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

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Abstract

TAK1 (MAP3K7) mediation of the I- κ B kinase (IKK) complex–nuclear factor- κ B (NF- κ B) 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- κ B activity is suggested to be involved in the regulation of receptor editing. Thus, NF- κ B 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 exhibited significant reduction of BM Ig λ -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* overexpression of exogenous *Bcl-2* enhanced the survival of TAK1-deficient Ig λ -positive B cells. TAK1–IKK–NF- κ B signaling contributes to the survival of λ -chain-positive B cells through NF- κ B-dependent anti-apoptotic *Bcl-2* expression.

Introduction

TAK1 (MAP3K7) is a member of the MAPK kinase kinase (MAP3K) family and a pivotal signaling component that mediates the activation of the I- κ B kinase (IKK) complex–NF- κ B 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 demonstrated that NF- κ B 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 complex activates IKK, enabling it to phosphorylate I κ Bs for their subsequent degradation (Thome *et al.* 2010; Kaileh & Sen 2012). The release of NF- κ B from I κ B enables NF- κ B translocation into the nucleus to induce target gene expressions. IKK β -mediated phosphorylation of CARMA1 at Ser-578 results in a TAK1–IKK positive-feedback loop to amplify and maintain the NF- κ B activation dynamics (Shinohara *et al.* 2007; Shinohara *et al.* 2014).

Key molecular events that control the activation threshold, rate, and amplitude of NF- κ B signaling have been described (Shinohara *et al.* 2014). However, **early studies of B cell-specific TAK1 deletion mice showed contradictory results; one study demonstrated normal immune responses but impaired IKK–NF- κ B activation induced by BCR signaling (Schuman *et al.* 2009), while another study showed no reduction in IKK–NF- κ B 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- κ B 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- κ B signaling, including the role of NF- κ B signaling itself in early B cell development is yet to be defined. Early reports suggested that genetic disruptions of NF- κ B or the expression of a dominant negative form of I κ B α 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- κ B 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- κ B signaling (Verkoczy *et al.* 2005). The loss of IKK complex components showed impairment only in the development of Ig λ -positive B cell, suggesting that NF- κ B signaling was dispensable for editing (Derudder *et al.* 2009). However, a subsequent study reported the activation of NF- κ B during receptor editing (Cadera *et al.* 2009). These observations suggested the possibility that the signaling pathway leading to NF- κ B activation in BM B cells may be distinct from peripheral signaling. In fact, Bcl-10 was indispensable for NF- κ B activation in mature B cells, but not for Ig λ -positive B cell development (Derudder *et al.* 2009).

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

Results

TAK1 deficiency impaired Ig λ 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 and 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). Our results demonstrated 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- κ B 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- κ B activity (Verkoczy *et al.* 2005; Cadera *et al.* 2009). To evaluate the V κ –J κ recombination of BCRs in splenic and BM B cells, we performed polymerase chain reaction (PCR) analyses and found normal use of J κ elements in TAK1-deficient B cells (Fig. 2A). We assessed rearrangements involving the recombining sequence (RS) element and DNA breaks during RAG-mediated Ig κ rearrangement, which serve as editing markers (Constantinescu & Schlissel 1997; Cadera *et al.* 2009), in pre-B cells (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 **Ig κ germline transcript (κ GT)** 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 κ C κ ⁻/J κ C κ ⁻, 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 Ig κ -deficient cells (Fig. 2E and F), demonstrating that λ -chain-positive B cell development in the absence of κ gene rearrangements was also regulated by TAK1.

TAK1 mediated NF- κ B 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 employed 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 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 displayed fewer λ-chain-positive B cells than those from control TAK1^{+/+}Mb1^{cre/+} BM (Fig. 3E and 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 and 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); hence, we demonstrated that this *in vitro* BM culture system can recapitulate B cell development steps *in vivo*.

We subsequently addressed the perturbation of IKK–NF-κB signaling in TAK1-deficient BM B cells, which was proposed in a previous study that demonstrated 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 demonstrated 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 (Fig. 4E and S2B). This result suggested that λ-chain-positive B cell development may require the upregulation of pro-survival factors through TAK1–IKK–NF-κB-mediated signaling.

