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Upregulated expression of the IL-9 receptor on TRAF3-deficient B lymphocytes confers the immunoglobulin isotype switching responsiveness to IL-9 in the presence of antigen receptor engagement and IL-4

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Abstract

The pleiotropic cytokine interleukin-9 (IL-9) signals to target cells by binding to a heterodimeric receptor consisting of the unique subunit IL-9 receptor (IL-9R) and the common subunit γ chain shared by multiple cytokines of the γ chain family. In the present study, we found that the expression of IL-9R was strikingly up-regulated in mouse naïve follicular B cells genetically deficient in TRAF3, a critical regulator of B cell survival and function. The highly up-regulated IL-9R on *Traf3*$^{-/-}$ follicular B cells conferred responsiveness to IL-9, including IgM production and STAT3 phosphorylation. Interestingly, IL-9 significantly enhanced class switch recombination (CSR) to IgG1 induced by B cell antigen receptor (BCR) crosslinking plus IL-4 in *Traf3*$^{-/-}$ B cells, which was not observed in littermate control (LMC) B cells. We further demonstrated that blocking the JAK-STAT3 signaling pathway abrogated the enhancing effect of IL-9 on CSR to IgG1 induced by BCR crosslinking plus IL-4 in *Traf3*$^{-/-}$ B cells. Our study thus revealed a novel pathway that TRAF3 suppresses B cell activation and Ig isotype switching by inhibiting IL-9R-JAK-STAT3 signaling. Taken together, our findings provide new insights into the TRAF3-IL-9R axis in B cell function and have significant implications for the understanding and treatment of a variety of human diseases involving aberrant B cell activation such as autoimmune disorders.

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Disclosures

The authors declare that they have no competing financial interests.

Introduction

Interleukin-9 (IL-9) is a γ chain family cytokine that has pleiotropic functions in the immune system (1–4). IL-9 binds to a heterodimeric receptor consisting of the unique subunit IL-9 receptor (IL-9R) and the common subunit γ chain shared by multiple cytokines of the γ chain family, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (1–3). Under physiological and pathological conditions, IL-9 is produced by a variety of activated immune cell subsets (1, 2). Examples are Th9 cells (which originally received the numerical designation from IL-9), follicular helper T (T_{FH}) cells, type 2 innate lymphoid cells (ILC2), mast cells, iNKT cells, mucosal-associated invariant T (MAIT) cells, NK cells, macrophages, basophils, eosinophils, CD8 cytotoxic T (Tc9) cells, Th2 and Th17 cells (1, 2, 4, 5). When produced by these immune cell subsets, IL-9 plays diverse roles in the host ranging from protective immunity to immunopathology depending on the specific setting and microenvironment (2, 4–8).

It has been demonstrated that IL-9 has important functions in protective immunity against helminthic and mycobacterial infections (4, 9–12). However, IL-9-mediated inflammation exacerbates the damaging effects of some other infectious pathogens such as the respiratory syncytial virus-induced pathology, *Staphylococcus aureus*-induced pneumonia, *Helicobacter pylori*-induced gastritis, and barrier function loss caused by fungal infections (13–18). Furthermore, increasing evidence obtained from both human patients and mouse models indicates that IL-9 plays multiple pathogenic roles in autoimmune disorders (such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, systemic sclerosis, and psoriasis), allergic diseases (such as asthma, dust mite allergy, and food allergy), inflammatory diseases (such as ulcerative colitis, atherosclerosis, vasculitis, hepatic and pulmonary fibrosis), and cancers (such as lymphomas and leukemias) (4, 5, 8, 19–29). On the other hand, IL-9 and Th9/Tc9 exhibit potent anti-tumor properties in solid tumors (such as melanoma, lung cancer, breast cancer, and colon cancer), which has recently attracted extensive interest in this cytokine (2, 5, 30–35).

The diverse roles of IL-9 in protective immunity, immunopathology, and anti-tumor immunity are realized through the activities of its various target cells expressing IL-9R constitutively or inducibly in specific stimulatory settings and microenvironments (4, 5, 20, 26). A wide variety of immune and non-immune cell types can respond to IL-9 under different physiological and pathological conditions. These include B lymphocytes, T lymphocytes, ILCs, mast cells, polymorphonuclear cells, DCs, platelets, hematopoietic progenitor cells, epithelial cells, vascular smooth muscle cells, keratinocytes, hepatic stellate cells, astrocytes, oligodendrocytes, and microglia (8, 19, 20, 22, 23, 26, 36, 37). By inducing the JAK-STAT pathway, IL-9 can regulate the survival, proliferation, differentiation, migration, activation or effector function of different target cells under specific conditions (8, 19, 20, 22, 26, 36, 37).

Among the target cells of IL-9, B lymphocytes are the central player of humoral immunity and the only cell type in mammals that can produce antibodies against various pathogens (38–40). IL-9 was initially reported to potentiate IL-4-induced production of IgE and IgG, but not IgM, in crude B lymphocytes prepared from human blood, human tonsils, and

mouse spleens (41–44). Subsequent evidence reveals that IL-9R is not expressed on naïve follicular (FO) B cells, although it is detected on B1 B cells, marginal zone (MZ) B cells, and some germinal center (GC) B cells (44–47). More recently, it has been shown that IL-9R is selectively induced on GC-derived memory precursor cells and memory B cells (6, 45). IL-9 accelerates the exit of memory precursor cells from the GC and induces the development, proliferation, and differentiation of memory B cells (6, 45). Interestingly, *Il9r*$^{-/-}$ mice exhibit normal T-dependent primary antibody responses but impaired recall antibody responses (45). Thus, the effects of IL-9 on potentiating IL-4-induced production of IgE and IgG observed in the initial studies are most likely attributable to memory B cells, B1 B cells, or MZ B cells included in the crude preparation of human and mouse B lymphocytes (41–43), as naïve FO B cells do not express IL-9R and are not responsive to IL-9.

In the present study, we found that the expression of IL-9R was strikingly up-regulated in mouse naïve FO B cells genetically deficient in TRAF3, a critical regulator of B cell survival and function (48–56). The highly up-regulated IL-9R on *Traf3*$^{-/-}$ FO B cells conferred responsiveness to IL-9, including IgM production and STAT3 phosphorylation. Interestingly, IL-9 significantly enhanced class switch recombination (CSR) to IgG1 induced by B cell antigen receptor (BCR) crosslinking plus IL-4 in *Traf3*$^{-/-}$ FO B cells, which was not observed in littermate control (LMC) B cells. This evidence reinforces the notion that in the absence of TRAF3, B cell activation and CSR no longer require cognate T cell help (53). Furthermore, we demonstrated that blocking the JAK-STAT3 signaling pathway abrogated the enhancing effect of IL-9 on CSR to IgG1 induced by BCR crosslinking plus IL-4. Our study thus revealed a novel pathway that TRAF3 suppresses B cell activation and Ig isotype switching by inhibiting the IL-9R-JAK-STAT3 signaling axis.

Materials and methods

Mice

Traf3$^{flox/flox}$CD19$^{+/Cre}$ (B-*Traf3*$^{-/-}$) and *Traf3*$^{flox/flox}$ (littermate control, LMC) mice were generated as previously described (49). All experimental mice for this study were produced by breeding of *Traf3*$^{flox/flox}$ mice with *Traf3*$^{flox/flox}$CD19$^{+/Cre}$ mice. All mice were kept in specific pathogen-free conditions in the Animal Facility at Rutgers University and were used in accordance with NIH guidelines and under an animal protocol (PROTO999900371) approved by the Animal Care and Use Committee of Rutgers University. Equal numbers of male and female mice were used in this study.

