Genes to Cells

TAK1 maintains the survival of immunoglobulin λ-chain-positive B cells

Hisaaki Shinohara[1]*, Takeshi Nagashima[2], Marilia I. Cascalho[3,4] and Tomohiro Kurosaki[5,6]*

[1]*Laboratory for Integrated Cellular Systems, RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan*
[2]*Division of Cell Proliferation, ART, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980-8575, Japan*
[3]*Department of Surgery, University of Michigan, Ann Arbor, MI 48109, USA*
[4]*Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA*
[5]*Laboratory for Lymphocyte Differentiation, RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan*
[6]*Laboratory for Lymphocyte Differentiation, Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan*

TAK1 (MAP3K7) mediation of the IκB kinase (IKK) complex–nuclear factor-κB (NF-κB) pathway is crucial for the activation of immune response and to perpetuate inflammation. Although progress has been made to understand TAK1 function in the B-cell receptor (BCR) signaling, the physiological roles of TAK1 in B-cell development, particularly in the bone marrow (BM), remain elusive. Previous studies suggested that the IKK complex is required for the development of immunoglobulin light chain λ-positive B cells, but not for receptor editing. In contrast, NF-κB activity is suggested to be involved in the regulation of receptor editing. Thus, NF-κB signaling in early B-cell development is yet to be fully characterized. Therefore, we addressed the role of TAK1 in early B-cell development. TAK1-deficient mice showed significant reduction of BM Igλ-positive B-cell numbers without any alteration in the BCR editing. Furthermore, the expression of survival factor *Bcl-2* was reduced in TAK1-deficient BM B cells as assessed by microarray and quantitative PCR analyses. *Ex vivo* over-expression of exogenous *Bcl-2* enhanced the survival of TAK1-deficient Igλ-positive B cells. TAK1–IKK–NF-κB signaling contributes to the survival of λ-chain-positive B cells through NF-κB-dependent anti-apoptotic *Bcl-2* expression.

Introduction

TAK1 (MAP3K7) is a member of the MAPK kinase (MAP3K) family and a pivotal signaling component that mediates the activation of the IκB kinase (IKK) complex–NF-κB pathway in response to various signaling by immunoregulatory receptors, such as the Toll-like receptor, tumor necrosis factor receptor, B-cell receptor (BCR) and T-cell receptor (Karin 2009; Hayden & Ghosh 2012). TAK1 function in these signaling pathways is essential for immune response, inflammation and consequently tumor development (Shinohara & Kurosaki 2009; Sakurai 2012).

A combination of experimental and computer modeling analyses of BCR signaling showed that NF-κB activation was triggered by a positive-feedback loop involving TAK1–IKK and CARMA1 (CARD11). BCR stimulation activates protein kinase C (PKC)-β, which in turn phosphorylates CARMA1 at Ser-668, enabling the recruitment of other signaling molecules such as Bcl-10, IKK and TAK1. TAK1–IKK interaction in the CARMA1-scaffolding

Communicated by: Sho Yamasaki
**Correspondence:* hisaaki.shinohara@riken.jp or tomohiro.kurosaki@riken.jp
Competing interests: The authors declare no competing interest.

DOI: 10.1111/gtc.12442

complex activates IKK, enabling it to phosphorylate IκBs for their subsequent degradation (Thome *et al.* 2010; Kaileh & Sen 2012). The release of NF-κB from IκB enables NF-κB translocation into the nucleus to induce target gene expressions. IKKβ-mediated phosphorylation of CARMA1 at Ser-578 results in a TAK1–IKK positive-feedback loop to amplify and maintain the NF-κB activation dynamics (Shinohara *et al.* 2007, 2014).

Key molecular events that control the activation threshold, rate and amplitude of NF-κB signaling have been described (Shinohara *et al.* 2014). However, early studies of B-cell-specific TAK1 deletion mice showed contradictory results; one study showed normal immune responses but impaired IKK–NF-κB activation induced by BCR signaling (Schuman *et al.* 2009), while another study showed no reduction in IKK–NF-κB activation but reduced humoral immune response (Sato *et al.* 2005). These discrepancies were likely due to insufficient gene deletion attributed to the use of CD19-Cre (Hobeika *et al.* 2006). Using the mb1 (CD79a)-Cre system, which has a higher recombination efficiency than the CD19-Cre, recent studies showed that TAK1 was essential for NF-κB activation in BCR signaling and immune responses in mice (Shinohara *et al.* 2014; Shinohara & Kurosaki 2016). However, the physiological significance of TAK1-dependent regulation of IKK–NF-κB signaling, including the role of NF-κB signaling itself in early B-cell development is yet to be defined. Early reports suggested that genetic disruptions of NF-κB or the expression of a dominant negative form of IκBα can affect B-cell development in the bone marrow (BM); however, single gene deletion of IKK signaling components showed no effect (Gerondakis & Siebenlist 2010). The role of NF-κB signaling in receptor editing that generates functional BCR through the rearrangement of immunoglobulin (Ig) light chain is unclear. The expression of recombination-activating gene (*Rag*) 1 and 2 that manage receptor editing was suggested to be regulated by NF-κB signaling (Verkoczy *et al.* 2005). The loss of IKK complex components showed impairment only in the development of Igλ-positive B cell, suggesting that NF-κB signaling was dispensable for editing (Derudder *et al.* 2009). However, a subsequent study reported the activation of NF-κB during receptor editing (Cadera *et al.* 2009). These observations suggested the possibility that the signaling pathway leading to NF-κB activation in BM B cells may be distinct from peripheral signaling. In fact, Bcl-10 was indispensable for NF-κB activation in mature B cells, but not for Igλ-positive B-cell development (Derudder *et al.* 2009).

To dissect possible roles of TAK1–IKK–NF-κB signaling in B-cell development, we examined the effect of B-cell-specific TAK1 deletion on the development and survival of Igλ L-chain (LC)-positive B cells. We showed that TAK1 signaling preserved the viability of λ-chain-positive B cells by maintaining *Bcl-2* expression through IKK–NF-κB signaling. Thus, our study elucidated the role of NF-κB signaling in early B-cell development.

Results

TAK1 deficiency impaired Igλ LC-positive B-cell development

We first examined the requirement for TAK1 in λ-chain-positive B-cell development in the BM. As determined by flow cytometry, a significant reduction in λ-chain-positive immature B-cell ($B220^{lo}IgM^{+}$) proportion was observed in the BM-derived B cells from $TAK1^{F/F}Mb1^{cre/+}$ mice compared to those from the control $TAK1^{+/+}Mb1^{cre/+}$ mice (Fig. 1A, B). Furthermore, the proportions of λ-chain-positive splenic follicular ($AA4.1^{-}CD21\ m^{ed}CD23^{+}$; Fo), marginal zone ($AA4.1^{-}CD21^{hi}CD23^{-}$; MZ) and transitional [$AA4.1\ (CD93)^{+}B220^{+}$] B cells were greatly reduced in TAK1-deficient mice (Fig. 1C). However, the total number of splenic and BM B220-positive cells in $TAK1^{F/F}Mb1^{cre/+}$ mice remained normal (Shinohara & Kurosaki 2016) (Fig. S1 in Supporting Information). Our results showed that TAK1 was required for λ-chain-positive B-cell development in the BM.