Microarray analysis demonstrated 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 factors *Bcl-2* was downregulated in TAK1 deficient cells (Fig. 5B). In contrast, the 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 demonstrated that *Bcl-2* transgenic overexpression 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 upregulating 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 and 4E, respectively). *Bcl-2* transgene overexpression also restored λ-chain-positive B cell survival (Fig. S3). Furthermore, the proportion of cells displaying 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). 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 (Fig. 6C and S5). 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- κ B activation signal in early B cell development. Importantly, we found that TAK1-mediated signaling in the BM was important for the maintenance of Ig λ -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 Ig λ -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 Ig λ -positive cell development (Derudder *et al.* 2009). These observations suggested that TAK1-IKK-NF- κ B signaling in the BM was probably activated by different signaling cascades from BCR in mature B cells. While we cannot exclude the role of other signaling pathways in the Ig λ -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 demonstrated TAK1-mediated regulation of Ig λ -positive cell survival by utilizing 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), the culture system was able to detect a significant difference in cell survival between the control and TAK1-deficient Ig λ -positive cells (Fig. 4E). In this system, anti-IgM mimics BCR-induced receptor editing. Indeed, anti-IgM was able to induce the Ig λ -positive B cell population even in the absence of TAK1 (Fig. 6C). Consistently, TAK1-deficient cells displayed normal editing markers (Fig. 2). This finding suggested that the receptor editing machinery was intact in TAK1-deficient cells.

Since we could not obtain compelling transcriptome data from Ig λ -positive cells, we assessed signaling and gene expression in cell population that was composed almost entirely of Ig κ -positive B cells. Although the net cell number was not altered in either the culture system or in the BM (Fig. 3B and S1, respectively), these cells displayed defective NF- κ B activation and anti-apoptotic Bcl-2 expression in both Ig κ - and Ig λ -positive cells (Fig. 4C and 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, Ig λ -positive cells cannot overcome the defective signaling. Hence, we postulated that Ig λ -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- κ B 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- κ B signaling components and expression of IKK–NF- κ B 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- κ B 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 κ C κ ⁻/J κ C κ ⁻, 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-6G⁺, Ly71⁺, IgM⁺, and IgD⁺ cells with magnetic beads using the AutoMACS system (Miltenyi Biotec, 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, Japan) in the presence of mouse recombinant IL-7 (BioLegend, San Diego, CA). Anti-mouse IgM F(ab')₂ fragments were obtained from Jackson ImmunoResearch (West Grove, PA).

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, -2, and -3 (BD Biosciences, Franklin Lakes, NJ). Stained cells were analyzed on a FACSCanto flow cytometer (BD Biosciences). Cytoplasmic immunoglobulins were stained using the Cytotfix/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) according to the manufacturer's instructions.

Gene expression analysis

BM B cells were separated into Igκ⁺ 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, 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). 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, 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'), κ GT (5' -GGACGTTCCGGTGGAGGC-3'
and 5' -GGAAGATGGATACAGTTGGTGCA -3') (Cadera *et al.* 2009), *Mcl1* (5' -
CGAACCATAGCAGAACTATCACAG-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 performed 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 J κ usage, V κ -J κ rearrangements
were detected by PCR using the high-fidelity polymerase KOD-plus (Toyobo Life
Science), the V κ -forward 1 degenerate primer [5' -
AGCTTCAGTGGCAGTGG(A/G)TC(A/T)GG(A/G)AC-3'], and C κ reverse primer
(5' -CTTCCACTTGACATTGATGTC-3'). The PCR conditions were: 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 detection of V κ -RS rearrangements and RS-IRS recombination, genomic DNA was
amplified by PCR with the V κ -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: 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 performed using the BW linker oligonucleotides (5' - GCGGTGACCCGGGAGATCTGAATTC-3') and (5' -GAATTCAGATC-3'). The linker primer (5' -CCGGGAGATCTGAATTCAC-3') was used with primer ko5 (5' -GCCCAAGCGCTTCCACGCATGCTTGGAG-3') to detect primary breaks and with $V\kappa$ -forward 2 to detect secondary breaks. Touchdown PCR was performed 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, 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 3,000 rpm 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, I κ B α (Santa Cruz Biotechnology, Dallas, TX), anti-phospho-I κ B α , anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-phospho-NF- κ B1, and anti-phospho-RelA (Cell Signaling Technology, Danvers, MA). The ECL Plex fluorescent Western blotting system and ImageQuant LAS 4000 (GE Healthcare, Wauwatosa, WI) were used for protein band detection and quantification.