Reagents and antibodies

Tissue culture supplements including stock solutions of sodium pyruvate, L-glutamine, non-essential amino acids, and HEPES (pH 7.55) were from Invitrogen (Carlsbad, CA). IL-9 and IL-4 were purchased from Peprotech (Rocky Hill, NJ). Goat anti-mouse IgM F(ab′)2 was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Agonistic anti-CD40 (HM40–3) was purchased from eBioscience (San Diego, CA). Peficitinib, baricitinib, decernotinib, stattic, and AS1517499 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). EDTA-free Protease Inhibitor Cocktail Tablets were obtained from Roche

Diagnostics Corp (Indianapolis, IN). Phosphatase Inhibitor Mini Tablets were purchased from Pierce (Rockford, IL). DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Polyclonal or monoclonal rabbit Abs against total or phosphorylated STAT3, STAT6, STAT1, RelA, RelB, c-Rel, NF-κB1, NF-κB2, YY1, AID, Bcl6, and Blimp1 were from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit Abs to TRAF3 (H122) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin Ab was from Chemicon (Temecula, CA). HRP-labeled secondary Abs were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Splenic B cell purification, culture, and stimulation

Mouse splenic B cells were purified using anti-mouse CD43-coated magnetic beads and a MACS separator (Miltenyi Biotec Inc.) following the manufacturer's protocols as previously described (49). The purity of the isolated B cell population was monitored by FACS analysis, and cell preparations of >98% B220+CD3- purity were used for culture experiments as well as RNA or protein preparation. Purified splenic B cells were cultured *ex vivo* with mouse B cell medium (15, 25) in the absence or presence of IL-9, IL-4, anti-IgM (surface B cell receptor, BCR), anti-CD40, LPS, CpG or different inhibitors, alone or in combination, for 1 to 5 days before preparation of RNA or proteins and analysis of Ig isotype switching.

Flow cytometry

Single cell suspensions were made from mouse spleens. Immunofluorescence staining and FACS analyses were performed as previously described (49, 57). Erythrocytes from spleens were depleted with ACK lysis buffer. Cells (1×10^6) were blocked with rat serum and FcR blocking Ab (2.4G2), and then incubated with various Abs conjugated to different fluorochromes for multiple color fluorescence surface staining. Intracellular proteins were stained after fixation and permeabilization of cells using an Intracellular or Phospho-Flow Staining kit (BD Biosciences, San Jose, CA). Intracellular staining of Ig isotypes, including IgG1, IgG2a, IgG2b, IgG3, and IgE, was performed as previously described (58). Analyses of cell surface markers included antibodies to CD45R (B220), CD3, CD21, CD23, IgM, CD138, IL-4R, IL-21R, and IL-6R (BioLegend, San Diego, CA). FACS Ab specific for mouse IL-9R was previously described (45). FACS Abs specific for Phospho-STAT3 (pY705), Phospho-STAT1 (pY701), Phospho-STAT5 (pY694), Phospho-STAT6 (pY641), and Phospho-ERK1/2 (pT202/pY204) were purchased from Becton Dickinson (Mountain View, CA). FACS data were acquired on a Northern Lights spectral flow cytometer (Cytek, Fremont, CA). The results were analyzed using the FlowJo software (TreeStar, San Carlos, CA). Forward light scatter (FSC-A and FSC-H) and side light scatter (SSC-A and SSC-H) gatings were used to gate single live cells.

Real-time PCR analysis of the transcript levels of specific genes

Total cellular RNA was extracted from splenic B cells using RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. RNA concentration and quality were assessed using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was prepared from RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). TaqMan assay of mouse *Il9r* was performed using specific

TaqMan primers and probe (FAM-labeled) (Applied Biosystems) as previously described (59, 60). Each reaction also included the probe (VIC-labeled) and primers for mouse *Actb*, which served as an endogenous control. Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primers specific for mouse *Cγ1* germline transcript (GLT, Forward: 5'-GGC CCT TCC AGA TCT TTG AG-3'; Reverse: 5'-CAG GGT CAC CAT GGA GTT AGT T-3'), *Aicda* (Forward: 5'-CGC GCC TCT ACT TCT GTG AA-3'; Reverse: 5'-GTC TGG TTA GCC GGA CAG AA-3'), and *Actb* (Forward: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; Reverse: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3') as described (58). Reactions were performed on a QuantStudio™ 3 Real-Time PCR System (96-well, 0.1ml Block, Applied Biosystems). Reaction specificity of each gene was monitored by post-amplification melting curve analyses. Real-time PCR data were analyzed using the QuantStudio™ Design and Analysis Software v1.5.2 (Applied Biosystems). Specific mRNA levels of each gene were normalized to that of the housekeeping gene *Actb*. Relative mRNA expression levels of each gene were calculated using the comparative threshold cycle (Ct) method (ΔΔCt) as previously described (59, 60).

Detection of isotype-specific switch circle transcripts (SCTs)

The γ1 SCTs were amplified from cDNA samples using Iγ1F primer (5'-GGC CCT TCC AGA TCT TTG AG-3') paired with CμR primer (5'-AAT GGT GCT GGG CAG GAA GT-3') and PCR cycle conditions as reported by Kinoshita et al (61). *Gapdh* transcripts were also amplified as a control using specific primers (Forward: 5'-ACC ACA GTC CAT GCC ATC AC-3'; Reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3'). The PCR products were separated by PAGE gel electrophoresis followed by ethidium bromide staining for size assessment and imaging analysis.

Protein extraction and immunoblot analysis

Total protein lysates were prepared by lysing cell pellets in 2X SDS sample buffer (62.5 mM Tris, pH6.8, 1% SDS, 15% glycerol, 2% β-mercaptoethanol and 0.005% bromophenol blue), sonicated for 30 pulses, and then boiled for 10 minutes (52, 59). Cytoplasmic and nuclear extracts were prepared as previously described (49, 62). Proteins were separated by SDS-PAGE and immunoblotted with antibodies to phosphorylated (P-) or total STAT3, STAT6, STAT1, RelA, RelB, c-Rel, NF-κB1, p100/p52 NF-κB2, AID, Blimp1, Bcl6, TRAF3, YY1, or actin, followed by HRP-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse IgG) (52, 60). A chemiluminescent substrate (Pierce) was used to detect HRP-labeled Abs on the immunoblots. Chemiluminescence images of the immunoblots were acquired using a low-light imaging system (LAS-4000 mini, FUJIFILM Medical Systems USA, Inc., Stamford, CT) (52, 57, 62).

Statistics

Statistical analyses were performed using the Prism software (GraphPad, La Jolla, CA). For direct comparison of the levels of IL-9R expression between LMC and *Traf3*$^{-/-}$ B cells, statistical significance was determined with the unpaired *t* test for two-tailed data. For comparison of three or more groups of data, a one-way analysis of variance (ANOVA) was used to determine the statistical significance. *P* values less than 0.05 are considered

significant (*), *P* values less than 0.01 are considered very significant (**), and *P* values less than 0.001 are considered highly significant (***).

Results

Up-regulation of IL-9R on *Traf3*$^{-/-}$ B lymphocytes

To elucidate the mechanisms of TRAF3-mediated regulation of B cell survival and function, we recently performed a transcriptome profiling to identify genes differentially expressed between LMC and *Traf3*$^{-/-}$ splenic B cells purified from gender-matched young adult mice (NCBI GEO accession number: GSE113920) (https://www.ncbi.nlm.nih.gov/geo/) (51). Interestingly, we found that the expression of *Il9r* was strikingly up-regulated in *Traf3*$^{-/-}$ splenic B cells (GSE113920). Given the recent recognition of IL-9R in memory B cell response (6, 45), we verified its striking up-regulation (> 30 fold) in *Traf3*$^{-/-}$ B cells at the mRNA level using TaqMan quantitative real-time PCR (qRT-PCR) (Fig. 1A). To investigate the potential importance of *Il9r* up-regulation in *Traf3* deficiency-initiated B cell malignant transformation, we examined the transcript levels of *Il9r* in splenic B lymphomas spontaneously developed in 7 different individual B-*Traf3*$^{-/-}$ mice using the Taqman assay. Our results showed that *Il9r* expression was moderately up-regulated (2 – 5-fold) in 6 of the 7 examined splenic B cell lymphomas (Fig. 1B). Thus, it appeared that the highly up-regulated *Il9r* expression observed in non-malignant *Traf3*$^{-/-}$ B cells is not stringently maintained during or after B cell malignant transformation. Based on these results, we selected to focus on the *Il9r* up-regulation in non-malignant *Traf3*$^{-/-}$ B lymphocytes in the present study.