Receptor editing in TAK1-deficient B cells

Because TAK1 mediates IKK–NF-κB signaling in splenic B cells (Schuman *et al.* 2009; Shinohara *et al.* 2014), reduction in the number of λ-chain-positive B cells may be caused by a defect in BCR editing, thereby altering IKK–NF-κB activity (Verkoczy *et al.* 2005; Cadera *et al.* 2009). To evaluate the Vκ–Jκ recombination of BCRs in splenic and BM B cells, we carried out polymerase chain reaction (PCR) analyses and found normal use of Jκ elements in TAK1-deficient B cells (Fig. 2A). We assessed rearrangements involving the recombining sequence (RS) element and DNA breaks during RAG-mediated Igκ rearrangement, which serve as editing markers (Constantinescu & Schlissel 1997; Cadera *et al.* 2009), in pre-B cells

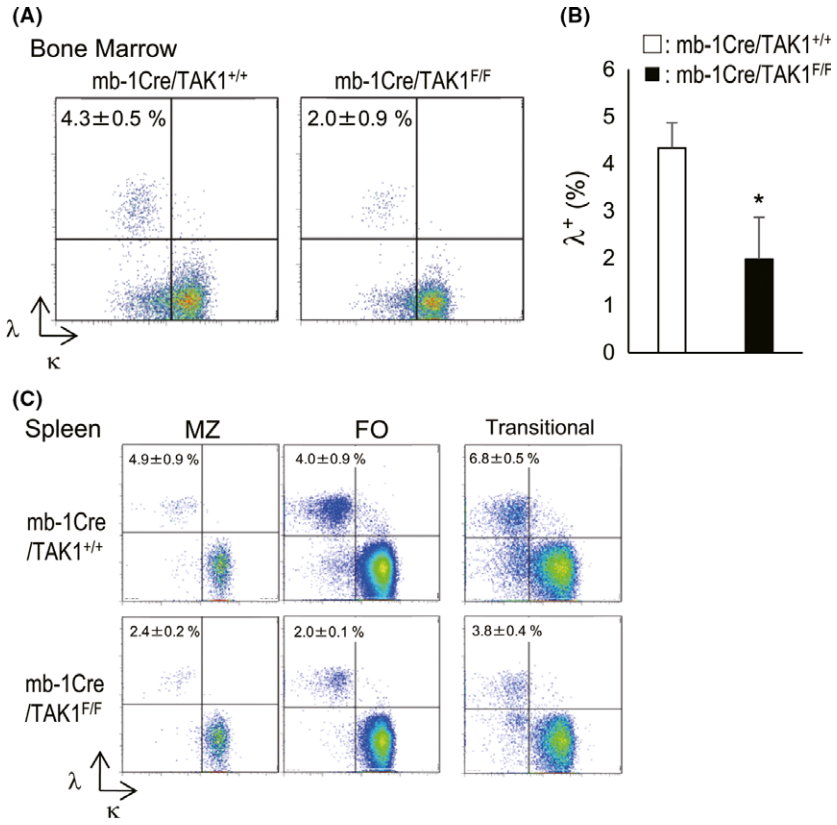

Figure 1 TAK1 deficiency impaired the development of λ-chain-positive B cells. (A) Immature B cells ($B220^{lo}IgM^{+}$) from control $TAK1^{+/+}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{+/+}$) and $TAK1^{F/F}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{F/F}$) mouse BM were stained for receptor κ-chain (κ) and λ-1-, λ-2- and λ-3-chain expressions. Number in the upper left corner of each plot indicates the mean % proportion ± standard deviation (SD) of λ-chain-positive cells ($n = 8$ mice) and is shown in the bar graph (B). $*P < 0.0001$ relative to mb-1Cre/$TAK1^{+/+}$ by Welch's *t*-test. (C) Splenic marginal zone B cells ($B220^{+}CD93^{-}CD21^{hi}CD23^{-}$-gated cells; MZ), follicular B cells ($B220^{+}CD93^{-}CD21\ m^{ed}CD23^{+}$-gated cells; Fo) and transitional B cells ($B220^{+}CD93^{+}$-gated cells; Transitional) from control $TAK1^{+/+}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{+/+}$) and $TAK1^{F/F}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{F/F}$) mice stained for κ-chain (κ) and λ-1-, λ-2- and λ-3-chain (λ) are shown. Number in the upper left corner of each plot indicates the mean % λ-chain-positive cells ± SD ($n = 3$ mice).

($B220^{lo}IgM^{-}CD25^{+}$) from $TAK1^{F/F}Mb1^{cre/+}$ and $TAK1^{+/+}Mb1^{cre/+}$ mouse BM. Neither editing markers were significantly altered (Fig. 2B–D). Furthermore, the expression of Igκ germline transcript (κGT) and *Rag* transcript in TAK1-deficient pre-B cells was comparable to that in control $TAK1^{+/+}Mb1^{cre/+}$ B cells (Fig. 2D). These results indicated that receptor editing was not altered by the loss of TAK1-mediated signaling.

To confirm this, we bred $TAK1^{F/F}Mb1^{cre/+}$ mice with quasi-monoclonal (QM) mice (Cascalho *et al.* 1996). QM mouse is an established mouse strain in which a J_H locus was replaced with the 17.2.25 V_HDJ_H segment (V_HT) encoding a 4-hydroxy-3-nitrophenylacetyl-specific mAb and the other J_H and both κ loci were deleted (V_HT/J_H^{-}, $J\kappa C\kappa^{-}/J\kappa C\kappa^{-}$, λ^{+}/λ^{+}). Therefore, QM-derived B cells exclusively express the λ-chain with a V_HT-encoded heavy chain (Kanayama *et al.* 2002). TAK1-deficient IgM-positive B-cell proportion was significantly reduced in the Igκ-deficient cells (Fig. 2E,F), showing that λ-chain-positive B-cell development in the absence of κ gene rearrangements was also regulated by TAK1.