Statistical analysis

Data are presented as the average \pm standard deviation. Group means were compared by Welch's *t*-test using Microsoft Excel software (Redmond, WA).

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Competing interests

The authors declare no competing interest.

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Figure legends

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-, 2-, and 3-chain expressions. Number in the upper left corner of each plot indicates the mean % proportion \pm standard deviation (SD) of λ -chain-positive cells (n = 8 mice) and is presented 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^{med}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-, 2-, and 3-chain (λ) were shown. Number in the upper left corner of each plot indicates the mean % λ -chain-positive cells \pm SD (n = 3 mice).

Figure 2. TAK1 deficient B cells displayed normal receptor editing. (A–C) Effects of TAK1 deletion on receptor editing at the Ig κ locus. Samples were diluted (1, 2, 4, or 8 \times) 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 J κ usage, V κ –J κ rearrangements (V κ –C κ) involving J κ 1 (1 \rightarrow), J κ 2 (2 \rightarrow), J κ 4 (4 \rightarrow), and J κ 5 (5 \rightarrow) 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 phosphoribosyl transferase (*Aprt*) was used as loading control. (B) Endogenous V κ –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 Ig κ 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 Ig κ locus. (D) Evaluation of Ig λ gene rearrangement. V λ 1–J λ 1 excision products were assessed by quantitative PCR. Relative expression values were normalized to *Aprt* (a.u., arbitrarily unit) and presented as bar graphs (mean \pm 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 (κ GT, 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 \pm SD). (G, H) Effects of TAK1 deletion on the development of λ -chain-positive B cells without rearrangements at the Ig κ loci. Flow cytometry results of BM cells from Ig κ -deficient (TAK1^{F/F}, V_HT/J_H⁻, J κ C κ ⁻/J κ C κ ⁻, λ ⁺/ λ ⁺; TAK1^{F/F}/ κ ^{-/-}) and Ig κ /TAK1-double deficient mice (TAK1^{F/F}Mb1^{cre/+}, V_HT/J_H⁻, J κ C κ ⁻/J κ C κ ⁻, 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 Ig κ -deficient and Ig κ /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.

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 μ g/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 \pm 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 \pm SD) (D). NS, not significant by Welch's *t*-test. (E) Surface expression level of κ - (κ), λ -1-, 2-, and 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 \pm SD). **P* < 0.03 relative to mb-1Cre/TAK1^{+/+} by Welch's *t*-test.

Figure 4. TAK1 deficiency impaired the survival of λ -chain-positive B cells. BM cells were cultured with or without crosslinking 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-Igk 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 \pm SD) (B). NS, not significant by Welch's *t*-test. (C, D) Phosphorylation of I κ B α , NF- κ B1, 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 Ig λ -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). Viable cells are presented as bar graphs (mean \pm SD). * $P < 0.005$; NS, not significant relative to mb-1Cre/TAK1^{+/+} by Welch's t -test.

