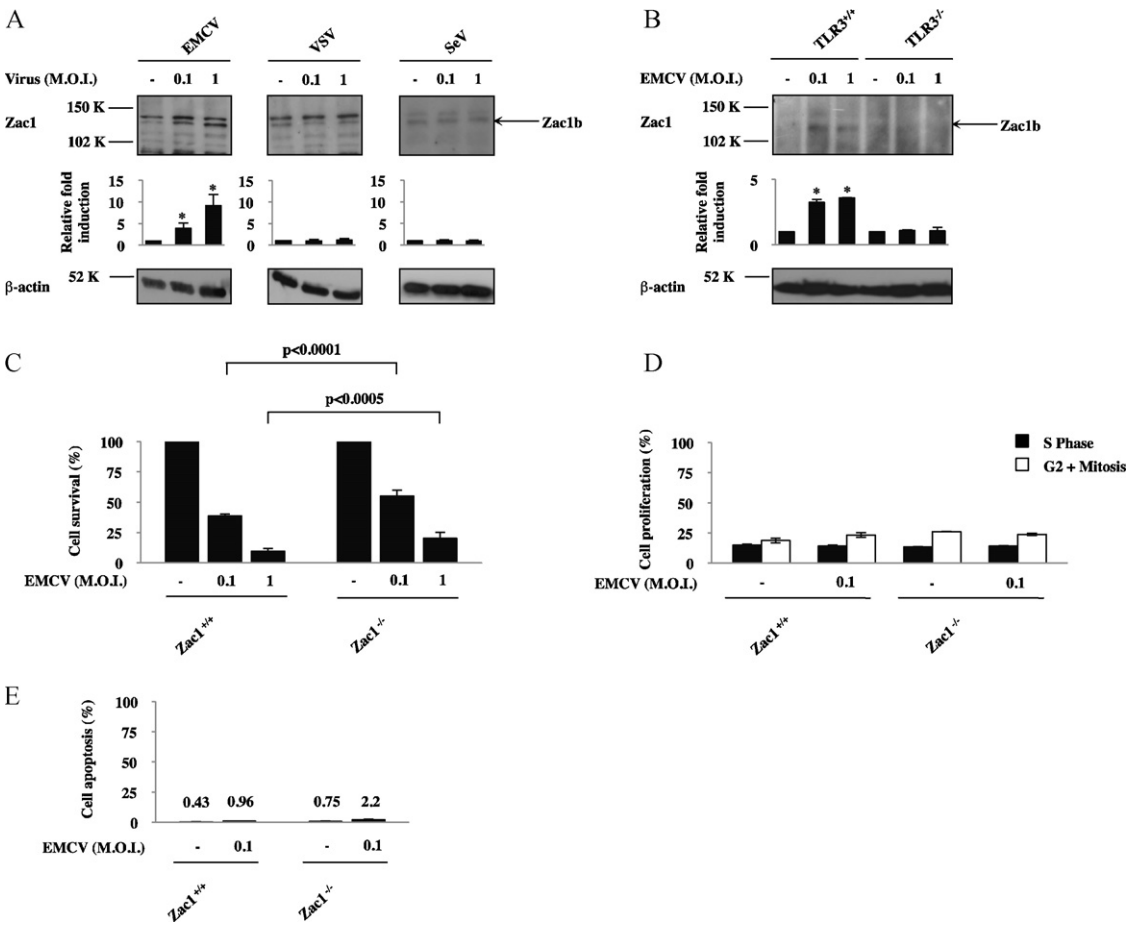

Fig. 6. Zac1$^{-/-}$ MEFs are less sensitive to EMCV-induced cell death than Zac1$^{+/+}$ MEFs. (A) WT MEFs were infected for 6 h with EMCV, VSV or SeV virus [0.1 or 1 Multiplicity of Infection (M.O.I.)]. Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. Comparable results were obtained in three independent experiments. (B) TLR3$^{+/+}$ and TLR3$^{-/-}$ MEFs were infected for 6 h with EMCV (0.1 or 1 M.O.I.). Total protein extracts were analyzed for Zac1 expression by Western blotting. β-Actin was used as loading control. Comparable results were obtained in three independent experiments. (*) Significantly different from untreated control. (C) Zac1$^{+/+}$ and Zac1$^{-/-}$ MEFs were infected for 12 h with EMCV (0.1 or 1 M.O.I.). Cell viability was determined by MTT assay. Data are expressed as mean percentage of uninfected MEFs ± SD. Comparable results were obtained in three independent experiments. (D and E) Zac1$^{+/+}$ and Zac1$^{-/-}$ MEFs were infected for 6 h with EMCV (0.1 or 1 M.O.I.). (D) Cell proliferation was determined by BrdU assay and (E) cell apoptosis was determined by TUNEL assay. Data are expressed as means ± SD of three independent experiments.

Zac1b expression (Fig. 5C and D). Thus, Zac1b expression upon TLR3 stimulation is independent on type I IFN production. We also assessed whether Zac1b induction by poly(I:C) impacted on type I IFN production by comparing IFN-α and IFN-β production in WT and Zac1$^{-/-}$ MEFs. As shown in Fig. 5E and F, there was no effect of Zac1 deletion on poly(I:C)-induced type I IFN production.

3.3. *Zac1$^{-/-}$ MEFs are less sensitive to EMCV-induced cell death than Zac1$^{+/+}$ MEFs*