TAK1 mediated NF-κB signaling and regulated the survival of λ-positive B cells in culture

To elucidate the mechanisms behind impaired λ-chain-positive B-cell development in TAK1-deficient mice, we preferred an *in vitro* BM culture system (Rolink *et al.* 1991; Flemming *et al.* 2003; Aiba *et al.* 2008). Pro-B cells (IgM^{-} isolated BM B cells) were

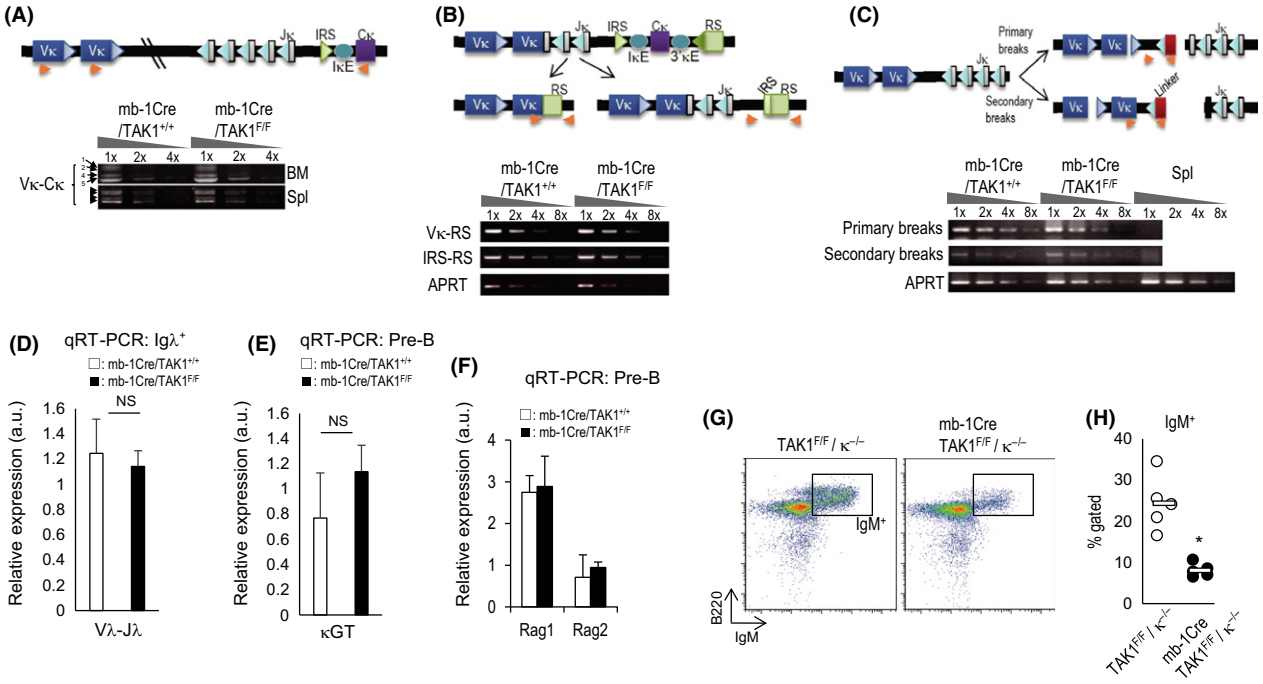

Figure 2 TAK1-deficient B cells showed normal receptor editing. (A–C) Effects of TAK1 deletion on receptor editing at the Igκ locus. Samples were diluted (1, 2, 4, or 8×) to monitor assay sensitivity. Analysis strategies and representative data from two independent experiments using pooled cells (three mice per experiment) are shown. (A) To evaluate Jκ usage, Vκ–Jκ rearrangements (Vκ–Cκ) involving Jκ1 (1→), Jκ2 (2→), Jκ4 (4→) and Jκ5 (5→) in splenic or BM B cells from control TAK1$^{+/+}$Mb1$^{cre/+}$ (mb-1Cre/TAK1$^{+/+}$) and TAK1$^{F/F}$Mb1$^{cre/+}$ (mb-1Cre/TAK1$^{F/F}$) mice were assessed by PCR. (B, C) Adenine phosphoribosyltransferase (*Aprt*) was used as loading control. (B) Endogenous Vκ-RS and IRS-RS rearrangements in pre-B cells (B220^{lo}IgM^{-}CD25^{+}) from control and TAK1$^{F/F}$Mb1$^{cre/+}$ mice were examined by PCR. (C) Primary and secondary breaks at the Igκ locus in pre-B cells from control and TAK1$^{F/F}$Mb1$^{cre/+}$ mouse BM were assessed by a ligation-mediated PCR analysis. Spleen (Spl) DNA was used as negative control, showing no break at the Igκ locus. (D) Evaluation of Igλ gene rearrangement. Vλ1–Jλ1 excision products were assessed by quantitative PCR. Relative expression values were normalized to *Aprt* (a.u., arbitrarily unit) and shown as bar graphs (mean ± SD). The data are from two independent experiments. NS, not significant relative to mb-1Cre/TAK1$^{+/+}$ by Welch's *t*-test. (E, F) The germline κ transcripts (κGT, E) and *Rag1* and *Rag2* (F) expressions in pre-B cells (B220^{lo}CD93^{+}IgM^{-}CD25^{+}) from control and TAK1$^{F/F}$Mb1$^{cre/+}$ mice normalized to *Gapdh* (a.u., arbitrarily unit). Results are from two representative experiments using pooled cells (three mice per experiment; mean ± SD). (G, H) Effects of TAK1 deletion on the development of λ-chain-positive B cells without rearrangements at the Igκ loci. Flow cytometry results of BM cells from Igκ-deficient (TAK1$^{F/F}$, V$_H$T/J$_H^{-}$, JκCκ^{-}/JκCκ^{-}, λ^{+}/λ^{+}; TAK1$^{F/F}$/κ$^{-/-}$) and Igκ/TAK1-double-deficient mice (TAK1$^{F/F}$Mb1$^{cre/+}$, V$_H$T/J$_H^{-}$, JκCκ^{-}/JκCκ^{-}, λ^{+}/λ^{+}; κ$^{-/-}$; mb-1Cre/TAK1$^{F/F}$/κ$^{-/-}$) stained with anti-B220 and anti-IgM. Box in flow cytometry plots depicts the IgM^{+} population (G). (H) IgM-positive B-cell proportions gated in (E). White (○) and black circles (●) represent Igκ-deficient and Igκ/TAK1-double-deficient mice, respectively. Horizontal bars indicate the mean (n = 5 mice). *P < 0.001 relative to TAK1$^{F/F}$/κ$^{-/-}$ by Welch's *t*-test.

cultured with IL-7 for 4 days for expansion. Withdrawal of IL-7 after culturing led to immature IgM^{+} B-cell development (Fig. 3A–D). As expected, cultures from TAK1$^{F/F}$Mb1$^{cre/+}$ BM showed fewer λ-chain-positive B cells than those from control TAK1$^{+/+}$Mb1$^{cre/+}$ BM (Fig. 3E,F). Using this system, we also examined the effects of anti-IgMs (α–μ), which mimic the trigger signals for receptor editing, on cytoplasmic Igκ level (Hertz & Nemazee 1997; Cadera *et al.* 2009). Cytoplasmic Igκ was induced in IL-7-depleted, anti-IgM-treated, and IL-7-depleted and anti-IgM-treated B cells from both TAK1-deficient and control mice (Fig. 4A,B). The normal cytoplasmic Igκ induction suggested that TAK1-deficient B cells underwent a normal receptor editing. The level of TAK1 protein in TAK1$^{F/F}$Mb1$^{cre/+}$ cells was efficiently reduced (Fig. S2A in Supporting Information); hence, we showed that this *in vitro* BM culture system can recapitulate B-cell development steps *in vivo*.