We next verified the drastic up-regulation of IL-9R on non-malignant *Traf3*$^{-/-}$ B cells at the protein level using flow cytometric analysis. We found that IL-9R was strikingly up-regulated in both the splenic FO and MZ B cell subsets of young adult B-*Traf3*$^{-/-}$ mice (Fig. 1C). In contrast, the expression of IL-9R was not altered in splenic T cells and the expression of another cytokine receptor, IL-4 receptor (IL-4R), was not changed in FO or MZ B cell subsets of B-*Traf3*$^{-/-}$ mice (Fig. 1C). Our results thus revealed a novel link between TRAF3 and IL-9R in mature B lymphocytes, including both the FO and MZ B cell subsets.

To investigate the potential involvement of IL-9R in the activation of B lymphocytes, we analyzed the expression levels of IL-9R on cultured LMC and *Traf3*$^{-/-}$ splenic B cells after stimulation with various B cell stimuli, including agonistic anti-CD40 Abs, LPS (an agonist of TLR4), CpG2084 (an agonist of TLR9), B cell receptor (BCR) crosslinking Abs, IL-4, and IL-9. Our FACS data demonstrated that among the B cell stimuli examined, CD40 engagement most robustly induced IL-9R up-regulation on the surface of LMC FO and MZ B cells (Fig. 1D, 1E, and Supplementary Fig. 1A), confirming the previous reports that IL-9R expression is induced by CD40 stimulation in B cells (44, 45). We noticed that LPS or CpG also clearly induced IL-9R up-regulation on LMC FO and MZ B cells (Fig. 1D, 1E, and Supplementary Fig. 1A). Each of these 3 stimuli further increased IL-9R expression on the surface of *Traf3*$^{-/-}$ FO and MZ B cells (Fig. 1D, 1E, and Supplementary Fig. 1A). In contrast, anti-BCR plus IL-4, in the absence or presence of IL-9, did not induce IL-9R expression on LMC or *Traf3*$^{-/-}$ FO and MZ B cells (Fig. 1D, 1E, and Supplementary

Fig. 1A). Addition of IL-4 could not potentiate IL-9R up-regulation induced by CD40 engagement in both genotypes of FO and MZ B cells (Fig. 1D, 1E, and Supplementary Fig. 1A). These data suggest that IL-9R up-regulation may have certain functional significance in B cell activation.

To understand how *Il9r* expression is up-regulated, we analyzed the promoter region of the mouse *Il9r* gene and identified potential binding sites for the transcription factors AP1, Sp1, and NF-κB. This is consistent with the initial report that the 5' flanking region of the human *IL9R* gene also contains potential binding motifs for AP1, AP2, AP3, Sp1, and NF-κB (63). We previously reported constitutively elevated levels of nuclear p52 NF-κB2 and RelB but normal levels of phosphorylated ERK, JNK and p38 in $Traf3^{-/-}$ B cells (49). NF-κB1 activation induced by CD40, LPS or BCR signaling is also enhanced in $Traf3^{-/-}$ B cells (53–55). In this context, we reasoned that NF-κB1 or NF-κB2 may be responsible for the up-regulation of *Il9r* expression in B cells. We thus searched and analyzed available RNA-seq datasets for the expression of *Il9r* in splenic B cells genetically deficient in different NF-κB subunits in comparison to WT B cells, in the absence or presence of stimulation with BAFF, CD40 or CD40 plus IgM (64–66). These include $Nfkb2^{-/-}$ splenic B cells (GSE75761 and GSE75762: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75761 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75762), $Rela^{-/-}$ splenic B cells (GSE58972: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58972), and $cRel^{-/-}$ and $Relb^{-/-}cRel^{-/-}$ FO B cells (GSE62559: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62559) B cells (64–66). The search results of these RNA-seq datasets revealed that BAFF or CD40-induced *Il9r* expression was not compromised in $Nfkb2^{-/-}$ splenic B cells (GSE75761 and GSE75762), while CD40+IgM-induced *Il9r* expression was moderately decreased in $Rela^{-/-}$ splenic B cells (GSE58972) (Supplementary Fig. 1B). Interestingly, BAFF-induced *Il9r* expression was completely abolished in FO B cells doubly deficient in both *c-Rel* and *Relb* ($Relb^{-/-}cRel^{-/-}$), although $cRel^{-/-}$ FO B cells exhibited increased *Il9r* expression as compared to WT cells (GSE62559) (Supplementary Fig. 1B). Together, these data suggest that the NF-κB subunits RelB and RelA contribute to the up-regulation of *Il9r* expression in $Traf3^{-/-}$ B cells or following B cell activation.

Up-regulation of IL-9R conferred responsiveness to IL-9 in $Traf3^{-/-}$ B lymphocytes

To explore the functional impacts of IL-9R up-regulation on $Traf3^{-/-}$ B cells, we first compared the effects of IL-9 stimulation on the survival, proliferation, and Ig production between LMC and $Traf3^{-/-}$ B cells purified from gender-matched, young adult mice. We found that IL-9 stimulation did not promote the survival or proliferation in both genotypes of B lymphocytes (Supplementary Fig. 2A). However, IL-9 induced IgM production in $Traf3^{-/-}$, but not LMC, B lymphocytes as analyzed by both intracellular FACS staining of the cultured FO B cells and ELISA of the culture supernatants (55, 58, 67) (Fig. 2A and 2B). We did not detect any effects of IL-9 on the production of other Ig isotypes in cultured $Traf3^{-/-}$ or LMC B cells, including IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE (data not shown), which often requires T-dependent signals. Thus, IL-9 specifically induced IgM production, a T-independent effect, in $Traf3^{-/-}$ B lymphocytes.

In light of the early evidence that IL-9 potentiates IL-4-induced production of IgG and IgE by B lymphocytes (41–43), we determined the effects of IL-9 and IL-4, alone or in combination, on LMC and *Traf3*$^{-/-}$ B cells. We noticed that IL-4 markedly promoted the survival of LMC B cells and slightly helped the survival of *Traf3*$^{-/-}$ B cells, which was not potentiated by addition of IL-9 (Supplementary Fig. 2B). IL-9 and IL-4 did not induce IgG1 or IgE production in LMC B cells (Supplementary Fig. 2C and 2D). In contrast, addition of IL-9 augmented the frequency of IL-4-induced IgG1-producing, but not IgE-producing, *Traf3*$^{-/-}$ B cells (Supplementary Fig. 2C and 2D). However, the combined treatment with IL-9 plus IL-4 only induced a very small population of IgG1-producing *Traf3*$^{-/-}$ B cells in culture (<1.5%), suggesting that these two cytokines are not sufficient to induce a robust Ig isotype switching response in IL-9R^{high} B cells.

