IκBζ facilitates protective immunity against *Salmonella* infection via Th1 differentiation and IgG production

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Inhibitor of kappa B (IκB)ζ transcription is rapidly induced by stimulation with TLR ligands and IL-1. Despite high IκBζ expression in inflammation sites, the association of IκBζ with host defence via systemic immune responses against bacterial infection remains unclear. Oral immunisation with a recombinant attenuated *Salmonella* vaccine (RASV) strain did not protect IκBζ-deficient mice against a lethal *Salmonella* challenge. IκBζ-deficient mice failed to produce *Salmonella* LPS-specific IgG, especially IgG2a, although inflammatory cytokine production and immune cell infiltration into the liver increased after oral RASV administration. Moreover, IκBζ-deficient mice exhibited enhanced splenic germinal centre reactions followed by increased total IgG production, despite IκBζ-deficient B cells having an intrinsic antibody class switching defect. IκBζ-deficient CD4⁺ T cells poorly differentiated into Th1 cells. IFN-γ production by CD4⁺ T cells from IκBζ-deficient mice immunised with RASV significantly decreased after restimulation with heat-killed RASV in *vivo*, suggesting that IκBζ-deficient mice failed to mount protective immune responses against *Salmonella* infection because of insufficient Th1 and IgG production. Therefore, IκBζ is crucial in protecting against *Salmonella* infection by inducing Th1 differentiation followed by IgG production.

Inhibitor of kappa B (IκB)ζ is a protein encoded by the NF-kappa-B inhibitor zeta (*NFKBIZ*) gene, and it contains ankyrin repeat domains and is a member of the IκB family of nuclear proteins. Activation of IκBζ in macrophages leads to IL-6 production, which is known to be mediated by the Myd88 (myeloid differentiation primary response 88) adaptor molecule. The depletion of IκBζ reduces the production of Myd88-dependent IL-6 production in various cell types, including macrophages, mouse embryonic fibroblasts, and epithelial cells. The transcription of IκBζ is rapidly induced by TLR (Toll-like receptor) stimulation and IL-1 signalling. Interestingly, it was reported that there were multiple *NFKBIZ* polymorphisms associated with susceptibility to *Streptococcus pneumoniae*-mediated invasive pneumococcal disease. Moreover, *Legionella pneumophila* infection was found to induce IκBζ-dependent IL-6 expression in lung epithelial cells.

It is well known that IκBζ is highly expressed in inflammation sites; however, the association between the IκBζ molecule and systemic immune response has not been thoroughly investigated. Several reports have suggested a cell-specific role of IκBζ. The lack of IκBζ in B cells reduces the antibody response, especially

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Results
Attenuated Salmonella vaccines did not protect IκBζ−/− mice from virulent Salmonella infection.

To evaluate the susceptibility of IκBζ-deficient (IκBζ−/−) mice against Salmonella infection, we administered virulent Salmonella typhimurium (UK-1) to wild-type and IκBζ−/− mice. In non-vaccinated mice, the survival rate of IκBζ−/− mice was not significantly different from that of WT ($p = 0.4550$, log-rank test), suggesting that innate immunity is critical for the early survival of non-vaccinated mice after Salmonella infection regardless of the existence of IκBζ (Fig. 1A). Further, we conducted assay determining Salmonella CFU from liver and spleen of the UK-1-infected mice. At day 9 after oral administration of 10^7 CFU per mice, all mice administered with UK-1 showed Salmonella colony formation in liver and spleen of IκBζ−/− mice and WT mice (Supplementary Fig. 1).

Next, to determine whether adaptive immunity could render protection against virulent Salmonella challenge in IκBζ−/− mice, we adopted a vaccine model using an attenuated Salmonella vaccine strain, RASV, in accordance with a previous study. RASV was orally administered to wild-type or IκBζ−/− mice at a dose of 10^9 CFU/mouse. After 14 d, each mouse was secondly immunised with the same dose of RASV.
immunisation, mice were orally challenged with 10^7 CFU of virulent Salmonella UK-1. Although oral vaccination with RASV successfully protected wild-type mice against virulent Salmonella infection, only 40% of IκBζ−/− mice survived (Fig. 1B) and the survival of RASV-vaccinated mice was significantly different (p = 0.0003, log-rank test). This result suggested that IκBζ−/− mice failed to mount sufficient protective immunity against Salmonella infection.