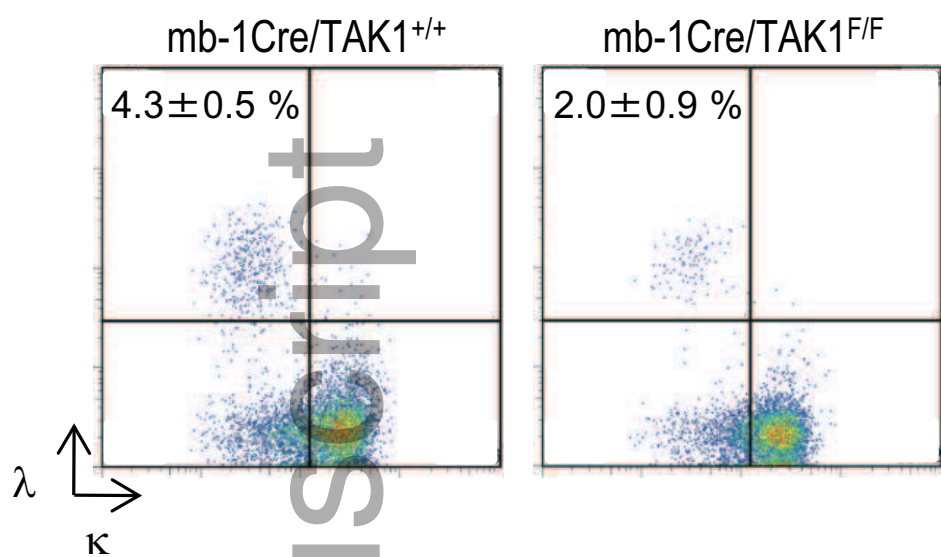
Figure 5. TAK1-deficient bone marrow B cells showed impaired pro-survival factor gene *Bcl-2* expression. (A–D) BM B cells from control (mb-1Cre/TAK1^{+/+}) and TAK1 B cell deficient (mb-1Cre/TAK1^{F/F}) mice were harvested as described. (A) Gene expression analysis with normalized expression levels shown as a heat map. (B) Expression level of NF- κ B target genes *Cd86*, *IkBa*, *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 represents two independent experiments (mean \pm 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- κ B signaling and restoration of λ -chain-positive B cell viability by *Bcl-2* overexpression. (A–D) The effect of *Tak1* or *Bcl-2* overexpression 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, were 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 \pm 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-, 2-, and 3-chain (λ) was assessed by flow cytometry. Cell populations with positive λ surface expression are presented as the mean \pm SD. * $P < 0.03$ relative to mock IL-7 (–) by Welch's t -test. (C) Cytoplasmic expression of λ -1-, 2-, and 3-chain (λ) was assessed by flow cytometry. NS,

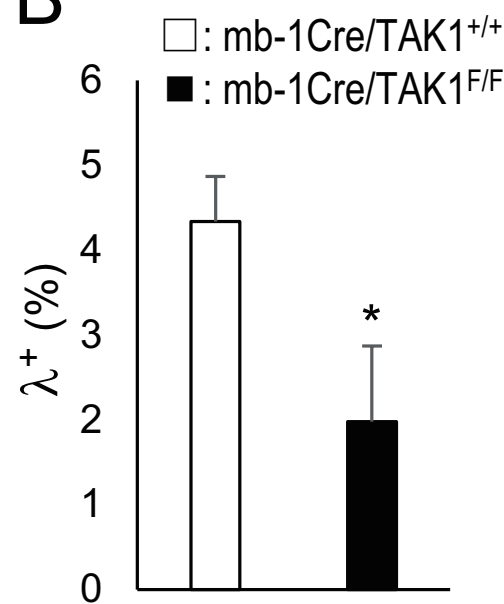
not significant compared to IL-7 (-) / anti-IgM (α - μ)-treated cells by Welch's *t*-test.