Chen *et al*. recently reported that BCR crosslinking plus IL-4 can significantly induce IgG1 CSR in *Traf3*$^{-/-}$ B lymphocytes (53). We thus investigated the CSR in LMC and *Traf3*$^{-/-}$ B cells after treatment with BCR crosslinking in combination with IL-9 or IL-4. Our results showed that addition of IL-9 to BCR crosslinking could not induce CSR in both genotypes of B cells. Interestingly, addition of IL-9 to BCR crosslinking plus IL-4 significantly potentiated Ig isotype switching to IgG1 in *Traf3*$^{-/-}$ but not LMC B cells (Fig. 2C and 2D), although it could not promote the survival or proliferation in these B cells (Supplementary Fig. 3A). We verified that IL-9 potentiated BCR plus IL-4-induced CSR to IgG1 in a dose-dependent manner (Supplementary Fig. 3B and 3C). We also analyzed the effects of IL-9 on CSR induced by CD40 engagement plus IL-4, a combinatory stimulation commonly used in CSR studies. We found that addition of IL-9 to CD40 engagement plus IL-4 significantly potentiated Ig isotype switching to IgG1 in both genotypes of B cells and that this potentiation effect was more robust in *Traf3*$^{-/-}$ than in LMC B cells (Fig. 2E and 2F). These results are consistent with the observation that IL-9R up-regulation was induced by CD40 engagement plus IL-4 but not by BCR crosslinking plus IL-4 in both genotypes of B cells (Fig. 1D and 1E). In contrast, IL-9 did not have obvious effects on CSR to IgE, IgG2a, IgG2b or IgG3 in the presence of BCR crosslinking plus IL-4 or CD40 engagement plus IL-4 in both genotypes of B cells (Fig. 2C, 2E, and data not shown). Taken together, our results indicate that up-regulation of IL-9R is functionally important and confers the responsiveness to IL-9 in *Traf3*$^{-/-}$ B lymphocytes, including IgM production induced by this cytokine alone as well as its potentiation of CSR to IgG1 induced by IL-4 in combination with BCR crosslinking or CD40 engagement. Thus, IL-9 alone is sufficient to induce a T-independent effect – IgM production in *Traf3*$^{-/-}$ B cells, while IL-9 together with BCR crosslinking plus IL-4 are able to stimulate a T-dependent response – IgG1 CSR in *Traf3*$^{-/-}$ B cells.

IL-9 augmented the expression of the Cγ1 germline transcript, γ1 switch circle transcript, and AID

CSR in B lymphocytes is preceded by germline transcription of specific switch (S) regions in the IgH locus and is accompanied by excision of specific switch circles (61, 68). To elucidate how IL-9 could potentiate CSR to IgG1 induced by BCR crosslinking plus IL-4, we first examined the expression levels of the Cγ1 germline transcript (GLT) in LMC and *Traf3*$^{-/-}$ B cells. Interestingly, we found that even in the absence of IL-4, BCR crosslinking

plus IL-9 induced significantly higher levels of the Cγ1 GLT in *Traf3*$^{-/-}$ B cells than in LMC B cells (Fig. 3A). Addition of IL-4 drastically further increased the levels of the Cγ1 GLT in both genotypes of B cells (Fig. 3A). We also tested the possibility that IL-9 alone could induce the expression of the Cγ1 GLT in *Traf3*$^{-/-}$ B cells. Our results demonstrated that this indeed was the case (Fig. 3B). We next analyzed the induction of the γ1 switch circle transcript (SCT), a hallmark of active *de novo* CSR in B cells, using reverse transcription PCR (61). Our results showed that BCR crosslinking plus IL-4 clearly induced the expression of the γ1 SCT and that addition of IL-9 robustly further increased the induction of the γ1 SCT expression in *Traf3*$^{-/-}$ B cells (Fig. 3C), indicating the presence of active *de novo* CSR to IgG1 in these stimulated *Traf3*$^{-/-}$ B cells.

CSR and production of switch circles require the critical enzyme activation-induced cytidine deaminase (AID), which is encoded by the *Aicda* gene (61, 69). We next determined the expression of AID at the mRNA and protein levels using qRT-PCR and Western blot analyses, respectively. We found that addition of IL-9 remarkably potentiated BCR crosslinking plus IL-4-induced expression of AID at both the mRNA and protein levels in *Traf3*$^{-/-}$ but not LMC B cells (Fig. 3D and 3E). Fawaz *et al.* previously suggested that IL-9 induces the up-regulation of Bcl-6 in GC B cells and Takatsuka *et al.* recently reported that IL-9 regulates the expression of Blimp-1 in *in-vitro*-induced GC-like B cells (44, 45, 70). Given that Bcl-6 and Blimp-1 are two transcriptional repressors especially crucial for B cell differentiation (71), we investigated their expression in our experimental model using Western blot analysis. We observed that addition of IL-9 did not further enhance the expression of Bcl-6 and Blimp-1 induced by BCR crosslinking plus IL-4 in both LMC and *Traf3*$^{-/-}$ B cells (Fig. 3E), arguing against a role for Bcl-6 or Blimp-1 in IL-9-induced effect on CSR. We noticed that the levels of *Aicda* and AID expression induced by IL-9 together with BCR crosslinking plus IL-4 (Fig. 3D and 3E) appear to be discordant with the extent of Cγ1 GLT induction (Fig. 3A), suggesting that Cγ1 GLT induction does not strictly require high protein levels of AID. Indeed, an early study by Berton *et al.* demonstrated that IL-4 alone can induce Cγ1 germline transcript expression in resting wild type (WT) B cells (72). In contrast, the effects of IL-9 together with BCR crosslinking plus IL-4 on the *Aicda* transcript and AID protein expression (Fig. 3D and 3E) are consistent with their effect on the γ1 SCT expression (Fig. 3C), reinforcing the notion that AID is critically required for CSR and production of switch circles (61, 69). Taken together, our results revealed that IL-9, in the presence of BCR crosslinking plus IL-4, induces the expression of AID and *de novo* CSR to IgG1 in *Traf3*$^{-/-}$ B cells.

IL-9 induced the activation of STAT3 in *Traf3*$^{-/-}$ B lymphocytes

It has been reported that IL-9 induces rapid activation of STAT3, STAT1, STAT5, and ERK1/2 in various target cells (2, 6, 28, 44, 45, 47). We analyzed and compared these proximal signaling events of IL-9R between LMC and *Traf3*$^{-/-}$ B cells. Our results of Phospho-Flow Cytometry demonstrated that IL-9 alone drastically induced the phosphorylation of STAT3 and also markedly induced the phosphorylation of STAT1 in *Traf3*$^{-/-}$ FO B cells, but it barely induced any phosphorylation of STAT3 or STAT1 in LMC FO B cells (Fig. 4A, 4B, and Supplementary Fig. 4A). Consistent with the previous observations that WT MZ B cells express IL-9R and respond to IL-9 treatment (1, 45, 47),

we detected a robust induction of STAT3 and STAT1 phosphorylation by IL-9 alone in the MZ B cell subset of LMC mice (Fig. 4C, 4D, and Supplementary Fig. 4A). In addition, IL-9-induced STAT1 phosphorylation, but not STAT3 phosphorylation, was significantly higher in *Traf3*$^{-/-}$ than in LMC MZ B cells (Fig. 4C and 4D), which correlates with the elevated expression level of IL-9R detected on the surface of *Traf3*$^{-/-}$ MZ B cells (Fig. 1C). In contrast, IL-9 alone did not induce the phosphorylation of STAT5 or ERK1/2 in FO and MZ B cell subsets of both genotypes (Fig. 4A–4D, and Supplementary Fig. 4B, 4C). Our results thus identify STAT3 and STAT1 as two major signaling components of IL-9R in *Traf3*$^{-/-}$ B lymphocytes.

We wondered whether the enhanced phosphorylation of STAT3 and STAT1 induced by IL-9 can be attributed entirely to the IL-9R up-regulation or whether there is an additional effect on signaling conferred by *Traf3* deficiency. To address this, we examined the phosphorylation of STAT3 and STAT1 induced by another cytokine of the γ chain family, IL-21, using Phospho-Flow Cytometry. Our results demonstrated that IL-21-induced phosphorylation of STAT3 and STAT1 was modestly enhanced in *Traf3*$^{-/-}$ FO B cells (Supplementary Fig. 5A and 5B). In addition, Lin *et al*. previously reported that IL-6-induced STAT3 phosphorylation and plasma cell differentiation are significantly increased in *Traf3*$^{-/-}$ B cells (56). However, we did not detect any significant difference in the expression levels of IL-21R and IL-6R between *Traf3*$^{-/-}$ and LMC FO B cells (Supplementary Fig. 5C), suggesting an effect of *Traf3* deficiency on IL-21R and IL-6R signaling in B cells. In this regard, Lin *et al*. revealed that TRAF3 inhibits IL-6R signaling by facilitating the association of the phosphatase PTPN22 with the kinase Jak1 to restrain STAT3 phosphorylation (56). Based on the above evidence, we speculate that the actions of IL-9 observed in *Traf3*$^{-/-}$ FO B cells can be attributed mainly to the striking up-regulation of IL-9R, while there is likely an additional effect of *Traf3* deficiency on IL-9R signaling in B cells.