Because Zac1 expression is regulated by TLR3 and IRF3, and possibly RLHs, which are central activators of antiviral immunity, the previous results unexpectedly suggested that Zac1b might be implicated in antiviral responses. We thus first examined whether viral infection induced Zac1b expression. We focused on RNA viruses, as TLR3 and RLHs primarily recognize their dsRNA replication intermediates (Alexopoulou et al., 2001; Yoneyama et al., 2004; Gitlin et al., 2006; Kato et al., 2006). MEFs were infected with three RNA viruses, namely Encephalomyocarditis virus (EMCV), Vesicular stomatitis virus (VSV) and Sendaï virus (SeV), and assessed for Zac1 expression. Western blot analyses showed that Zac1b was induced by infection with EMCV, but not with VSV or SeV (Fig. 6A). The EMCV-induced upregulation of Zac1b was totally abolished in TLR3$^{-/-}$ MEFs (Fig. 6B), indicating that TLR3 is the primary inducer of Zac1b upon EMCV infection.

As Zac1 has mainly been described as a pro-apoptotic factor (Spengler et al., 1997; Varrault et al., 1998), we next investigated whether the upregulation of Zac1 would impact on the survival of EMCV-infected cells. Survival tests showed that Zac1$^{-/-}$ MEFs indeed were modestly but significantly less sensitive to EMCV-induced cell death than WT MEFs (Fig. 6C). However, this increased survival of Zac1$^{-/-}$ MEFs was due neither to an increase in cell proliferation, nor to a decrease in apoptosis (Fig. 6D and E). The identification of the exact mechanisms by which Zac1 may regulate cell survival will thus require further experimentation. Of note, similar cell death rates were observed when WT or Zac1$^{-/-}$ MEFs were infected with VSV or SeV, or treated with the DNA damaging agents H_2O_2 and doxorubicin (Fig. 7A–D). indicating that the effects observed with EMCV were specifically due to differential Zac1 expression. Again, Zac1 expression did not impact on type I IFN production upon EMCV infection, as WT and Zac1$^{-/-}$ MEFs expressed similar levels of IFN-α and IFN-β (Fig. 8A and B).