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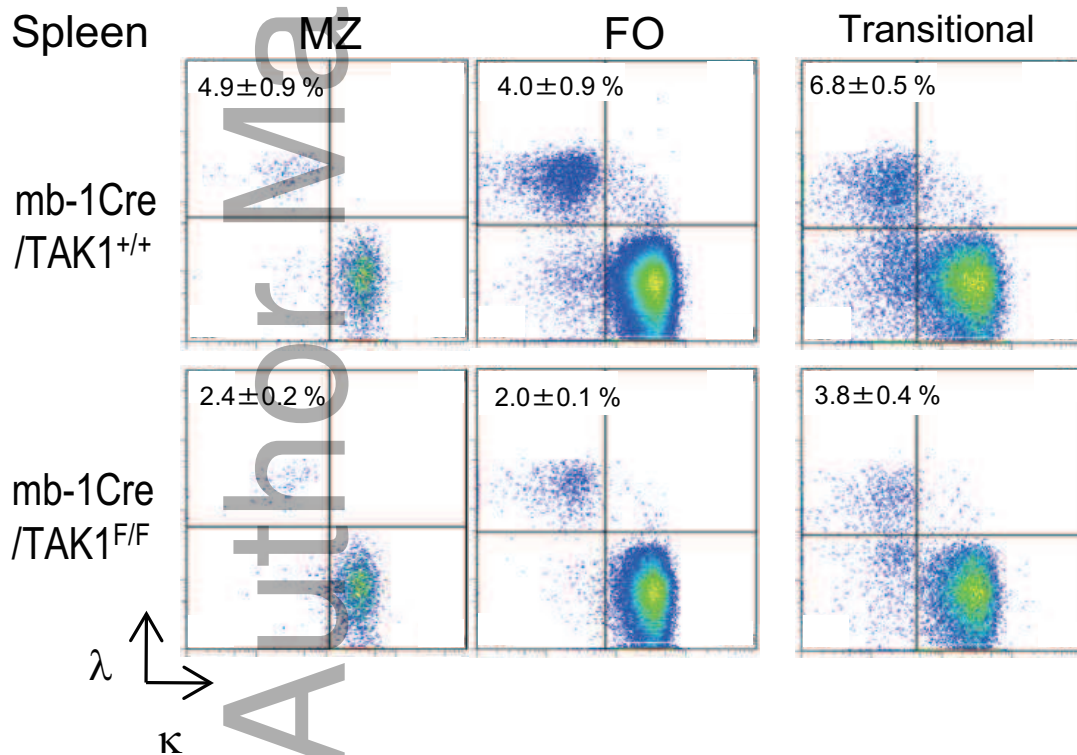
A Bone Marrow



B

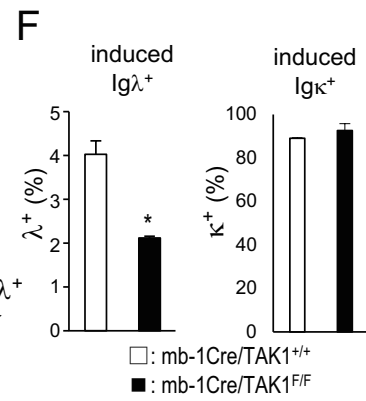
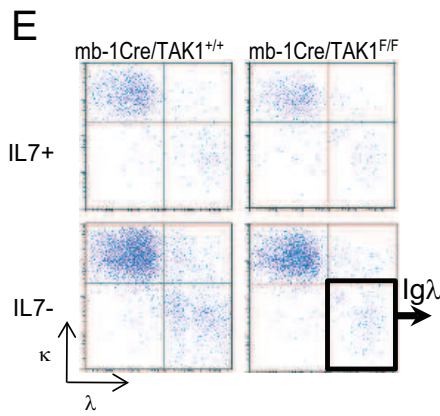
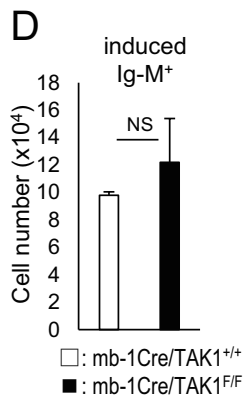
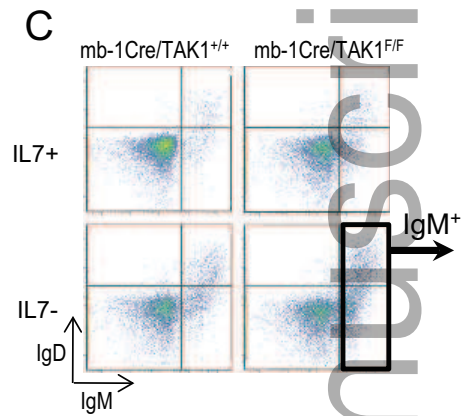
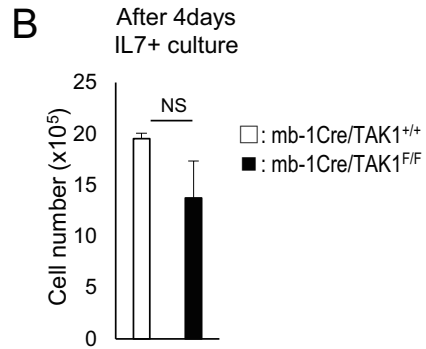
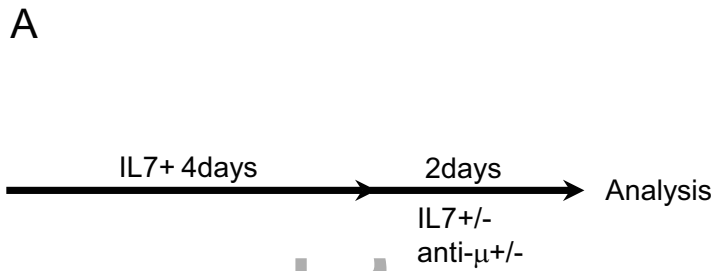