Despite the high attenuation of the vaccine strain, immune-compromised hosts contain defects in the clearance of live vaccine strains and sustain continued colonisation. Likewise, the residual attenuated RASV was identified in the spleen and liver at unimmunised and 14 d (D14) after the first administration with RASV and 14 d after the second administration (D28). There was more colonisation of Salmonella in the liver at 14 d after the first RASV administration in IκBζ−/− mice than in wild-type mice (Fig. 1C). Furthermore, at 14 d (D28) after the second RASV administration, Salmonella was still detected in liver and spleen tissues in IκBζ−/− mice (Fig. 1C,D). These results suggested that IκBζ−/− mice could not efficiently eliminate attenuated Salmonella after oral administration, possibly resulting in the insufficient protective efficacy of RASV in IκBζ−/− mice.

**IκBζ−/− led to chronic inflammation after oral administration of the RASV strain.** Because it was previously reported that IκBζ−/− mice have highly elevated levels of cytokines, including IL-6 and IL-17, the levels of inflammatory cytokines in the serum of IκBζ−/− mice were determined after RASV administration. The levels of TNF-α and IL-6 were significantly increased in IκBζ−/− mice after oral administration with RASV (Fig. 2A,B). We assumed that high level of inflammatory cytokine (TNF-α and IL-6) of IκBζ−/− mice could be associated with higher RASV burden due to insufficient induction of RASV-induced immune responses.
To compare the severity of inflammation after oral administration of the RASV strain, liver tissues from wild-type and IκBζ−/− mice were collected at unimmunised and 14 d post-administration with RASV (D14) and 14 d after the second administration (D28). The histology of liver tissues showed inflammatory foci comprising infiltrated immune cells following administration of RASV (Fig. 2C). The inflammatory foci could be found in both wild-type and IκBζ−/− mice at D14 but only in IκBζ−/− mice at D28 (Fig. 2D). Taken together, these results suggested that IκBζ−/− mice could not efficiently eliminate attenuated Salmonella after oral infection, although IκBζ−/− mice exhibited increased inflammation following RASV administration.

Oral immunisation with RASV failed to elicit an LPS-specific IgG response in IκBζ−/− mice. Previous study reported that IκBζ plays a vital role in B cells, especially the LPS-mediated T cell-independent B cell class switching response (CSR)19. Thus, we analysed the levels of antigen-specific Ab production in IκBζ−/− mice after oral RASV administration. Salmonella LPS-specific IgG levels, especially the IgG2a level, were significantly lower in IκBζ−/− mice than in wild-type mice following RASV immunisation (Fig. 3A,B). Consistent with this, the numbers of Salmonella LPS-specific IgG- or IgG2a-secreting cells significantly decreased in the spleen and MLN (Fig. 3C,D). Taken together, these results suggested that IκBζ−/− mice could not efficiently eliminate attenuated Salmonella after oral infection, although IκBζ−/− mice exhibited increased inflammation following RASV administration.

Figure 3. Salmonella LPS-specific IgG responses were significantly decreased in IκBζ−/− mice following oral administration of a RASV strain. Wild-type and IκBζ−/− mice were immunised orally with 10⁹ CFU of a RASV strain per mouse twice at 2-week intervals (n > 3 per group). (A,B) The level of Salmonella LPS-specific Ab in serum was measured at 14 d after the second immunisation. To titrate the levels of LPS-specific IgG and IgG2a, LPS-specific IgG and IgG2a antibodies were measured from the serum of WT/PBS, IκBζ−/−/PBS, WT/RASV and IκBζ−/−/RASV mice 14 d after the second immunisation. **P < 0.01 and ***P < 0.001 based on ANOVA with Bonferroni’s multiple comparison test. (C,D) The number of LPS-specific Ab-secreting cells isolated from spleen (C) and mesenteric lymph node (D) was measured by ELISPOT assay 14 d after the second immunisation with the RASV strain. (E) Splenic resting B cells were cultured in the presence of 12.5 μg/ml LPS plus 25 nM of retinoic acid (RA) and/or 0.2 ng/ml of TGF-β for 7 d to induce Ab class switching. Total IgG levels were measured by ELISA in the culture supernatant. Data are representative of three independent experiments. **P < 0.01 and ***P < 0.001 based on unpaired t-test.
switching. Therefore, the decreased production of LPS-specific IgG Ab in IκBζ−/− mice following RASV immunisation could not control an infectious pathogen or even an attenuated vaccine strain.

Germinal centre reaction after oral administration of RASV strain in IκBζ−/− mice. In our previous study, oral administration of RASV in Myd88-deficient mice resulted in chronic infection accompanied by enlarged germinal centres and hypergammaglobulinemia15, with increased levels of LPS-specific IgG responses14. Because Myd88 plays a crucial role in the stabilisation of IκB-ζ mRNA20, we checked the germinal centres and total IgG levels in IκBζ−/− mice after RASV administration. Although IκBζ−/− mice could not produce sufficient LPS-specific IgG Ab (Fig. 3A–D), the size of the germinal centres found in the spleen increased in IκBζ-deficient mice compared with those in wild-type mice after RASV administration (Fig. 4A,B). Moreover, the levels of total IgG also highly increased in the serum of IκBζ-deficient mice after RASV administration (Fig. 4C). Taken together, IκBζ−/− mice failed to produce protective Ag-specific Ab and were finally infected even by an attenuated bacterial strain despite exhibiting a high degree of inflammation.