3.4. *Zac1$^{+/-pat}$ mice do not show significant changes in their survival rates following EMCV infection*

Because Zac1 is maternally imprinted, Zac1$^{+/-pat}$ heterozygotes mice inheriting a wild-type allele from their mother are deficient

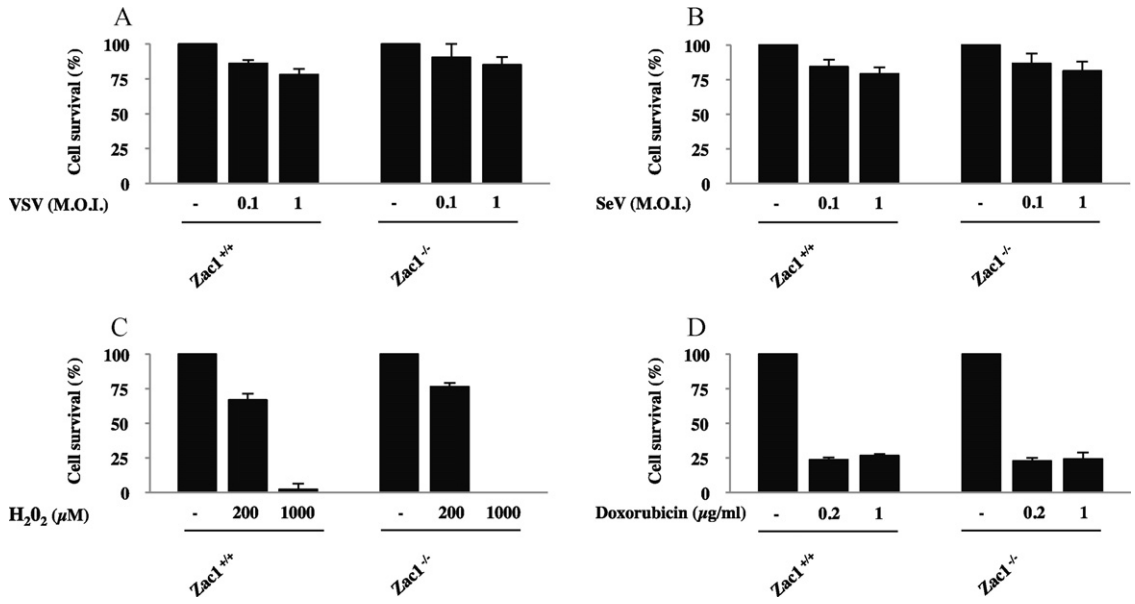

Fig. 7. Zac1$^{-/-}$ MEFs are not less sensitive to VSV-, SeV-, H_2O_2- and doxorubicin-induced cell death than Zac1$^{+/+}$ MEFs. (A–D) Zac1$^{+/+}$ and Zac1$^{-/-}$ MEFs were infected for 24 h with (A) VSV or (B) SeV (0.1 or 1 M.O.I.), or treated for 24 h with (C) H_2O_2 (200 or 1000 μM) or (D) doxorubicin (0.2 or 1 μg/ml). Cell viability was determined by MTT assay. Data are expressed as mean percentage of untreated MEFs ± SD. Comparable results were obtained in three independent experiments.

for Zac1 expression (Varrault et al., 2006). In agreement with previous reports (Varrault et al., 2006), we also observed that Zac1 mRNA was not expressed in several organs from Zac1$^{+/-pat}$ mice such as liver or lung (data not shown). Zac1$^{+/-pat}$ heterozygous were thus considered equivalent to null mutants throughout this study.

To observe the effect of the Zac1 gene inactivation on the survival of mice, we have infected Zac1$^{+/+}$ and Zac1$^{+/-pat}$ mice with EMCV. As shown in Fig. 9A–C, Zac1$^{+/-pat}$ mice did not show significant changes in their survival rates following EMCV infection. Also the production of type I IFNs upon EMCV infection was similar between Zac1$^{+/+}$ and Zac1$^{+/-pat}$ mice (Fig. 9D and E).