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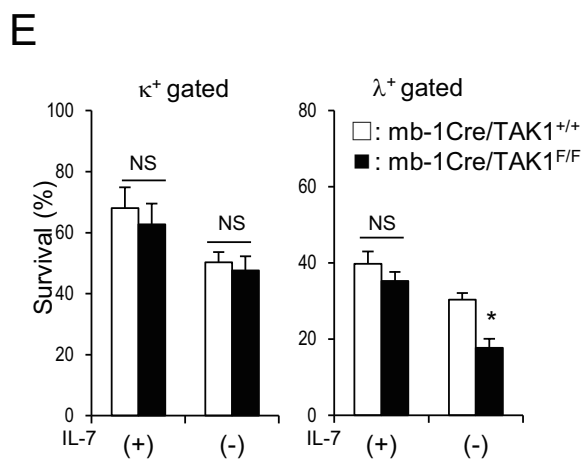
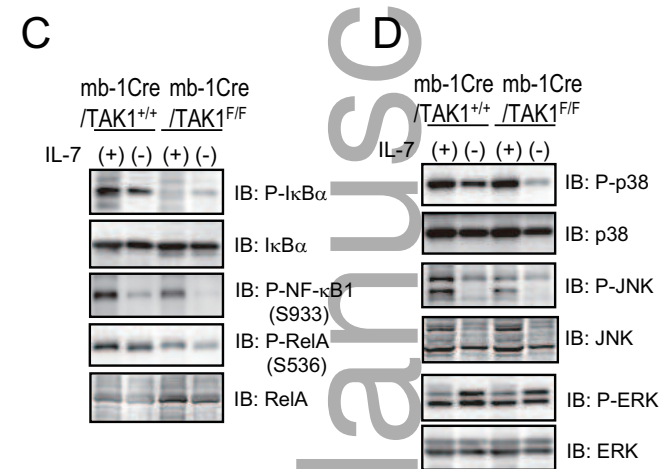
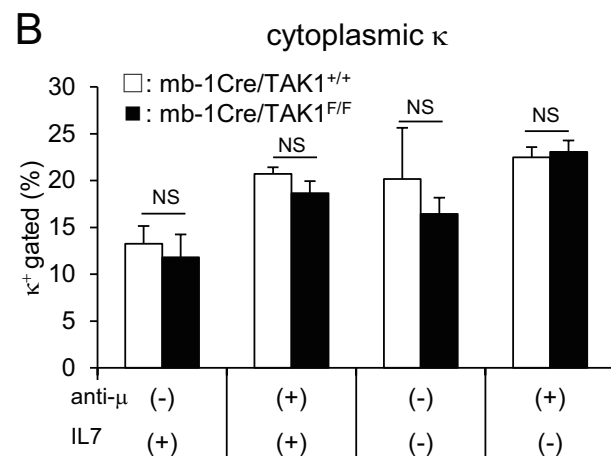
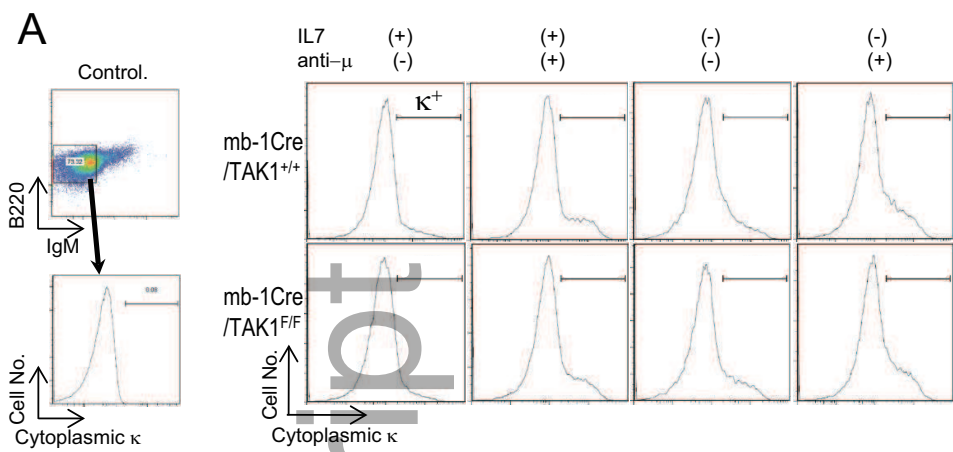