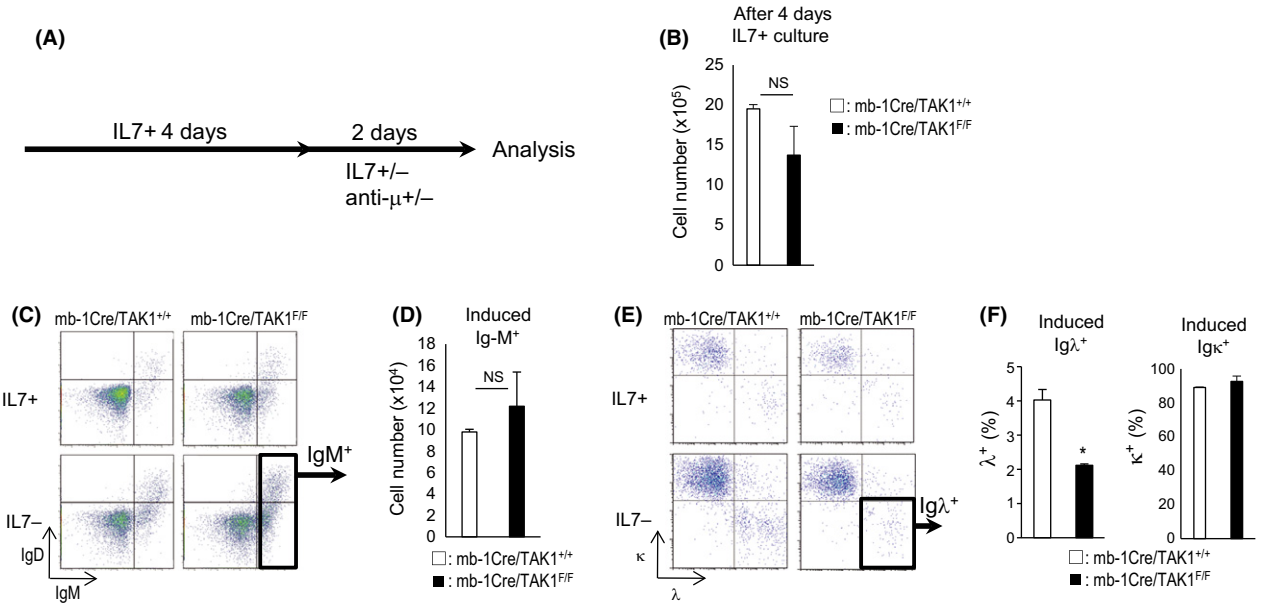

Figure 3 Generation of λ-chain-positive B cells from BM culture. (A–E) B cells (IgM^-) from control $TAK1^{+/+}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{+/+}$) and $TAK1^{F/F}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{F/F}$) mice BM (5×10^5) were cultured in the presence of 20 ng/mL murine recombinant IL-7 for 4 days. The cells were washed with medium twice and cultured in the absence (−) or presence of IL-7 (+) with or without anti-IgM $F(ab')_2$ (anti-μ, 10 μg/mL) for additional 2 days (A). The cells were recovered after 6 days of culture and analyzed. Data are from two independent experiments of triplicate cultures (three mice). (B) Cell numbers were counted after 4 days of culture in the presence of IL-7. Data represent the means of recovered cell number ± SD. NS, not significant by Welch's *t*-test. (C) The recovered cells were stained with anti-IgM and anti-IgD antibodies and subjected to flow cytometric analysis. Numbers of induced IgM^+ cells are indicated in the bar graph (mean ± SD) (D). NS, not significant by Welch's *t*-test. (E) Surface expression level of κ- (κ), λ-1-, λ-2- and λ-3-chain of IgM^+ in cells recovered from the control $TAK1^{+/+}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{+/+}$) and $TAK1^{F/F}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{F/F}$) mice was assessed by flow cytometry as described in Fig. 1A. (F) The bar graphs illustrate the surface λ- or κ-single positive B-cell population shown in (E) (the mean ± SD). $*P < 0.03$ relative to mb-1Cre/$TAK1^{+/+}$ by Welch's *t*-test.

We subsequently addressed the perturbation of IKK–NF-κB signaling in TAK1-deleted BM B cells, which was proposed in a previous study that showed TAK1 involvement in the splenic BCR signaling (Shinohara *et al.* 2014). Phosphorylation of IκBα, NF-κB1 and RelA was impaired in TAK1-deficient BM B cells regardless of the developmental derivation by IL-7 withdrawal (Fig. 4C). Although p38 and JNK phosphorylations were reduced in TAK1-deficient B cells, ERK phosphorylation was comparable to that in control $TAK1^{+/+}Mb1^{cre/+}$ B cells (Fig. 4D). Thus, TAK1 was likely to be essential for the IKK–NF-κB signaling induced by IL-7 withdrawal, which mimics the induction of BM B-cell differentiation or alternatively for a ligand-independent signaling termed the tonic signal (Lam *et al.* 1997; Meffre & Nussenzweig 2002; Monroe 2006). Previous studies showed that an efficient generation of λ-positive B cells (but not receptor editing) is dependent on IKK–NF-κB signaling, possibly by enhancing cell survival (Derudder *et al.* 2009). In the current study, TAK1-deficient λ-positive B cells were found to be more susceptible to cell death by IL-7 depletion than control $TAK1^{+/+}Mb1^{cre/+}$ cells (Figs 4E,S2B in Supporting Information). This result suggested that λ-chain-positive B-cell development may require the up-regulation of pro-survival factors through TAK1–IKK–NF-κB-mediated signaling.