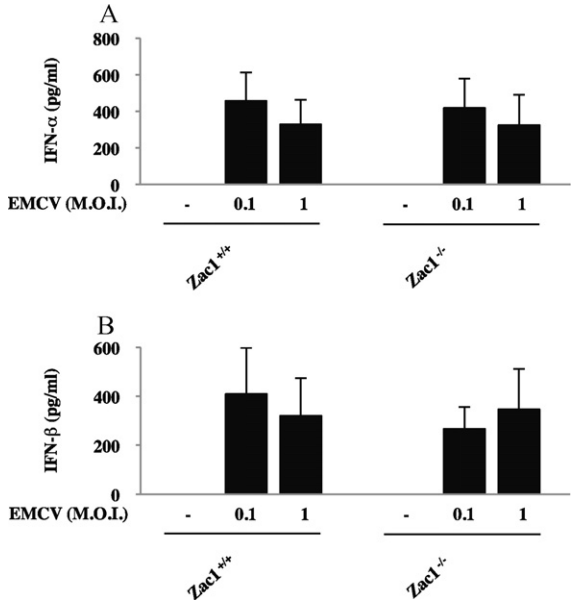

Fig. 8. Zac1 does not regulate type I IFN production upon EMCV infection. (A and B) Zac1$^{+/+}$ and Zac1$^{-/-}$ MEFs were infected for 24 h with EMCV (0.1 or 1 M.O.I.). (A) IFN-α and (B) IFN-β concentrations in cell supernatants were measured by ELISAs. Data are expressed as means ± SD of three independent experiments.

4. Discussion

In spite of the important potential functions attributed to Zac1, little is known of its physiological regulation and its biological function. Although Zac1 is quite ubiquitously expressed at the mRNA level, detection of its protein product does not always follow the expression pattern of its mRNA. Furthermore, it remained unknown whether Zac1 expression could be regulated transcriptionally, under which circumstances this regulation occurs, and what the functional consequences of this regulation, if any, could be.

Zac1 and p53 share the ability to induce concurrently apoptosis and cell cycle arrest through different and independent pathways (Spengler et al., 1997). Because of its functional parallelism with p53, and because p53 is highly expressed in response to DNA damage-inducing agents such as H_2O_2, γ-irradiations, doxorubicin, nutlins and UV irradiations (Van Dyke, 2007), we initially tested the effect of DNA damage inducers on Zac1 expression in MEFs. Surprisingly, Zac1 expression was not induced under any of the conditions tested, whereas p53 was potently induced as expected. These results thus indicate that in spite of their similar functions, Zac1 undergoes a different regulation than p53.

Some pro-inflammatory stimuli may act as potent inducers of apoptosis (Rath and Aggarwal, 1999; Ma et al., 2007; Grunnet et al., 2009). We thus sought to determine whether strong pro-inflammatory stimuli such as TNF-α, IL-1β, or TLR ligands could regulate Zac1 expression. The pro-inflammatory cytokines had no effect on Zac1 induction. Unique among the TLR ligands tested, poly(I:C), a specific TLR3 ligand, induced Zac1 protein in MEFs. Analysis of the protein and transcript of Zac1 in poly(I:C)-treated MEFs indicated that mainly the Zac1b isoform was induced. As MEFs are embryonic cells, which could limit the interpretation of the results obtained in these cells, we studied the induction of Zac1b by poly(I:C) in primary adult cells. We showed that peritoneal macrophages from mice treated intraperitoneally with poly(I:C) expressed Zac1b, thus ruling out cell-type specific effects of the regulation.