We further investigated the combined effects of IL-9 and BCR crosslinking plus IL-4 on these proximal signaling events. We found that BCR crosslinking plus IL-4 could not further increase IL-9-induced phosphorylation of STAT3 and STAT1 in *Traf3*$^{-/-}$ FO and MZ B cell subsets (Fig. 4A–4D). Interestingly, BCR crosslinking plus IL-4 remarkably induced the phosphorylation of STAT6, a key player of the IL-4 signaling pathway (73), in FO and MZ B cell subsets of both genotypes (Fig. 4A–4D). However, addition of IL-9 could not further increase the phosphorylation of STAT6 induced by BCR crosslinking plus IL-4 (Fig. 4A–4D). These data suggest the potential importance of STAT3, STAT1, and STAT6 in CSR induced by IL-9 in the presence of BCR crosslinking plus IL-4 in *Traf3*$^{-/-}$ B cells.

We next employed Western blot analysis of cytoplasmic and nuclear extracts to verify our findings obtained from Phospho-Flow Cytometry. Our results demonstrated that addition of IL-9 markedly potentiated the phosphorylation and nuclear translocation of STAT3 induced by BCR crosslinking plus IL-4 in *Traf3*$^{-/-}$ B cells (Fig. 5A). The results of Western blot analysis also verified that IL-9 could not further increase the phosphorylation and nuclear translocation of STAT6 induced by BCR crosslinking plus IL-4 in both genotypes of B cells (Fig. 5A). In contrast, addition of IL-9 did not obviously increase the phosphorylation (pY701) and nuclear translocation of STAT1 induced by BCR crosslinking plus IL-4 in

Traf3$^{-/-}$ B cells as determined by Western blot analysis (Supplementary Fig. 6), which did not reflect the data of Phospho-Flow Cytometry (Fig. 4A–4D) for an unknown reason. We previously reported the constitutive NF-κB2 activation in *Traf3*$^{-/-}$ B cells (49). Here we showed that IL-9 together with BCR crosslinking plus IL-4 did not further increase the levels of nuclear NF-κB2 subunits, including p52 NF-κB2 and RelB, in *Traf3*$^{-/-}$ B cells (Supplementary Fig. 6). It is known that BCR crosslinking induces NF-κB1 activation and that NF-κB1 plays a critical role in CSR (55, 58, 74). We found that addition of IL-9 did not potentiate the increase of cytoplasmic or nuclear NF-κB1 subunits, including p50 NF-κB1, RelA, and c-Rel, induced by BCR crosslinking plus IL-4 in both genotypes of B cells (Supplementary Fig. 6). Collectively, our results suggest that IL-9 and BCR crosslinking plus IL-4 activate distinct early signaling players such as STAT3, NF-κB1 and STAT6, which may cooperate at downstream signaling components to synergistically induce CSR in *Traf3*$^{-/-}$ B cells.

JAK and STAT inhibitors blocked the expression of AID induced by IL-9 in combination with BCR crosslinking plus IL-4

It has been shown that IL-9R binds to JAK1 constitutively and the common γ chain receptor binds to JAK3 constitutively. Upon IL-9 engagement, IL-9R and γ chain form a heterodimeric complex, and thus bring JAK1 and JAK3 to close proximity to induce their cross-activation, which in turn induces the phosphorylation of STAT3 (2, 28, 47). Similarly, IL-4 induces the phosphorylation of STAT6 via activating the IL-4R-associated JAK1 and γ chain-associated JAK3 in lymphocytes (73). To elucidate if these JAK/STAT signaling pathways are required for the up-regulation of AID expression induced by IL-9 in combination with BCR crosslinking plus IL-4, we examined the effects of several pharmacological inhibitors specific for JAK or STAT proteins. These include peficitinib (a pan JAK inhibitor), baricitinib (a JAK1/JAK2 inhibitor), decernotinib (a JAK3 inhibitor), stattic (a STAT3 inhibitor), and AS1517499 (a STAT6 inhibitor) (75, 76). As demonstrated by both the qRT-PCR data of the transcript level and Western blot results of the protein level, the induction of AID expression stimulated by IL-9 in combination with BCR crosslinking plus IL-4 was almost completely abolished by each of the three examined JAK inhibitors (peficitinib, baricitinib, and decernotinib) as well as the STAT6 inhibitor AS1517499, and was also substantially blocked by the STAT3 inhibitor stattic in *Traf3*$^{-/-}$ B cells (Fig. 5B and 5C). Hence, these results indicate that the JAK1/3-STAT3/6 signaling pathways are required for BCR+IL-4+IL-9-induced expression of AID, which is vital for mediating *de novo* CSR in activated B lymphocytes.

The STAT3 inhibitor abrogated the CSR to IgG1 induced by BCR+IL-4+IL-9, but not that induced by CD40+IL-4, in *Traf3*$^{-/-}$ B lymphocytes

Given the blocking effects of the JAK and STAT inhibitors on AID expression observed in *Traf3*$^{-/-}$ B cells stimulated with IL-9 in combination with BCR crosslinking plus IL-4, we next sought to investigate their subsequent functional impacts on Ig isotype switching. We found that the CSR to IgG1 induced by IL-9 in combination with BCR crosslinking plus IL-4 was almost completely abolished by each of the three examined JAK inhibitors (peficitinib, baricitinib, and decernotinib), was severely impaired by the STAT6 inhibitor AS1517499, and was also substantially suppressed by the STAT3 inhibitor stattic in *Traf3*$^{-/-}$

B cells (Fig. 6A and 6B). To further dissect out the IL-9-specific signaling components from those of IL-4, we determined the effects of these pharmacological inhibitors on CSR induced by CD40 engagement plus IL-4 in LMC and *Traf3*$^{-/-}$ B cells. Interestingly, the CSR to IgG1 induced by CD40 engagement plus IL-4 was almost abolished by each of the three examined JAK inhibitors (peficitinib, baricitinib, and decernotinib) as well as the STAT6 inhibitor AS1517499, but was not inhibited at all by the STAT3 inhibitor stattic in both genotypes of B cells (Fig. 6A and 6B). We also verified that the JAK or STAT3 inhibitors did not suppress the expression of IL-9R on *Traf3*$^{-/-}$ or LMC B cells, in the absence or presence of stimulation by CD40 engagement plus IL-4 (Supplementary Fig. 7). Taken together, our findings suggest that the JAK-STAT6 signaling pathway is induced by IL-4 and is therefore required for CSR induced by both BCR+IL-4+IL-9 and CD40+IL-4. In contrast, JAK-STAT3 signaling is specifically induced by IL-9 and is thus required for CSR induced by BCR+IL-4+IL-9, but not CD40+IL-4, in *Traf3*$^{-/-}$ B lymphocytes.