IFN-γ producing Th1 responses were impaired in IκBζ−/− mice. According to a recent study, IκBζ−/− T cells cannot differentiate to the Th17 subset because the interaction between the transcription factor RORγ complex and IκBζ is needed for differentiation to Th17 cells5. When we analysed T cells and B cells, the percentages of T cells and B cells in IκBζ−/− mice are not significantly different with that of WT mice (Supplementary Fig. 2). To determine whether IκBζ−/− CD4+ T cells have some defect in differentiating into the helper T cell lineage, naïve CD4+ T cells were cultured in vitro with conditioned media for each helper T cell lineage. IκBζ−/− CD4+ T cells exhibited decreases in both IFN-γ-producing Th1 cells compared to the levels in wild-type cells (Fig. 5A,B). The Th1 response, as well as Salmonella LPS-specific Ab production, have been revealed to be essential for successful protection against Salmonella infection21. IκBζ−/− mice produced markedly decreased levels of LPS-specific IgG2a Ab following RASV immunisation (Fig. 3C). To determine whether the reduced IgG2a production could be due to a reduced Th1 response, the levels of IFN-γ secreting CD4+ T cells were analysed following heat-killed RASV stimulation of splenocytes isolated from RASV immunised mice. Wild-type CD4+ T cells successfully induced IFN-γ production following heat-killed RASV re-stimulation (Fig. 5B,C). However, the IFN-γ-producing CD4+ T cells were significantly decreased in the IκBζ−/− mice (Fig. 5B,C). These results suggested that IκBζ is crucial for the Ag-specific Th1 cell response and further production of Ag-specific IgG, especially IgG2a Ab. IκBζ deficiency failed to resist Salmonella infection even with an attenuated strain due to a decreased Th1 response followed by decreased levels of IgG2a.

Figure 4. Germinal centre reaction after oral administration of a RASV strain in vivo. Wild-type and IκBζ−/− mice were orally immunised with 10^9 CFU of a RASV strain per mouse twice at 2-week intervals. (A) Representative H&E image of the spleen from an unimmunized mouse, 14 d after the first RASV oral administration (D14) and 14 d after the second RASV oral administration (D28). (B) Follicle size measured from histologic images. (C) Fourteen days after the second oral immunisation with RASV, the total IgG Ab level was measured in the serum. Data are representative of three independent experiments. ns, not significant; *P < 0.05 and ***P < 0.001 based on ANOVA with Bonferroni’s multiple comparison test.
κB marrow derived macrophages obtained from WT and IκBζ−/− mice. The levels of LPS-specific Ab responses in RASV-vaccinated IκBζ−/− mice were detected in IκBζ−/− mice. However, the levels of total IgG were markedly increased in RASV-vaccinated WT mice. But the percentages of IFN-γ producing CD4+ T cells were measured. (B) Representative intracellular staining results of IFN-γ produced by CD4+ T cells were measured. (C) The percentages of IFN-γ-producing CD4+ T cells are shown. Data are representative of three independent experiments. *P < 0.05 and **P < 0.01 based on unpaired t-test. (B, C) Splenocytes isolated from RASV-immunised wild-type and IκBζ−/− mice were re-stimulated by co-culturing with 10 heat-killed RASV per cell for 3 d. IFN-γ produced by CD4+ T cells were measured. 

Discussion

IκBζ−/− mice exhibit severe and chronic head and cervical inflammation, especially in aged mice17,22. Thus, they are widely used as an animal model of Sjögren syndrome22. One of the most common symptoms of Sjögren syndrome is mucosal dryness, which disrupts the mucosal barrier function. Because of this impaired host barrier function, mice with IκBζ deficiency may be more susceptible to infection by pathogenic organisms18. We hypothesised that the persistent invasion of exogenous microorganisms could be a trigger for the chronic inflammation. Furthermore, failure in innate and adaptive immunity could contribute to the chronic inflammation in IκBζ−/− mice.

IκBζ plays a critical role in eradicating pathogens such as Streptococcus pneumoniae and Legionella pneumophila23. Individuals with a single nucleotide polymorphism in the IκBζ allele are more likely to succumb to pneumococcal infection7, and IκBζ regulates the expression of IL-6 in human monocytes in response to D39, a wild-type strain of Streptococcus pneumoniae24. In the current study, we found that mice with IκBζ deficiency developed chronic inflammation but failed to mount specific immune responses, including LPS-specific Ab production and Salmonella-specific T cell responses.