We next dissected the signaling pathway triggered by poly(I:C) to induce Zac1b expression. Poly(I:C) may activate TLR3 signaling, as well as intracellular recognition pathways involving RLHs,

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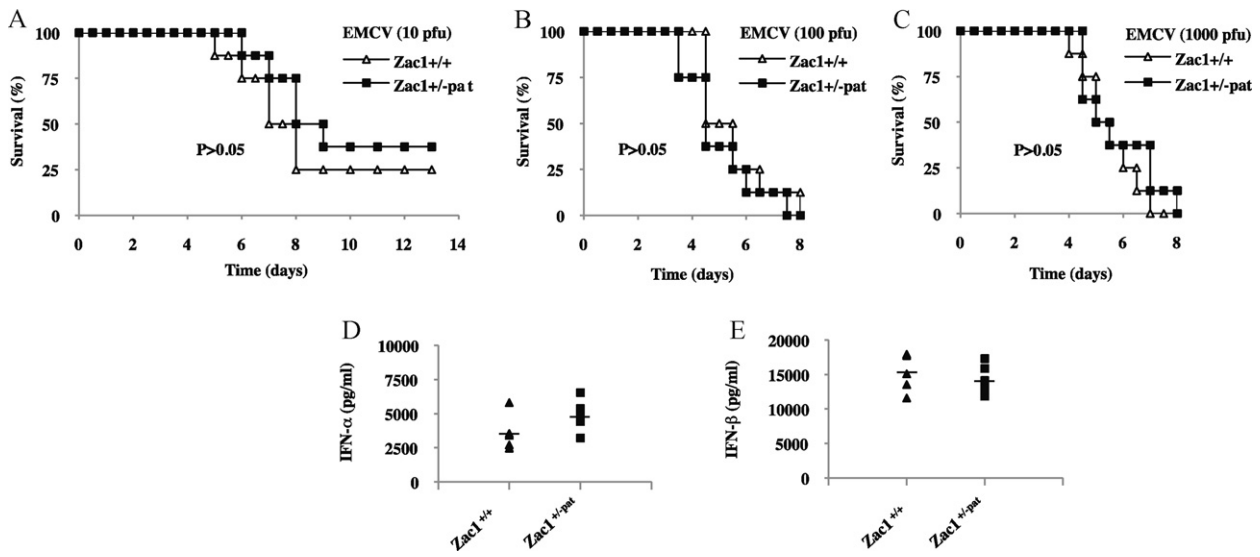

Fig. 9. Zac1$^{+/-pat}$ mice do not show significant changes in their survival rates or type I IFN production following EMCV infection. (A–C) The survival of Zac1$^{+/+}$ and Zac1$^{+/-pat}$ mice infected intraperitoneally with (A) 10, (B) 100 or (C) 1000 plaque-forming units (pfu) of EMCV was monitored through time (n = 8/group). A generalized Wilcoxon test was used to test for differences in survival. Comparable results were obtained in two independent experiments. (D–E) Zac1$^{+/+}$ and Zac1$^{+/-pat}$ mice were injected intraperitoneally with 10^7 pfu of EMCV (n = 6/group). Sera were collected 6 h after injection. (D) IFN-α and (E) IFN-β production levels were determined by ELISA. Data are expressed as means ± SD of three independent experiments.

depending on its cellular localization in endosomal vesicles or in the cytoplasm, respectively (Alexopoulou et al., 2001; Yoneyama et al., 2004; Gitlin et al., 2006; Kato et al., 2006). We thus compared Zac1 induction upon exposure of MEFs to deposited or transfected poly(I:C), and used TLR3$^{-/-}$ MEFs to estimate the contribution of both pathways. We could show that poly(I:C) most potently induced Zac1b through a classical TLR3-dependent pathway, while intracellular recognition may also upregulate Zac1 expression, but with much less potency. Interestingly, induction of Zac1b was dependent on IRF3, as shown by its reduced expression in poly(I:C)-treated IRF3$^{-/-}$ MEFs, but independent on autocrine type I IFN signaling, as treatment of MEFs with recombinant IFN-α and IFN-β failed to induce its expression. Thus, Zac1b might be under the direct transcriptional control of IRF3. Notably, whereas Zac1b induction by deposited poly(I:C) was completely abolished in TLR3-deficient MEFs, it was still faintly induced in IRF3-deficient MEFs, at least at the mRNA level, indicating that mechanisms additional to IRF3 may regulate Zac1b expression downstream of TLR3. Of note as well, Zac1 did not impact on type I IFN induction by poly(I:C).