Microarray analysis showed TAK1 requirement for *Bcl-2* expression

To address whether the expression of pro-survival genes was affected by TAK1 deficiency, we examined the gene expression profile of Igκ-positive B cells from control $TAK1^{+/+}Mb1^{cre/+}$ and $TAK1^{F/F}Mb1^{cre/+}$ BM (Fig. 5A). The expression of IKK–NF-κB signaling target genes *Cd86*, *IκBα*, *A20* and *Birc3* and pro-survival factor *Bcl-2* was down-regulated in TAK1-deficient cells (Fig. 5B). In contrast, the

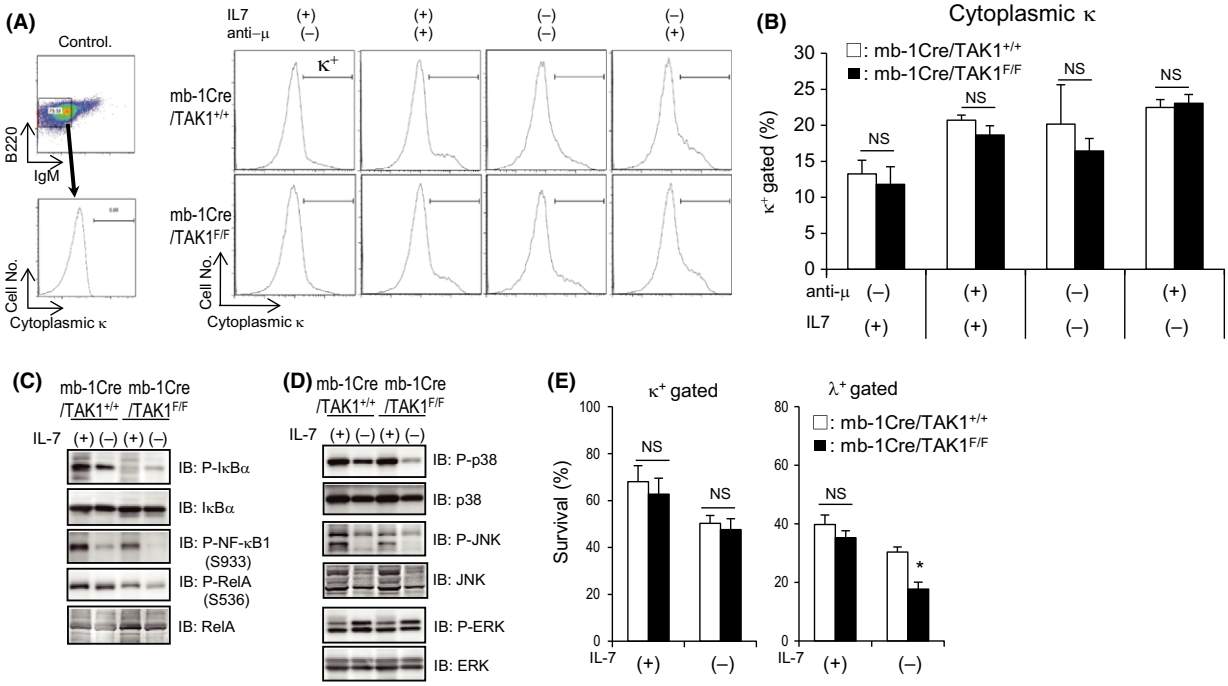

Figure 4 TAK1 deficiency impaired the survival of λ-chain-positive B cells. BM cells were cultured with or without cross-linking with anti-IgM (α–μ) to imitate a receptor-editing signal shown in Fig. 3A. Data are from two independent experiments of triplicate cultures (three mice). (A) Cells were surface-stained with anti-B220 and anti-IgM antibodies, and the cytoplasm was stained with anti-Igκ antibody. The flow cytometry plot (top left) shows B220 and IgM staining in control cells. Gated cells (box in the flow cytometry plot) are represented in the histogram (bottom left) to show cytoplasmic κ staining. The bar graph shows gated cytoplasmic κ staining of control $TAK1^{+/+}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{+/+}$) and $TAK1^{F/F}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{F/F}$) B cells. Numbers in the histograms indicate the cytoplasmic κ-positive cell numbers (mean ± SD) (B). NS, not significant by Welch's *t*-test. (C, D) Phosphorylation of IκBα, NF-κB1 and RelA (C), and p-38, JNK and ERK (D) were evaluated by Western blotting (from the recovered cells described in A). Representative data from two independent experiments are shown. (E) Survival of Igλ-positive cells from the recovered cells shown in A. The viability of κ-chain-positive ($κ^+$-gated) and λ-chain-positive ($λ^+$-gated) cells was assessed by staining with 7AAD and a fluorescent Caspase-3 substrate (gating is shown in Fig. S1 in Supporting Information). Viable cells are shown as bar graphs (mean ± SD). *$P < 0.005$; NS, not significant relative to mb-1Cre/$TAK1^{+/+}$ by Welch's *t*-test.

expression of pro-survival factors *Bcl-x*$_L$ and *Mcl-1* was not affected in these cells. These findings were confirmed by a quantitative reverse-transcriptional polymerase chain reaction analysis (qRT-PCR; Fig. 5C) and cytoplasmic staining of Bcl-2 protein (Fig. 5D).

A previous study showed that *Bcl-2* transgenic over-expression was able to restore the normal λ-chain-positive immature B-cell development from BM leukocytes lacking IKK–NF-κB signaling (Derudder *et al.* 2009). Consistent with this, *Bcl-2* transcript was reduced by TAK1 loss. These results suggested that TAK1–IKK–NF-κB signaling preserved the λ-chain-positive B-cell population by up-regulating the expression of the pro-survival factor *Bcl-2*.

TAK1-deficient λ-chain-positive B-cell survival was restored by *Bcl-2* expression

Given TAK1 requirement for the expression of pro-survival factor transcript expressions, we evaluated the ability of exogenous *Bcl-2* to rescue the viability of TAK1-deficient λ-chain-positive B cells. We also assessed the effects of transgenes introduced by retroviral transduction into TAK1-deficient BM B cells (as described in Fig. 3). TAK1 reconstitution restored the viability of TAK1-deficient λ-chain-positive B cells by ≥40%, which was comparable to that observed in control $TAK1^{+/+}Mb1^{cre/+}$ cells (Fig. 6A,4E, respectively). *Bcl-2* transgenic over-expression also restored λ-chain-positive B-cell survival (Fig. S3 in Supporting Information).