In addition to adaptive immunity induced by the RASV vaccination, innate immunity associated with IκBζ can affect on the protection against Salmonella. To confirm this, we performed Salmonella infection in bone marrow derived macrophages obtained from WT and IκBζ−/− mice, and found that higher bacterial colonies were detected in IκBζ−/− mice as compared to WT mice (Supplementary Fig. 7). This means that macrophage can play a role in Salmonella suppression in an IκBζ-dependent manner at early stage of infection.

To clarify the role of IκBζ in the induction of acquired immunity, we used a live attenuated recombinant Salmonella vaccine strain (RASV) to induce Salmonella-specific T cell and B cell responses. Typically, Salmonella invades via the gastro/oral route through contaminated food or water, causing salmonellosis accompanied by fever and diarrhoea14,15. Salmonella organisms possess a variety of antigenic molecules such as LPS, flagellin, and lipoprotein, which can also bind to TLR to trigger the innate immune responses24–26. Previously, we showed that Myd88 deficiency in mice mediated hypergammaglobulinemia with increased LPS-specific IgG Ab after oral administration of RASV15. Furthermore, we showed that WT mice which were adoptively transferred with sera from RASV-vaccinated Myd88−/− mice were partly protected against pathogenic Salmonella infection14. Contrary to Myd88, it was suggested that IκBζ is a critical regulator of TLR-mediated class switch recombination (CSR) in B cells, and mice with IκBζ deficiency have impaired type 1 T cell-independent Ab responses27; thus, we presumed that the LPS-specific response might be reduced after RASV administration in IκBζ−/− mice. Indeed, the levels of LPS-specific Ab responses in RASV-vaccinated IκBζ−/− mice were significantly lower than those in RASV immunised WT mice. However, the levels of total IgG were markedly increased in RASV-vaccinated mice.

Figure 5. IFN-γ producing Th1 responses were impaired in IκBζ−/− mice. (A) Naive CD4+ T cells were cultured for 5 d with 2 μg/ml anti-CD3 and anti-CD28 antibody as induced to differentiate into Th1 cell with 10 ng/ml of IL-12. Under Th1 differentiation conditions, the intracellular IFN-γ on CD4+ T cells were analysed. *P < 0.05 and **P < 0.01 based on unpaired t-test. (B, C) Splenocytes isolated from RASV-immunised wild-type and IκBζ−/− mice were re-stimulated by co-culturing with 10 heat-killed RASV per cell for 3 d. IFN-γ produced by CD4+ T cells were measured. (B) Representative intracellular staining results of IFN-γ synthesised by CD4+ T cells. (C) The percentages of IFN-γ-producing CD4+ T cells are shown. Data are representative of three independent experiments. *P < 0.05 and **P < 0.01 based on ANOVA with Bonferroni's multiple comparison test.

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In IκBζ−/− mice compared to those in RASV-vaccinated WT mice. Thus, we confirmed that the LPS-specific Ab response, which represents the type 1 T cells-independent Ab response, was impaired in IκBζ−/− mice after RASV vaccination. To check how much *Salmonella*-specific IgG was in the total IgG of RASV-vaccinated IκBζ−/− mice, we conducted ELISA using whole cells of RASV as an antigen. The levels of anti-RASV IgG were higher in the serum of RASV-vaccinated IκBζ−/− compared with those in the serum of RASV-vaccinated WT mice (Supplementary Fig. 3). These data suggest that T cell-dependent and type 2 T cell-independent antibody production responses may occur in IκBζ deficiency after RASV vaccination. However, the higher levels of total antibody and RASV-specific antibody did not confer a protective effect against lethal RASV challenge.

The type 1 helper T cell (Th1) response is strongly induced in the host after *Salmonella* infection, which secretes IFN-γ, which activates macrophages and monocytes and makes them more potent in capturing and digesting *Salmonella*, and RASV vaccination. To check how much *Salmonella* and RASV-specific antibody did not confer a protective effect against lethal RASV infection, we conducted ELISA using whole cells of RASV as an antigen. The levels of anti-RASV IgG were higher in the serum of RASV-vaccinated IκBζ−/− mice compared with those in the serum of RASV-vaccinated WT mice (Supplementary Fig. 3). These data suggest that T cell-dependent and type 2 T cell-independent antibody production responses may occur in IκBζ deficiency after RASV vaccination. However, the higher levels of total antibody and RASV-specific antibody did not confer a protective effect against lethal RASV challenge.