TLR3 and RLHs are central activators of antiviral immunity that are activated by viral dsRNA replication intermediates (Alexopoulou et al., 2001; Yoneyama et al., 2004; Gitlin et al., 2006; Kato et al., 2006). We thus examined whether infection with RNA viruses, namely EMCV (Picornaviridae), VSV (Rhabdoviridae) and SeV (Paramyxoviridae) induced Zac1b expression. We observed that only infection with EMCV induced Zac1b expression. One explanation for the differential effect of EMCV compared to VSV and SeV might lie in the fact that they trigger different RLH pathways. Indeed, EMCV is thought to be detected mainly by MDA5, while VSV and SeV would be sensed mostly by RIG-I (Gitlin et al., 2006; Kato et al., 2006; Saito and Gale, 2008). However, we observed that TLR3 was the main Zac1b inducer in EMCV-infected cells, which makes this explanation less likely. Another explanation could be that VSV and SeV inhibit the induction of type I IFNs in MEFs, through undefined mechanisms (Kato et al., 2006). Thus, like other viruses, VSV and SeV might inhibit IRF3 activity (Basler et al., 2003; Cardenas et al., 2006), which could explain that they do not induce Zac1b. In contrast, EMCV potently stimulates IRF3 activity (Kato et al., 2006), which we showed promotes Zac1b expression.

Finally, we investigated the potential functional significance of Zac1b expression during EMCV infection. As Zac1 has mainly been described as a pro-apoptotic factor (Spengler et al., 1997; Varrault et al., 1998), we studied whether the upregulation of Zac1 would impact on the survival of EMCV-infected cells. We showed that Zac1$^{-/-}$ MEFs were only mildly, but yet significantly, less sensitive to EMCV-induced cell death than Zac1$^{+/+}$ MEFs. However, we could not find a straightforward explanation for this observation. Indeed, Zac1 did not seem to impact on the proliferation and apoptosis rates of EMCV-infected cells. Of note, EMCV infection mostly leads to the necrosis of the cells (Schwarz et al., 1998; Huang et al., 2009), and apoptosis rates were accordingly very low in our experiments. We also could show that Zac1 does not impact on type I IFN expression upon EMCV infection. Thus, it is probable that Zac1 impacts on the survival of EMCV-infected cells through as yet unidentified mechanisms unrelated to apoptosis. We however could show that the survival advantage conferred to Zac1-deficient cells upon EMCV infection was not related to a general increase in resistance to cell death. Indeed, Zac1$^{-/-}$ MEFs displayed survival rates similar to Zac1$^{+/+}$ MEFs in response to H_2O_2 or doxorubicin.

In vivo, EMCV infects preferentially cardiomyocytes and induces their necrosis, which eventually leads to the death of the infected animal (Kato et al., 2006). On the other hand, death of infected cells can also limit viral replication and dissemination, increasing host resistance to infection (Takaoka et al., 2003). We thus wondered what the effect, if any, of the inactivation of the Zac1 gene might be on the survival of mice infected with EMCV. We did not observe any significant change between survival rate or type I IFN production of Zac1$^{+/+}$ and Zac1$^{+/-pat}$ mice following EMCV infection. Thus, although Zac1 has some mild effect on the course of EMCV infection *in vitro*, its role during viral infection *in vivo* remains undefined. A possible explanation may lie in differences in the biology of cardiomyocytes *in vivo* compared to MEFs, the latter indeed being cultured proliferating embryonic cells.

In conclusion, this study identifies and characterizes for the first time a physiological induction of Zac1b. Indeed, we show that Zac1b is regulated through TLR3-, IRF3-, and possibly RLHs-dependent pathways, and is induced by viral infection. Zac1 however does not impact on the course of viral infection *in*

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vivo. Its exact physiological function thus remains to be further investigated.

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