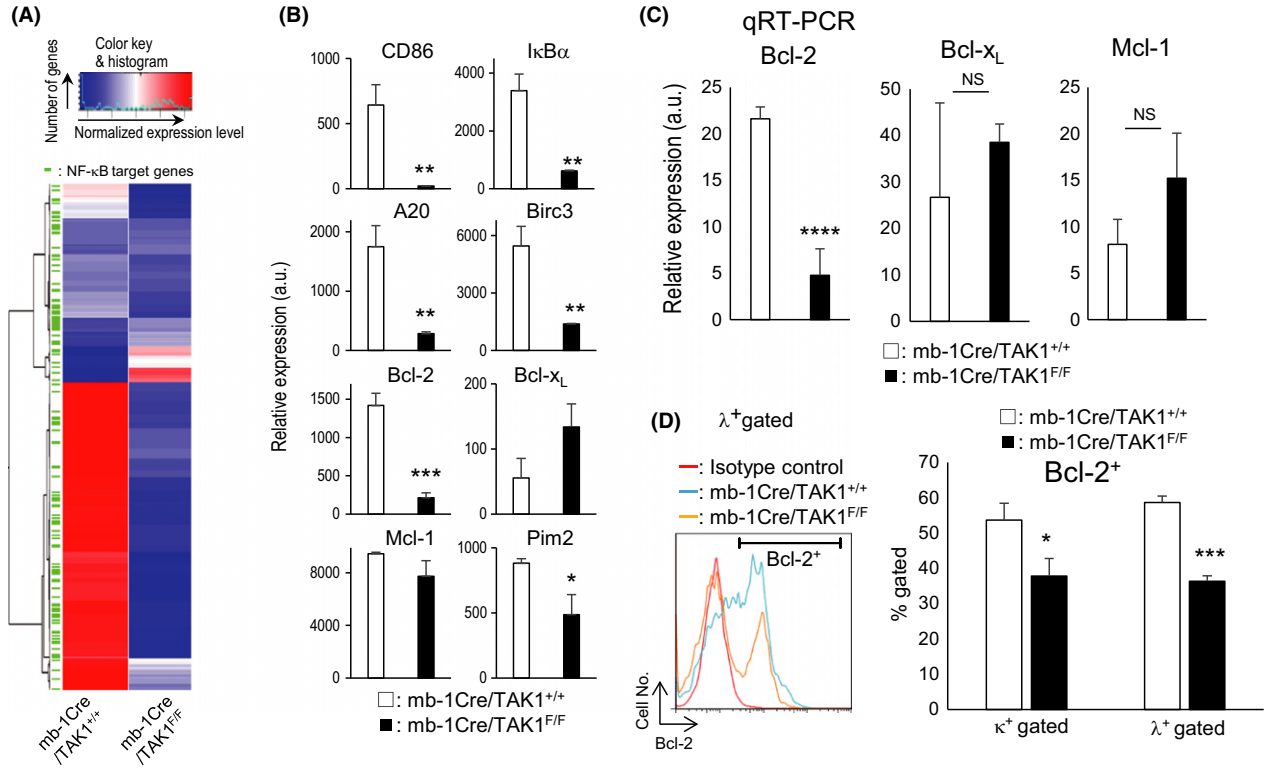

Figure 5 TAK1-deficient bone marrow B cells showed impaired pro-survival factor gene *Bcl-2* expression. (A–D) BM B cells from control (mb-1Cre/TAK1$^{+/+}$) and TAK1 B-cell-deficient (mb-1Cre/TAK1$^{F/F}$) mice were harvested as described. (A) Gene expression analysis with normalized expression levels shown as a heat map. (B) Expression level of NF-kB target genes *Cd86*, *IκBα*, *A20* and *Birc3*, and pro-survival genes *Bcl-2*, *Bcl-xL*, *Mcl-1* and *Pim2* relative to *Gapdh* (a.u.). (C) Expression level of *Bcl-2*, *Bcl-xL* and *Mcl-1* relative to *Gapdh* (a.u., arbitrary unit) as determined by qRT-PCR. (D) Bcl-2 protein expression was assessed by flow cytometry. (B–D) Data represent two independent experiments (mean ± SD). *$P < 0.05$; **$P < 0.03$; ***$P < 0.01$; ****$P < 0.001$; NS, not significant relative to mb-1Cre/TAK1$^{+/+}$ by Welch's *t*-test.

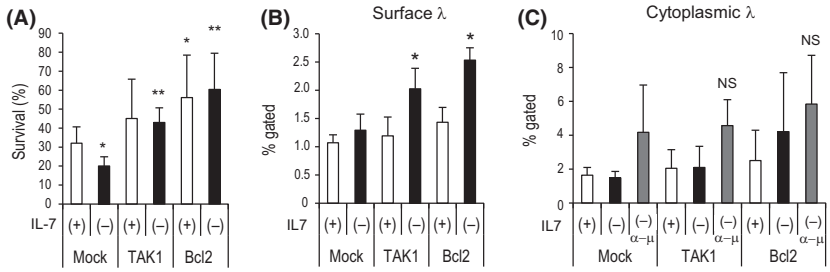

Figure 6 Role of Tak1 in IKK–NF-κB signaling and restoration of λ-chain-positive B-cell viability by *Bcl-2* over-expression. (A–D) The effect of *Tak1* or *Bcl-2* over-expression on the generation of λ-chain-positive B cells in the BM culture (described in Fig. 3A). TAK1-deficient (TAK1$^{F/F}$Mb1$^{cre/+}$) BM B cells were transduced with empty vector control (mock) or retroviral vector encoding the *Tak1* or *Bcl-2* gene. Assays were monitored by assessing the GFP-positive cell populations. Representative data from two independent experiments, each using pooled cells from three mice, are shown. (A) Survival of λ-positive cells. Viability of λ-chain-positive (λ^{+}-gated) cells was assessed using the Dead Cell Discrimination Kit. Number of viable cells is presented as the mean ± SD. *$P < 0.03$ relative to mock IL-7 (+), **$P < 0.03$ relative to mock IL-7 (−) by Welch's *t*-test. (B) Surface expression of λ-1-, λ-2- and λ-3-chain (λ) was assessed by flow cytometry. Cell populations with positive λ surface expression are presented as the mean ± SD. *$P < 0.03$ relative to mock IL-7 (−) by Welch's *t*-test. (C) Cytoplasmic expression of λ-1-, λ-2- and λ-3-chain (λ) was assessed by flow cytometry. NS, not significant compared to IL-7 (−)/anti-IgM (α–μ)-treated cells by Welch's *t*-test.

Furthermore, the proportion of cells showing surface λ-chain expression was restored by *Tak1* or *Bcl-2* expression (Fig. 6B). Consistently, *Tak1* expression restored Bcl-2 protein expression in TAK1-deficient cells (Fig. S4 in Supporting Information). The population of cells expressing cytoplasmic λ was also enhanced by *Tak1* or *Bcl-2* expression; however, these were not statistically significant even in the presence of anti-IgM (Figs 6C,S5 in Supporting Information). Anti-IgM was found to stimulate cytoplasmic λ induction in TAK1-deficient cells. Thus, these data suggested that TAK1 can regulate the survival of λ-positive B cells through the Bcl-2 expression independently from receptor editing.