In addition, IκBζ mediates mucosal barrier function through secretion of inflammatory cytokines in epithelial cells. Thus, it was supposed that the weaker barrier function of IκBζ−/− mice allowed the invasion of commensal bacteria, which might also influence the occurrence of chronic inflammation. In our study, we administered RASV via the oral route, and disseminated RASV via a leaky gut might induce systemic infection. In addition, we found that higher levels of IFN-γ-positive cells are found in non-vaccinated IκBζ−/− mice as compared to those in non-vaccinated WT mice. We presume that the impaired mucosal barrier function of IκBζ−/− mice could lead higher level of IFN-γ producing CD4+ T cell against some commensal bacteria which genetically close with *Salmonella* organism.

Nramp1 is a well-known factor for Fe2+-dependent reactive oxygen species production by phagocytes, and the 129 Sv mice having Nramp1G169D are more resistant to *Salmonella* than C57BL/6 mice having homozygous mutation of Nramp1G169D allele. Thus, in the current study, we confirmed that both of mixed-background 129Sv-ICR IκBζ−/− mice and littermate WT control mice have susceptible Nramp1G169D allele (Supplementary Fig. 8).

In the current study, we hypothesised that IκBζ−/− mice could be protected from pathogenic *Salmonella* infection through immunisation. However, the oral administration of RASV to IκBζ−/− mice did not result in the production of high levels of LPS-specific antibody, and the mice were more susceptible to challenge with a lethal strain of *Salmonella* because of impaired T cell and B cell responses. Therefore, we presume that the reduced expression or the defective function of IκBζ in some populations might be associated with their higher susceptibility to specific pathogenic infections and that they can also be infected by the administration of an attenuated strain of bacteria which is given for vaccination. Thus, the administration of live attenuated vaccine strains should be avoided or re-considered for individuals with insufficient induction of adaptive immune responses due to an IκBζ hypomorphic mutation. Overall, we suggest that IκBζ is critical to mediate vaccine-induced adaptive immunity as well as innate immunity.

**Materials and Methods**

**Mice and bacteria.** IκBζ−/− mice were provided by Prof. Shizuo Akira (IFReC, Osaka University, Japan). It was known that inbred C57BL/6 genetic background IκBζ−/− mice is embryonic lethal. Thus we used IκBζ−/− mice on a mixed 129Sv-ICR mice. All experiments were conducted with IκBζ−/− and their littermate WT control mice obtained from mating between mixed 129Sv-ICR IκBζ−/− mice and littermate WT control mice have susceptible IκBζ−/− mice. The mice used in this study were maintained in an experimental facility of Kangwon National University under specific pathogen-free conditions. All animal experiments, including the RASV immunisation and UK-1 challenge experiment, were approved by the Institutional Animal Care and Use Committees (IACUC) of Kangwon National University (Permit Number: KW-160201-2) and were performed in accordance with approved guidelines and regulations. Attenuated *Salmonella typhimurium* N9241 (ΔpabA1516 ΔpabB232 ΔasdA16 ΔaraBAD3 ΔrelA198: araC7RasJacl1 (ATG) TT containing pYA3620) and the virulent *Salmonella* strain UK-1 used in this study were kindly provided by Roy Curtiss, III (Arizona State University). All strain of bacteria was cultured in Luria-Bertani media at 37 °C in a shacking incubator, and prepared as previously reported. For immunisation, RASV (10⁸ CFU/mouse) was orally administered and secondly immunised at the same dose after 14 d as the second immunisation. UK-1 was challenged at a dose of 10⁷ CFU/mouse 14 d after the final RASV oral administration. We confirmed that all mice were killed by administration of 2 × 10⁶, 10⁷, and 5 × 10⁷ CFU per mouse (Supplementary Fig. 4). The administration of *Salmonella* doses was checked by plating serial dilutions onto XLD agar (Becton, Dickinson, MD, USA) plates.

**ELISA and ELISPOT.** ELISA and ELISPOT were conducted according to a previous study. Briefly, 5 μg/ml *Salmonella typhimurium*-derived LPS (Sigma-Aldrich) in 50 mM sodium bicarbonate (Sigma) was coated on 96-well immunoplates (Falcon) and incubated overnight at 4 °C. Next, the immunoplates were aspirated and washed 3–5 times using 1X PBS containing 0.05% Tween-20 (1X PBS-T). After the washing step, immunoplates were blocked with 1% BSA for 2 h at 37 °C, and samples were added to plates with samples diluted 2-fold serially starting with a 1/16 dilution in 0.1% BSA, followed by incubation for 2 h at 37 °C. Goat anti-mouse IgG, and IgG2a antibodies conjugated with HRP (Southern Biotechnology Associates) were diluted at a 1:5000 ratio in 1X
PBS-T and added to each well, followed by incubation at 37 °C. For colour development, the substrate solution (TMB, Surmodics) was added, and the reaction was stopped by adding 0.5 N HCl. The colour development was measured at 450 nm on an ELISA reader (Microplate spectrophotometer; Molecular Devices), and antibody titres were calculated as reciprocal log₂ titres.