Discussion

Regulation of B cell function is central to humoral immunity (38–40, 77). Inadequate B cell activation often leads to compromised antibody responses, infectious diseases, and chronic infections, while aberrant activation of B cells by innocuous or self antigens causes allergic and autoimmune diseases (38–40, 77). A better understanding of how precisely B cell function is regulated in different immune contexts is required for improving the management of these disease conditions resulting from abnormal B cell activation. In the present study, we provide novel insights into the molecular mechanisms of how an important cytokine IL-9 regulates B cell activation and Ig isotype switching in the context of genetic deficiency in TRAF3, a vital regulator of B cell survival and function (48–56). TRAF3 critically restrains the signaling events of multiple receptors essential for B cell physiology, including BAFF-R, CD40, BCR, Toll-like receptors (TLRs), and IL-6 receptor (48–50, 53–56, 78). In an effort to identify TRAF3 downstream target genes in B lymphocytes, we found that the expression of IL-9R was strikingly up-regulated in *Traf3*$^{-/-}$ FO B cells. The highly up-regulated IL-9R expression conferred responsiveness to IL-9 in naïve *Traf3*$^{-/-}$ FO B cells. Together, our evidence presented in this study revealed a novel pathway that TRAF3 suppresses B cell activation and Ig CSR by inhibiting the IL-9R-JAK-STAT3 signaling axis.

Interestingly, IL-9-induced IL-9R-JAK-STAT3 signaling (but not STAT5 or STAT6 phosphorylation) remarkably and specifically potentiated CSR to IgG1 induced by BCR crosslinking plus IL-4 or CD40 engagement plus IL-4 in *Traf3*$^{-/-}$ FO B cells. In line with our finding, STAT3 is known as a transcription factor that promotes IgG-specific plasma cell differentiation and inhibits CSR from IgG1 to IgE in GC B cells, which has been demonstrated by both mouse and human evidence (79–82). B cell-specific *Stat3*$^{-/-}$ mice display profound defects in T-dependent IgG1 responses, but elevated serum IgE levels (79, 80). In humans, dominant-negative mutations in the *STAT3* gene underlie sporadic and dominant forms of the hyper-IgE syndrome (HIES), a disease characterized by elevated serum IgE levels and recurrent infections (81, 82). Similar to IL-9, the follicular helper CD4 T (T_{FH}) cell-derived cytokine IL-21 also induces the JAK-STAT3 signaling pathway to potentiate IgG1 CSR, while inhibiting IgE CSR in GC B cells (83). The importance of STAT3 in this process is further highlighted by the evidence that naïve B cells from HIES

patients with *STAT3* mutations are unable to differentiate into IgG-producing cells after stimulation with IL-21 in the presence of CD40L (84, 85). However, IL-21R, but not IL-9R, is expressed on naïve WT FO B cells, therefore normal naïve FO B cells respond to IL-21 but are irresponsive to IL-9 (45, 84, 86).

For normal naïve FO B cells, full activation and Ig CSR require not only the engagement of BCR by specific antigens but also the signals provided by T_{FH} cells that recognize peptides presented in complex with MHC class II on the surface of B cells, including the membrane-bound CD40L expressed on the surface of T_{FH} cells and the cytokines such as IL-21 and IL-4 secreted by T_{FH} cells (84, 87–90). Such linked recognition of antigens by B cells and cognate T_{FH} cells serves to distinguish foreign antigens from self antigens (84, 87–90). Upon antigen binding, BCR signaling induces the activation of NF-κB1, Akt-FOXO1, and ERK1/2-AP1 (87, 88, 91). Engagement of CD40 by CD40L stimulates the activation of NF-κB1, NF-κB2, Akt, and ERK1/2 (87, 88, 91). IL-4 induces the principal IL-4R-JAK-STAT6 signaling pathway, while IL-21 primarily activates the IL-21R-JAK-STAT3 signaling pathway (84, 88–90). The concerted action of NF-κB1, NF-κB2, STAT6, and STAT3 potently induces the expression of AID, the enzyme that catalyzes CSR, thus mediating Ig isotype switching in activated B cells (84, 87–90). In the absence of CD40L and IL-21 provided by T_{FH} cells, BCR crosslinking by various antigens plus IL-4 can stimulate the BCR and IL-4R signaling pathways to induce the expression of certain transcription factors required for B cell differentiation such as Bcl-6 and Blimp1, but cannot induce the expression of AID due to lack of NF-κB2 activation and STAT3 signaling (Fig. 3D–E, 5A–C, and Supplementary Fig. 6). Consequently, normal naïve FO B cells cannot undergo CSR in response to BCR crosslinking triggered by self antigens even if inflammatory cytokines IL-4 and IL-9 are present in the microenvironment (Fig. 7A).

However, for TRAF3-deficient naïve FO B cells, full activation and Ig CSR do not stringently require the signals provided by T_{FH} cells (Fig. 7B). Previous evidence reveals that deletion of TRAF3 leads to constitutive activation of NF-κB2 and also results in elevated BCR signaling in B cells (49, 50, 53, 54). In response to BCR crosslinking plus IL-4, the induced activation of NF-κB1 and STAT6 together with the constitutive activation of NF-κB2 moderately promotes AID expression and CSR in *Traf3*$^{-/-}$ B cells (Fig. 2C–D, 3C–E, and 4A–B) (53). In the present study, we further demonstrated that loss of TRAF3 also causes strikingly up-regulated expression of IL-9R on FO B cells and that addition of IL-9 robustly potentiates AID expression and CSR induced by BCR crosslinking plus IL-4 via JAK-STAT3 signaling in *Traf3*$^{-/-}$ B cells (Fig. 2C–D, 3C–E, 4A–B, 5A, and Supplementary Fig. 6). Thus, in the absence of intracellular TRAF3, constitutive activation of NF-κB2 provides surrogate CD40 signaling to remove the requirement of CD40L expressed on the surface of T_{FH} cells (53), while up-regulated IL-9R confers IL-9-induced JAK-STAT3 signaling to replace the requirement of IL-21 produced by T_{FH} cells. As a consequence, full activation and Ig CSR of TRAF3-deficient naïve FO B cells could be induced by self antigens, as long as they can engage specific BCRs and the necessary cytokines such as IL-4 and IL-9 are present in the microenvironment (Fig. 7B). Consistent with this scenario, elevated serum levels of IL-4 and IL-9 have been detected in human patients with systemic lupus erythematosus (SLE) and other autoimmune disorders (8, 21, 92–94). Therefore, although it is recognized that T-dependent functions cannot be simply

reduced to NF-κB2 activation and cytokine production and that B-T cell cognate interactions are necessary for affinity maturation and high affinity antibody production which is often found in autoimmune conditions (95), the above findings suggest that loss of TRAF3 in B cells would lead to increased risks of autoimmunity.

Indeed, B-*Traf3*$^{-/-}$ mice exhibit autoimmune manifestations, including the presence of high levels of anti-dsDNA autoantibodies in the serum, spontaneous formation of GCs in the spleen, and deposition of immune complexes at glomeruli in the kidney (49). Relevant to the observed autoimmunity, *Traf3*$^{-/-}$ B cells display enhanced antibody production and Ig CSR responses to TLR agonists (55). In line with the notion that autoimmune conditions are strong predisposing factors for B lymphoma development (96), we observed that over 80% of B-*Traf3*$^{-/-}$ mice spontaneously develop B cell lymphomas at the age of 9–18 months (57). Deletions and inactivating mutations of the *TRAF3* gene are frequently detected in a variety of human B cell malignancies (97–100). Up-regulation of IL-9R has also been detected in malignant B cells of patients with diffuse large B-cell lymphoma (DLBCL) and Hodgkin lymphoma (HL), while elevated serum levels of IL-9 have been observed in patients with chronic lymphocytic leukemia (CLL), DLBCL, and HL (28, 101–105). Overexpression of IL-9 in transgenic mice results in a complex phenotype characterized by the development of autoantibodies and T cell lymphoma (19). Interestingly, IL-9 significantly increases *in vitro* production of anti-systemic scleroderma 70 (Scl70) autoantibodies by B cells isolated from the peripheral blood of patients with systemic sclerosis, suggesting a pathogenic role of IL-9-IL-9R on autoreactive B cells (22). Furthermore, the latent membrane protein LMP1 encoded by the Epstein-Barr virus (EBV), a risk factor of B cell oncogenesis and autoimmunity (106–108), sequesters TRAF3 in B lymphocytes and can render EBV-infected B cells functionally TRAF3 deficient without genetic alterations of the *TRAF3* gene (109, 110). In this context, our study lays the ground for further investigation on the TRAF3-IL-9R-JAK-STAT3 axis of B lymphocytes in the pathogenesis of autoimmunity, especially in human patients of autoimmune disorders associated with EBV or B cell malignancies.