Discussion

In this study, we provided evidence that may elucidate the role of NF-κB activation signal in early B-cell development. Importantly, we found that TAK1-mediated signaling in the BM was important for the maintenance of Igλ-positive cells. In mature B cells, IKK activation requires signaling components such as CARMA1, Bcl-10 and MALT1 (Thome *et al.* 2010). However, *Bcl-10* appears to be dispensable for the development of Igλ-positive cells. In contrast, the role of TNF receptor-associated factor 6 (TRAF6), which is known to mediate IKK activation in T-cell receptor signaling (Sun *et al.* 2004), is unclear in peripheral B cells (Kobayashi *et al.* 2009). Nevertheless, TRAF6 was found to regulate Igλ-positive cell development (Derudder *et al.* 2009). These observations suggested that TAK1–IKK–NF-κB signaling in the BM was probably activated by different signaling cascades from BCR in mature B cells. Although we cannot exclude the role of other signaling pathways in the Igλ-positive cell development, JNK and p38 were also impaired in TAK1-deficient B cells (Fig. 4D). The roles of these MAPKs in cell death response have been reported; however, their functions in B cells are unclear (Davis 2000; Chang & Karin 2001).

In the current study, we showed TAK1-mediated regulation of Igλ-positive cell survival using a well-established BM culture system (Hertz & Nemazee 1997; Aiba *et al.* 2008; Cadera *et al.* 2009). Although the dying cell population of freshly isolated BM cells was not well captured (Fig. S6 in Supporting Information), the culture system was able to detect a significant difference in cell survival between the control and TAK1-deficient Igλ-positive cells (Fig. 4E). In this system, anti-IgM mimics BCR-induced receptor editing. Indeed, anti-IgM was able to induce the Igλ-positive B-cell population even in the absence of TAK1 (Fig. 6C). Consistently, TAK1-deficient cells showed normal editing markers (Fig. 2). This finding suggested that the receptor-editing machinery was intact in TAK1-deficient cells.

As we could not obtain compelling transcriptome data from Igλ-positive cells, we assessed signaling and gene expression in cell population that was composed almost entirely of Igκ-positive B cells. Although the net cell number was not altered in either the culture system or in the BM (Figs 3B,S1 in Supporting Information, respectively), these cells showed defective NF-κB activation and anti-apoptotic Bcl-2 expression in both Igκ- and Igλ-positive cells (Figs 4C,5). The receptor editing restores nonfunctional receptors generated by the random gene rearrangement process (Nemazee 2006). As defect in signaling is considered equivalent to nonfunctional receptor, signaling-defective cells may undergo receptor editing because the receptor-editing machinery is intact. However, after editing, Igλ-positive cells cannot overcome the defective signaling. Hence, we postulated that Igλ-positive cells were more susceptible to cell death in the absence of TAK1.

Our findings suggest a role for TAK1 in sustaining λ-chain-positive B-cell survival through IKK–NF-κB signaling. The phenotype of TAK1-deficient λ-positive B cells was similar to that observed in IKK-deficient B cells (Derudder *et al.* 2009). Furthermore, the phosphorylation of several IKK–NF-κB signaling components and expression of IKK–NF-κB target genes were impaired in TAK1-deficient B cells. TAK1 deficiency was also found to reduce the survival of λ-positive B cells, which was restored by an exogenous expression of an anti-apoptotic gene *Bcl-2*. Together, these results indicated that TAK1–IKK–NF-κB signaling promoted λ-chain-positive B-cell survival by maintaining the expression of *Bcl-2*.

Experimental procedures

Mice

B-cell lineage-specific $TAK1^{F/F}$/mb-1 Cre knock-in (Hobeika *et al.* 2006; Shinohara *et al.* 2014) and QM (V_HT/J_H^-, $J\kappa C\kappa^-/J\kappa C\kappa^-$, λ^+/λ^+) mice were described previously (Cascalho *et al.* 1996). We generated $TAK1^{F/F}Mb1^{cre/+}$ and $TAK1^{F/F}Mb1^{cre/+}$QM mice by crossing $TAK1^{F/F}$ and/or $TAK1^{F/F}$QM mice with mb-1 Cre mice. Mice were maintained under specific pathogen-free conditions. Mice 8–12 weeks of age were used in the study. All mice experiments were conducted in accordance with guidelines approved by the RIKEN Animal Committee.

Cell culture and reagents

BM B cells were purified from raw BM cell suspensions by depleting $CD3^+$, $NK1.1^+$, $CD11b^+$, $CD71^+$, $Ly\text{-}6G^+$, $Ly71^+$, IgM^+ and IgD^+ cells with magnetic beads using the AutoMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany). The remaining B cells were cultured in Iscove's modified Dulbecco's medium that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Wako Pure Chemical Industries, Osaka, Japan) in the presence of mouse recombinant IL-7 (BioLegend, San Diego, CA, USA). Anti-mouse IgM F $(ab')_2$ fragments were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Flow cytometry

Lymphocytes were isolated from the spleen and BM of mice. The following antibodies were used for flow cytometry: anti-B220, anti-CD93, anti-CD21, anti-CD23, anti-Bcl-2, (BioLegend), anti-Igκ, anti-Igλ-1, anti-Igλ-2 and anti-Igλ-3 (BD Biosciences, Franklin Lakes, NJ, USA). Stained cells were analyzed on a FACSCanto flow cytometer (BD Biosciences). Cytoplasmic immunoglobulins were stained using the Cytofix/Cytoperm Kits (BD Biosciences) according to the manufacturer's instruction. B-cell survival was determined using the Fixation and Dead Cell Discrimination Kit (Miltenyi Biotec) or 7-amino-actinomycin D (7AAD) (BD Biosciences) staining and the Caspase-3 detection kit (FITC-DEVD-FMK) (Merck, Kenilworth, NJ, USA) according to the manufacturer's instructions.