For ELISPOT assays, 5 μg/ml Salmonella typhimurium-derived LPS (Sigma-Aldrich) in 50 mM sodium bicarbonate (Sigma) was coated on 96-well nitrocellulose immunoplates and incubated overnight at 4 °C. Next, the immunoplates blocked with RPMI 1640 (Gibco) supplemented with 10% foetal bovine serum (Gibco), and serially diluted mononuclear cells were plated. After incubation for 4 h at 37 °C in a 5% CO₂ incubator, 1:5000 diluted goat anti-mouse IgG, and IgG2a antibodies conjugated with HRP (Southern Biotechnology Associates) were applied to each well. For colour development, a peroxidase substrate (3-amino-9-ethylcarbazole kit; Moss) was applied, and the number of Ab-secreting cells was counted with the aid of a stereomicroscope (SZ2-ILST; Olympus).

**In vitro CD4⁺ T cell differentiation.** To prepare primary lymphocytes, spleen, mesenteric, inguinal, axillary, and superficial cervical lymph node were obtained from WT and Is-Bζ⁻/⁻ mice and mechanically ground through a nylon mesh. The ground tissue was added to ACK lysing buffer to remove red blood cells. CD4⁺ T cells were negatively selected using a CD4⁺ T cell isolation kit (Miltenyi Biotech) and stained with a fluorescence-conjugated antibody. CD25⁺CD4⁺CD44lowCD62Lhi naïve CD4⁺ T cells were sorted using an Aria II instrument (Becton, Dickinson, MD, USA) in the Central Laboratory of Kangwon National University. Gating strategy was shown in Supplementary Fig. 5. Then, 10⁵ naïve CD4⁺ T cells were seeded onto 96 well plates pre-coated with anti-CD3, and anti-CD28 antibody with RPMI 1640 media supplemented with 10% FBS (base condition media). Conditioned media contained 10 ng/ml IL-2, 10 ng/ml IL-12, and 10 μg/ml anti-IL-4 Ab for Th1. After 5 d of culture at 37 °C in a 5% CO₂ incubator, cells were stimulated with 50 ng/ml PMA, 1 μg/ml ionomycin, and brefeldin A for 4 h. Cells were analysed with a FACsverse instrument and Flowjo program.

**Stimulation with heat-killed RASV.** To inactivate RASV bacteria, cell suspension was incubated on 100 °C and 10 min. The spleens from unimmunised and RASV-immunised mice were harvested at 14 d after the second immunisation. Splenocytes were seeded at 2 × 10⁶ cells/well on 96 well cell culture plates and re-stimulated with 10 heat-killed bacteria per cell of RASV for 3 d at 37 °C in a 5% CO₂ incubator. Before harvest, 5 μg/ml of brefeldin A was added into cell culture media. Harvested cells were stained with PerCP-Cy5.5 conjugated anti-mouse CD4 antibody and LIVE/DEAD™ Fixable Near-IR Dead cell staining kit separating live and dead cell (Invitrogen™). After surface marker staining, intracellular cytokine staining was conducted with IC Fixation buffer and intra-cellular IFN-γ was stained with APC-conjugated anti-mouse IFN-γ antibody according to the manufacturer’s protocol (Invitrogen™).

**In vitro class switching response.** Splenic resting B cells were isolated using CD43 microbeads (Miltenyi Biotech) in accordance with the manufacturer’s procedure. A total of 5 × 10⁶ CD43⁺ B cells were seeded on a 24 well plate and cultured for 3 d with 12.5 μg/ml LPS or in a combination of 25 nM retinoic acid and/or 0.2 ng/ml TGF-β. Cultured supernatants were analysed for the concentration of polyclonal IgG Ab level by ELISA.

**Statistical analysis.** To compare the differences between the two experimental groups, we used the Student’s t-test. We compared multiple groups using a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). Values of P < 0.05 were considered significant at a 95% confidence interval. Survival (Kaplan-Meier) curves were compared using the log-rank test (GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA)).