Elevated levels of IL-9 and/or IL-4 are also detected in the specimens of patients with various allergic diseases, including asthma, atopic dermatitis, and food allergy (23, 111–113). Moreover, IL-9 promotes IL-4-induced IgE production in allergic B cells isolated from children with asthma (112), indicating that allergic B cells express IL-9R. Interestingly, the rs71421264 and rs56101042 SNPs of *TRAF3* are associated with increased susceptibility to asthma and atopic dermatitis, respectively, in human patients (114, 115). In light of our finding that up-regulated IL-9R on *Traf3*$^{-/-}$ B cells allows IL-9 to potentiate BCR+IL-4-induced CSR to IgG1, it is possible that the TRAF3-IL-9R-JAK-STAT3 axis plays a role in the pathogenesis of IgG1-dependent allergic diseases (116–118). Notably, IgG1+ memory B cells can undergo further CSR to generate IgE-producing plasma cells upon subsequent exposure to specific allergens (118–123). Based on the above evidence, we speculate that the TRAF3-IL-9R axis may be broadly implicated in the pathogenesis of both IgG1- and IgE-dependent allergic diseases in patients having repeated exposures to allergens, which requires further investigation in future studies.

In summary, our findings provide novel insights into the TRAF3-IL-9R axis in B cell function and bear significant implications for the understanding and treatment of a variety of human diseases involving aberrant B cell activation, including autoimmune disorders and allergic diseases. Our study identifies the TRAF3-IL-9R-JAK-STAT3 pathway components as potential therapeutic targets to intervene autoimmunity and allergy. Given the recent exciting developments of IL-9 and Th9/Tc9 in the immunotherapy of solid tumors (30, 31, 124, 125), our findings suggest that prudent considerations should be given on the potential risks of such therapy at inducing autoimmunity or allergy. This is particularly important for solid tumor patients with latent EBV infection, autoimmune or allergic history, or specific *TRAF3* inactivation or SNPs. Considering that autoreactive, allergic, and TRAF3-deficient B cells are responsive to IL-9 (5, 22, 26, 112), this knowledge will provide new perspectives to minimize adverse effects of IL-9 and Th9/Tc9 when treating solid tumors within different genetic and immune contexts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

Ab	antibody
AID	activation-induced cytidine deaminase
BAFF	B-cell activating factor
BAFF-R	BAFF receptor
BCR	B cell antigen receptor
B-*Traf3*$^{-/-}$ mice	B cell-specific TRAF3-deficient mice
CLL	chronic lymphocytic leukemia
CSR	class switch recombination
DLBCL	diffuse large B-cell lymphoma
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting

FO	follicular
GC	germinal center
GLT	germline transcript
HIES	hyper-IgE syndrome
HL	Hodgkin lymphoma
Ig	immunoglobulin
ILC2	type 2 innate lymphoid cells
IL-9	interleukin-9
IL-9R	IL-9 receptor
JAK	Janus kinase
LMC	littermate control
LPS	lipopolysaccharides
MZ	marginal zone
NF-κB	nuclear factor κ light chain enhancer of activated B cells
NF-κB2	noncanonical NF-κB
***P* value**	probability value calculated by a statistical method
qRT-PCR	quantitative real-time polymerase chain reaction
SCT	switch circle transcript
SD	standard deviation
SEM	standard deviation of the mean
SNP	single nucleotide polymorphisms
STAT	signal transducer and activator of transcription
Tc9	IL-9-producing CD8 cytotoxic T cells
T_{FH}	follicular helper CD4 T cells
Th9	IL-9-producing CD4 helper T cells
TLRs	Toll-like receptors
TNF-R	tumor necrosis factor-receptor
TRAF3	TNF-R-associated factor 3
WT	wild type

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Key points:

- IL-9R is highly up-regulated on mouse TRAF3-deficient B lymphocytes.
- IL-9 enhances CSR induced by BCR crosslinking + IL-4 in TRAF3-deficient B cells.
- JAK or STAT3 inhibitors abrogate the enhancing effect of IL-9 on CSR.

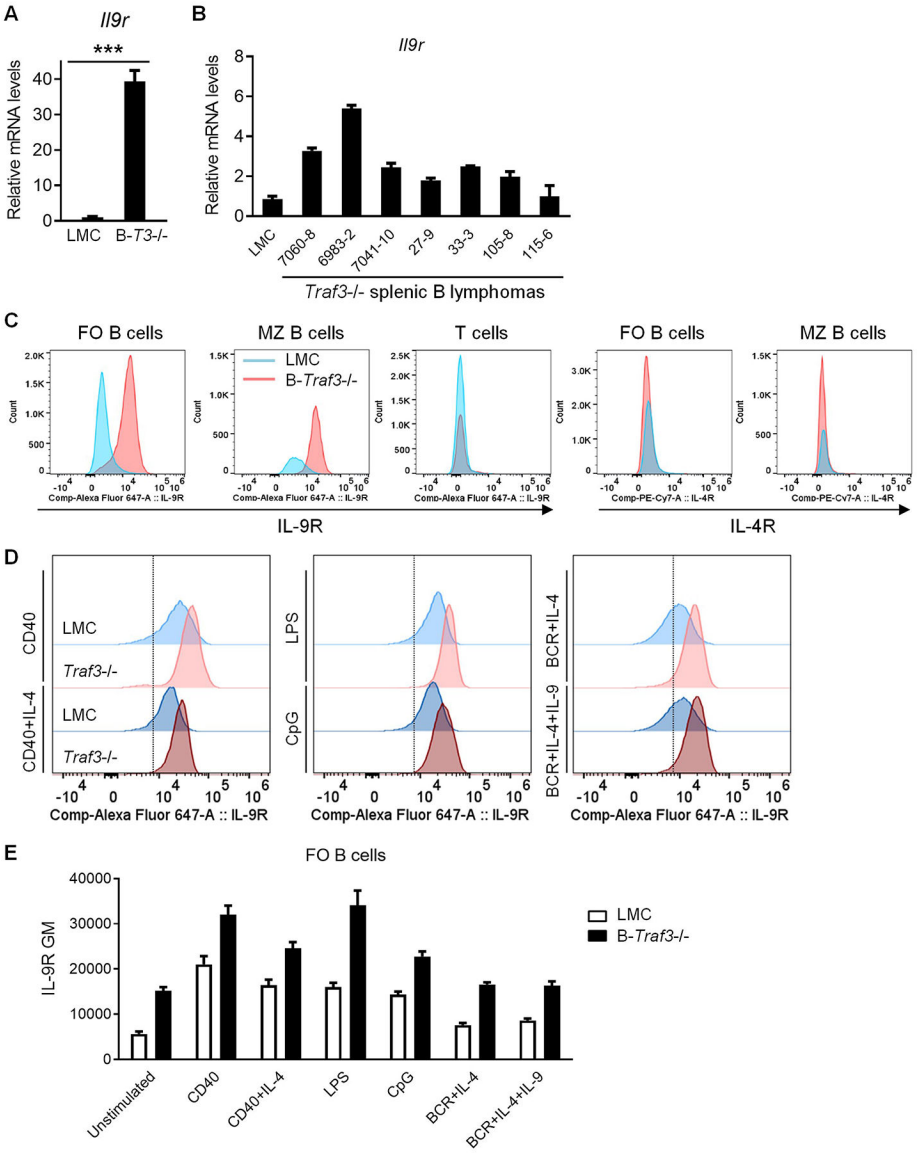

Figure 1. Up-regulation of IL-9R expression in B lymphocytes by *Traf3* deficiency and B cell stimuli.