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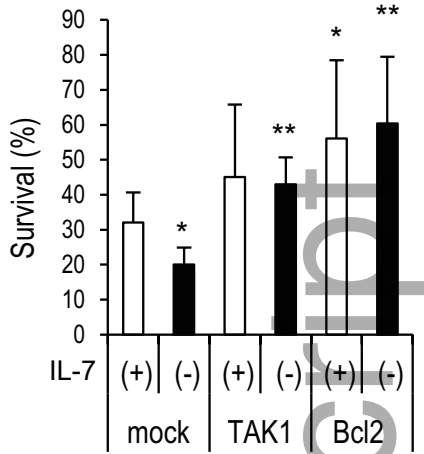
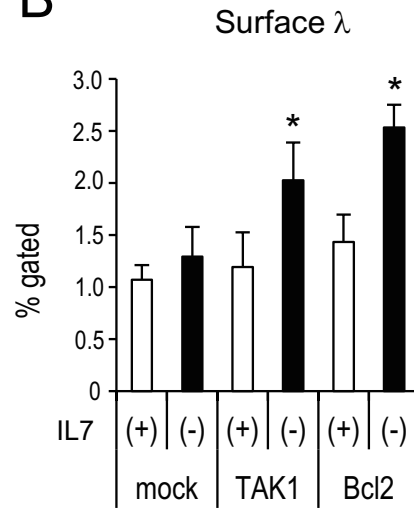
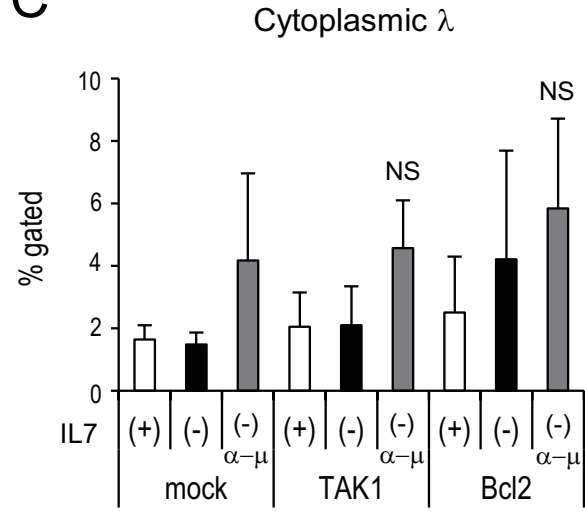


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