**References**

1. Schuster, M., Annemann, M., Plaza-Sirvent, C. & Schmitz, I. Atypical IkappaB proteins - nuclear modulators of NF-kappaB signaling. *Cell Commun Signal* **11**, 23, https://doi.org/10.1186/1478-811x-11-23 (2013).
2. Yamamoto, M. et al. Regulation of Toll/IL-1 receptor-mediated gene expression by the inducible nuclear protein IkappaBZeta. *Nature* **430**, 218–222, https://doi.org/10.1038/nature02738 (2004).
3. Okamoto, K. et al. IkappaBZeta regulates T(H)17 development by cooperating with ROR nuclear receptors. *Nature* **464**, 1381–1385, https://doi.org/10.1038/nature08922 (2010).
4. Yamazaki, S., Muta, T. & Takeshige, K. A novel IkappaB protein, IkappaBzeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem* **276**, 27657–27662, https://doi.org/10.1074/jbc.M103426200 (2001).
5. Setson, D. B. & Medzhitov, R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* **24**, 93–103, https://doi.org/10.1016/j.immuni.2005.12.003 (2006).
6. Chapman, S. J. et al. NFkBIZ polymorphisms and susceptibility to pneumococcal disease in European and African populations. *Genes Immun* **11**, 319–325, https://doi.org/10.1038/gene.2009.76 (2010).
7. Lorenz, J., et al. Legionella pneumophila-induced IkappaBZeta-dependent expression of interleukin-6 in lung epithelium. *Eur Respir J* **37**, 648–657, https://doi.org/10.1183/09031936.00200009 (2011).
8. Johansen, C. et al. IkappaBZeta is a key driver in the development of psoriasis. *Proc Natl Acad Sci USA* **112**, E5825–5833, https://doi.org/10.1073/pnas.1509971112 (2015).
9. Touma, M. et al. Impaired B cell development and function in the absence of IkappaBNS. *J Immunol* **187**, 3942–3952, https://doi.org/10.4049/jimmunol.1002109 (2011).
10. Miyake, T. et al. IkappaBZeta is essential for natural killer cell activation in response to IL-12 and IL-18. *Proc Natl Acad Sci USA* **107**, 17680–17685, https://doi.org/10.1073/pnas.1012977107 (2010).
11. Horber, S. et al. The Atypical Inhibitor of NF-kappaB, IkappaBZeta, Controls Macrophage Interleukin-10 Expression. *J Biol Chem* **291**, 12851–12861, https://doi.org/10.1074/jbc.M111.218825 (2016).
12. Hildebrand, D. G. et al. IkappaBZeta is a transcriptional key regulator of CCL2/MCP-1. *J Immunol* **190**, 4812–4820, https://doi.org/10.4049/jimmunol.1300089 (2013).
13. Park, S. M. et al. MyD88 signaling is not essential for induction of antigen-specific B cell responses but is indispensable for protection against Streptococcus pneumoniae infection following oral vaccination with attenuated Salmonella expressing PspA antigen. *J Immunol* **181**, 6447–6455 (2008).
14. Ko, H. J. et al. Innate immunity mediated by MyD88 signal is not essential for induction of lipopolysaccharide-specific B cell responses but is indispensable for protection against Salmonella enterica serovar Typhimurium infection. *J Immunol* **182**, 2305–2312, https://doi.org/10.4049/jimmunol.0801980 (2009).
15. Ko, H. J. et al. Expansion of Th1-like cells during chronic Salmonella exposure mediates the generation of autoimmune hypergammaglobulinemia in Myd88-deficient mice. *Eur J Immunol* **42**, 618–628, https://doi.org/10.1002/eji.201141748 (2012).
16. Lee, S. J. et al. Dual Immunization with SseB/Flagellin Provides Enhanced Protection against Salmonella Infection Mediated by Circulating Memory Cells. *J Immunol* **199**, 1335–1361, https://doi.org/10.4049/jimmunol.1601357 (2017).
17. Ueta, M. et al. Stat6-independent tissue inflammation occurs selectively on the ocular surface and perioral skin of I kappa B-zetamediated *Salmonella* infection. *Invest Ophthalmol Vis Sci* **49**, 3387–3394, https://doi.org/10.1167/iovs.08-2691 (2008).
18. Kim, Y. et al. The resident pathobiont Staphylococcus xylosus in Nfkbiz-deficient skin accelerates spontaneous skin inflammation. *Sci Rep* **7**, 6348, https://doi.org/10.1038/s41598-017-05740-z (2017).
19. Hanhira-Tatsuzawa, F. et al. Control of Toll-like receptor-mediated T-cell-independent type 1 antibody responses by the inducible nuclear protein IkappaB-zeta. *J Biol Chem* **289**, 30925–30936, https://doi.org/10.1074/jbc.M114.553230 (2014).