Gene expression analysis

BM B cells were separated into $Ig\kappa^+$ and pre-B-cell ($B220^{lo}IgM^-CD25^+$) populations using the BD FACSAria cell sorter (BD Biosciences). Total RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany). For microarray analysis, GeneChip (Affymetrix GeneChip Mouse Genome 430 2.0 Array) assays were conducted according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Microarray data were analyzed as previously described (Shinohara *et al.* 2014) and were deposited in the NCBI Gene Expression Omnibus database (GEO ID: GSE74248). cDNA synthesis and quantitative PCR were conducted using the KOD SYBR qPCR kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's instructions. Primers to detect transcripts were as follows: *Rag1* (5′-TCTCCAGTAGTTCCAGAGAAGCCTGGTGGTCAG-3′ and 5anTATCTCCGGCTGTGCCCGTCACTCTTGAAACG-3′), *Rag2* (5′-ACAGTCTTGCCAGGAGGAATCTCTGTC-3′ and 5′-TATCTGAGGTCCAGTCAGGAGTCTCCATCTCACTG-3′), *Bcl2* (5′-GCTGGGGATGACTTCTCTCG-3′ and 5′-GGTGAAGGGCGTCAGGTG-3′), *Bcl2l1* (5′-CCACCTATCTGAATGACCACCTAG-3′ and 5′-TGCTGCATTGTTCCCGTAGAG-3′), κGT (5′-GGACGTTCGGTGGAGGC-3′ and 5′-GGAAGATGGATACAGTTGGTGCA-3′) (Cadera *et al.* 2009), *Mcl1* (5′-CGAACCATTAGCAGAAACTATCACAG-3′ and 5′-TGCCGCCTTCTAGGTCCTG-3′) and *GAPDH* (as a housekeeping gene for normalization) (5′-CATGTTCCAGTATGACTCCACTCA C-3′ and 5′-CTTCTCCATGGTGGTGAAGACACCAGTAG-3′).

Analysis of editing markers

PCR was carried out as previously described (Schlissel *et al.* 1993; Tiegs *et al.* 1993; Hertz & Nemazee 1997; Derudder *et al.* 2009). Splenic B cells were purified by depleting $CD43^+$ cells from raw cell suspensions using the AutoMACS Pro Separator (Miltenyi Biotec). BM B cells were isolated as described above. PCR products were subjected to electrophoresis on agarose gels. To assess Jκ usage, Vκ–Jκ rearrangements were detected by PCR using the high-fidelity polymerase KOD-plus (Toyobo Life Science), the Vκ-forward 1 degenerate primer [5′-AGCTTCAGTGGCAGTGG(A/G)TC(A/T)GG(A/G)AC-3′] and Cκ reverse primer (5′-CTTCCACTTGACATTGATGTC-3′). The PCR conditions were as follows: 94 °C for 2 min and 26 cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 1.5 min.

For the detection of Vκ-RS rearrangements and RS-IRS recombination, genomic DNA was amplified by PCR with the Vκ-forward 2 degenerate primer [5′-CCGAATTCG(G/C)TTCAGTGGCAGTGG(A/G)TC(A/G)GG(A/G)AC-3′], RS reverse primer (5′-GGACATCTACTGACAGGTTATCACAGGTC-3′) and IRS forward primer (5′-ATGACTGCTTGCCATGTAGATACCATGG-3′). The PCR conditions were as follows: 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Primers for *Aprt* were forward (5′-TGCTAGACCAACCCGCACCCAGAAG-3′) and reverse (5′-TCGTGACCGCACCTGAACAGCAC-3′).

To assess Igλ gene rearrangement, Vλ1–Jλ1 excision products were detected by quantitative PCR using genomic DNA as template. Primers used were forward (5′-GCTGCATACATCACAGATGC-3′) and reverse (5′-CAATGATTCTATGTTGTGCC-3′). The relative expression values were normalized to *Aprt*. PCR conditions were described previously (Tiegs *et al.* 1993).

For the primary and secondary κ breaks, linker ligation-mediated PCR was carried out using the BW linker oligonucleotides (5′-GCGGTGACCCGGGAGATCTGAATTC-3′) and (5′-GAATTCAGATC-3′). The linker primer (5′-CCGGGAGATCTGAATTCCAC-3′) was used with primer ko5 (5′-GCCCAAGCGCTTCCACGCATGCTTGGAG-3′) to detect primary breaks and with Vκ-forward 2 to detect secondary breaks. Touchdown PCR was carried out as follows: 94 °C for 1 min and 19 cycles starting at 92 °C for 30 s and 70 °C for 40 s, with a 0.5 °C temperature drop with each successive cycle. This was followed by 19 cycles starting at 92 °C for 30 s and 60 °C for 40 s, with 1 s added with each successive cycle.

Retroviral transduction

Tak1 or *Bcl-2* genes were cloned into the pMX–IRES–EGFP retroviral vector (a gift from T. Kitamura, University of

Tokyo, Japan). Packaging cells EcoPack2 (BD Biosciences) were transfected with the vector using the FuGENE6 reagent (Roche, Basel, Switzerland) according to the manufacturer's protocols. Retroviruses were harvested from the culture supernatant at 48 h post-transfection. After 24 h of culturing with 20 ng/mL IL-7, isolated B cells were infected with the retrovirus by centrifuging at 2000 *g* for 2 h. The transduced cells (expressing EGFP) were monitored using flow cytometry after culturing for an additional 2–3 days in media with or without IL-7.

Western blot analysis

Western blot analysis was conducted as previously described (Shinohara *et al.* 2005) using antibodies against ERK, JNK, p38, IκBα (Santa Cruz Biotechnology, Dallas, TX, USA), anti-phospho-IκBα, anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-phospho-NF-κB1 and anti-phospho-RelA (Cell Signaling Technology, Danvers, MA, USA). The ECL Plex fluorescent Western blotting system and ImageQuant LAS 4000 (GE Healthcare, Wauwatosa, WI, USA) were used for protein band detection and quantification.

Statistical analysis

Data are presented as the average ± standard deviation. Group means were compared by Welch's *t*-test using MICROSOFT EXCEL software (Redmond, WA, USA).

Acknowledgements

We would like to thank our colleagues, M. Reth for providing the mb-1 Cre mice, S. Akira for the $TAK1^{F/F}$ mice and Ms Noriko Yumoto for technical assistance. We would like to thank Editage (<www.editage.jp>) for English language editing. This study was supported by Grants-in-Aid 15K08534 and 15H01195 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Mochida Memorial Foundation and Takeda Science Foundation to H.S.

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Received: 12 June 2016
Accepted: 8 September 2016

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 B cell number in the bone marrow.

Figure S2 Survival of Igλ-positive cells. Bone marrow B cells from the contro1 $TAK1^{+/+}Mb1^{cre/+}$ (mb-1 Cre/$TAK1^{+/+}$) and $TAK1^{F/F}Mb1^{cre/+}$ (mb-1Cre/$TAK1^{F/F}$) mice were cultured in the presence (+) or absence (−) of IL-7.

Figure S3 Survival of Igλ-positive cells. TAK1-deficient ($TAK1^{F/F}Mb1^{cre/+}$) bone marrow B cells were transduced with the empty vector control (mock) or retroviral vectors encoding the *Tak1* or *Bcl-2* genes.

Figure S4 The cytoplasmic Bcl-2 expression.

Figure S5 The cytoplasmic λ-chain expression.

Figure S6 Survival of λ-positive *ex vivo* BM cells.