20. MaruYama, T., Sayama, A., Ishii, K. I. & Muta, T. Screening of posttranscriptional regulatory molecules of IkappaB-zeta. *Biochem Biophys Res Commun* **469**, 711–715, https://doi.org/10.1016/j.bbrc.2015.12.068 (2016).
21. Pashine, A., John, B., Rath, S., George, A. & Bal, V. Th1 dominance in the immune response to live Salmonella typhimurium requires bacterial invasiveness but not persistence. *Int Immunol* **11**, 481–489 (1999).
22. Okuma, A. et al. Enhanced apoptosis by disruption of the STAT3-I kappa B-zeta signaling pathway in epithelial cells induces Sjogren's syndrome-like autoimmune disease. *Immunity* **38**, 450–460, https://doi.org/10.1016/j.immuni.2012.11.016 (2013).
23. Sundaram, K. et al. IkappaBzeta Regulates Human Monocyte Pro-Inflammatory Responses Induced by Streptococcus pneumoniae. *PLoS One* **11**, e0161931, https://doi.org/10.1371/journal.pone.0161931 (2016).
24. Stotts, C. J., Schembri, M. A., Sweet, M. J. & Kapetanovic, R. When bacterial infections persist: Toll-like receptor-inducible direct antimicrobial pathways in macrophages. *Journal of leukocyte biology* **103**, 35–51, https://doi.org/10.1007/s00780-015-1398-8 (2018).
25. Sivick, K. E. et al. Toll-like receptor-deficient mice reveal how innate immunity influences Salmonella virulence strategies. *Cell Host Microbe* **15**, 203–213, https://doi.org/10.1016/j.chom.2014.01.013 (2014).
26. Kwon, H. C. et al. Natural resistance to Salmonella typhimurium in different inbred mouse strains. *Immunology* **119**, 63–70, https://doi.org/10.1111/j.1365-2567.2006.02173.x (2006).
27. Hormaeche, C. E. Genetics of natural resistance to salmonellae in mice. *Immunity* **2**, 503–515, https://doi.org/10.1016/0899-8016(89)90035-3 (1989).
28. Brown, D. E. et al. House Dust Mite Allergens and the Induction of Monocyte Interleukin 1beta Production That Triggers an IkappaBzeta-Dependent Granulocyte Macrophage Colony-Stimulating Factor Release from Human Lung Epithelial Cells. *Am J Respir Cell Mol Biol* **53**, 400–411, https://doi.org/10.1165/rcmb.2014-0370OC (2015).
29. Brown, D. E. et al. Salmonella enterica causes more severe inflammatory disease in C57BL/6N Rnapg1169 mice than C57BL/6 mice. *Veterinary pathology* **50**, 867–876, https://doi.org/10.1177/0300985813478213 (2013).
30. Hormaeche, C. E. Genetics of natural resistance to salmonellae in mice. *Immunology* **37**, 319–327 (1979).
31. Hormaeche, C. E. Natural resistance to Salmonella typhimurium in different inbred mouse strains. *Immunology* **37**, 311–318 (1979).
32. White, J. K., Mastroeni, P., Popoff, J. F., Evans, C. A. & Blackwell, J. M. Scl1a1-mediated resistance to Salmonella enterica serovar Typhimurium and Leishmania donovani infections does not require functional inducible nitric oxide synthase or phagocyte oxidase activity. *Journal of leukocyte biology* **77**, 311–320, https://doi.org/10.1182/jlb.11073014 (2005).
33. Song, J. H. et al. Anti-viral and Anti-Inflammatory Activities of Pochonin D, a Heat Shock Protein 90 Inhibitor, against Rhinovirus Infection. *Biomol Ther (Seoul)*, 711–715, https://doi.org/10.25921/3.6 (2017).
34. Hormaeche, C. E. Natural resistance to Salmonella typhimurium in different inbred mouse strains. *Immunology* **37**, 311–318 (1979).
35. White, J. K., Mastroeni, P., Popoff, J. F., Evans, C. A. & Blackwell, J. M. Scl1a1-mediated resistance to Salmonella enterica serovar Typhimurium and Leishmania donovani infections does not require functional inducible nitric oxide synthase or phagocyte oxidase activity. *Journal of leukocyte biology* **77**, 311–320, https://doi.org/10.1182/jlb.11073014 (2005).
36. Song, J. H. et al. Antiviral and Anti-Inflammatory Activities of Pochonin D, a Heat Shock Protein 90 Inhibitor, against Rhinovirus Infection. *Biomol Ther (Seoul)*, 711–715, https://doi.org/10.25921/3.6 (2017).

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Author Contributions
J.H.A. performed all experiments and data analysis and wrote the manuscript; J.C., Y.I.K. and B.E.K. contributed to design experiments, analyse data, and review the manuscript; G.S.L., S.I.Y., S.G.K., P.H.K., M.N.K., H.Y. and B.A.V. conducted data collection and analysis; Y.S.C. and H.J.K. contributed to conception and design of the study and supervised study.

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