Splenic B cells were purified from gender-matched, young adult (8–12-week-old) LMC or B-*Traf3*$^{-/-}$ mice. (**A** and **B**) Increased transcript expression of *Il9r* in non-malignant *Traf3*$^{-/-}$ B cells and the majority of *Traf3*$^{-/-}$ B lymphomas. Total cellular RNA was prepared from purified splenic B cells (A) or splenic B lymphomas spontaneously developed in 7 different individual B-*Traf3*$^{-/-}$ mice (B). cDNA was synthesized by reverse transcription. Quantitative real-time PCR was performed using TaqMan assay specific for mouse *Il9r*. Relative mRNA levels were analyzed using the ΔΔCt method and normalized using *Actb* mRNA as an endogenous control. Results shown are mean ± SEM (n=6/group). ***, highly significantly different ($p < 0.001$ as determined by *t* test) between LMC and *Traf3*$^{-/-}$ B cells. (**C**) Up-regulated surface expression of the IL-9R protein on *Traf3*$^{-/-}$ B cells analyzed by FACS. Freshly prepared splenocytes were stained with fluorochrome-conjugated Abs specific for IL-9R, IL-4R, and markers of different mature B cell subsets and T cells. FO

B cells were gated as CD3-B220+CD138-IgM+CD21^{int}CD23^{hi} single lymphocytes, MZ B cells were gated as CD3-B220+CD138-IgM+CD21^{hi}CD23^{int} single lymphocytes, and T cells were gated as CD3+B220- single lymphocytes. (**D**) Regulation of IL-9R expression by B cell stimuli. Purified B cells were cultured *ex vivo* in the absence or presence of B cell stimuli, including 2 μg/ml anti-CD40 (CD40), 10 ng/ml IL-4, 20 μg/ml LPS, 100 nM CpG 2084, 5 μg/ml anti-BCR (BCR), and 10 ng/ml IL-9, alone or in combination as indicated in the figure. FACS analysis of IL-9R expression was performed at day 2 after stimulation and the results of gated FO B cells are shown. The dotted vertical line in each panel shows the peak of IL-9R staining intensity of LMC FO B cells analyzed directly *ex vivo* (day 0). FACS profiles shown in (C and D) are representative of at least 3 independent experiments. (**E**) Graphical results of the geometric mean (GM) of IL-9R staining intensity on gated FO B cells as determined by FACS (mean ± SEM, n=4/group).

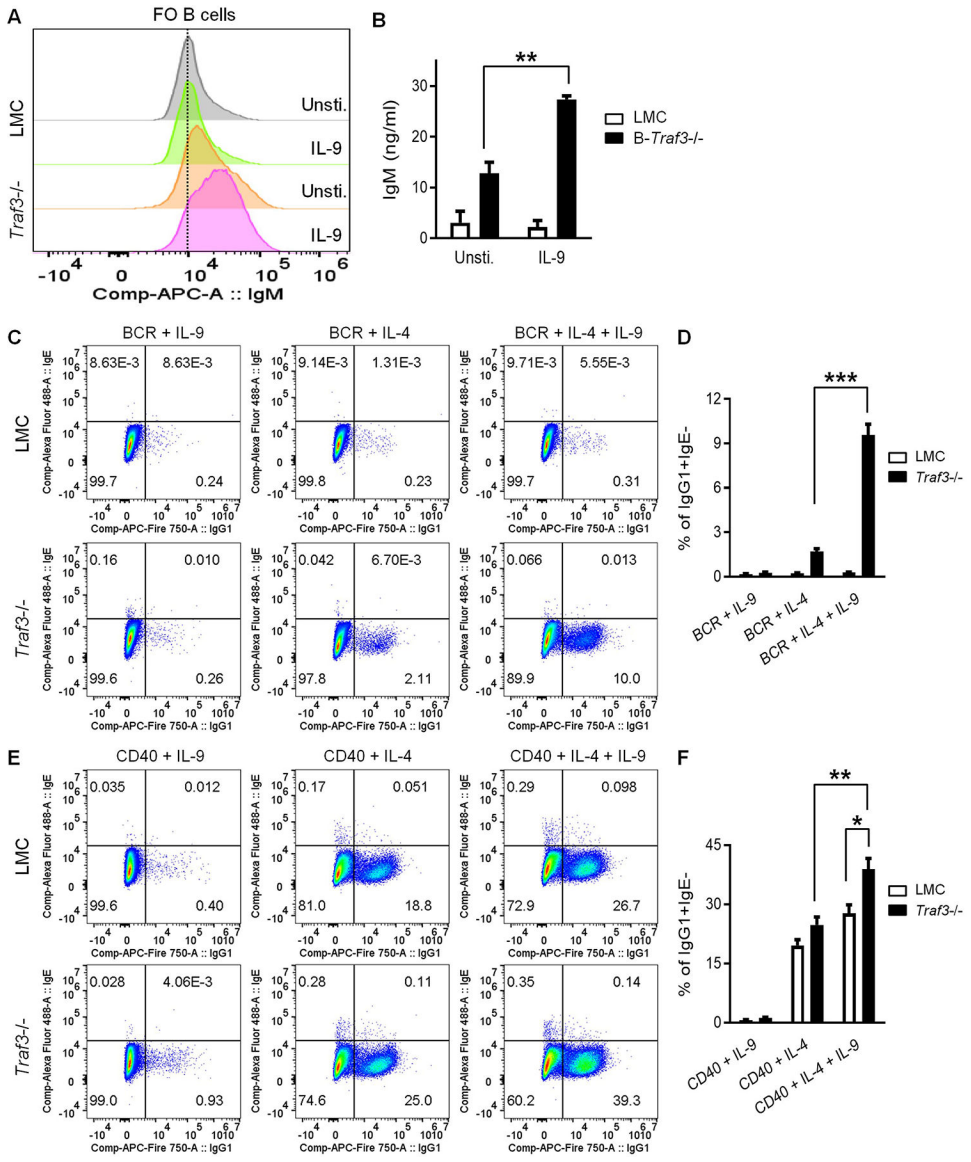

Figure 2. *Traf3*$^{-/-}$ B cells were responsive to IL-9.

Splenic B cells were purified from gender-matched, young adult LMC and B-*Traf3*$^{-/-}$ mice. (**A** and **B**) IL-9 promoted the production of IgM in *Traf3*$^{-/-}$ B cells. Cells were cultured in the absence (Unsti.) or presence of 10 ng/ml IL-9 for 5 days. The culture supernatants were harvested for ELISA, and the cells were treated with Golgi-Plug for 5 hours before intracellular staining of IgM and other Ig isotypes for FACS. (**A**) FACS histograms comparing IgM expression on IgE- gated splenic B cells. Results shown are representative of 3 independent experiments. (**B**) IgM secretion in the culture supernatants was analyzed by IgM-specific ELISA. Results shown are from 3 independent experiments with duplicate samples in each experiment (mean ± SEM). (**C** to **F**) IL-9 potentiated IgG1 CSR induced by anti-BCR plus IL-4 or anti-CD40 plus IL-4 in *Traf3*$^{-/-}$ B cells. Cells were cultured *ex vivo* for 4 days in the presence of 5 μg/ml anti-BCR (BCR), 10 ng/ml IL-9, 10 ng/ml IL-4, or 2 μg/ml anti-CD40 (CD40), in various combinations as indicated in the

figure, and then treated with Golgi-Plug for 5 hours before intracellular staining of IgG1 and other Ig isotypes for FACS. (**C** and **E**) FACS profiles of IgG1 *versus* IgE staining. Data shown are representative of 4 independent experiments. (**D** and **F**) Graphical results of the percentage of IgG1+IgE- B cells as determined by FACS (mean ± SEM, n=4/group). *, significantly different ($p < 0.05$); **, very significantly different ($p < 0.01$); ***, highly significantly different ($p < 0.001$) as determined by ANOVA (B, D and F).

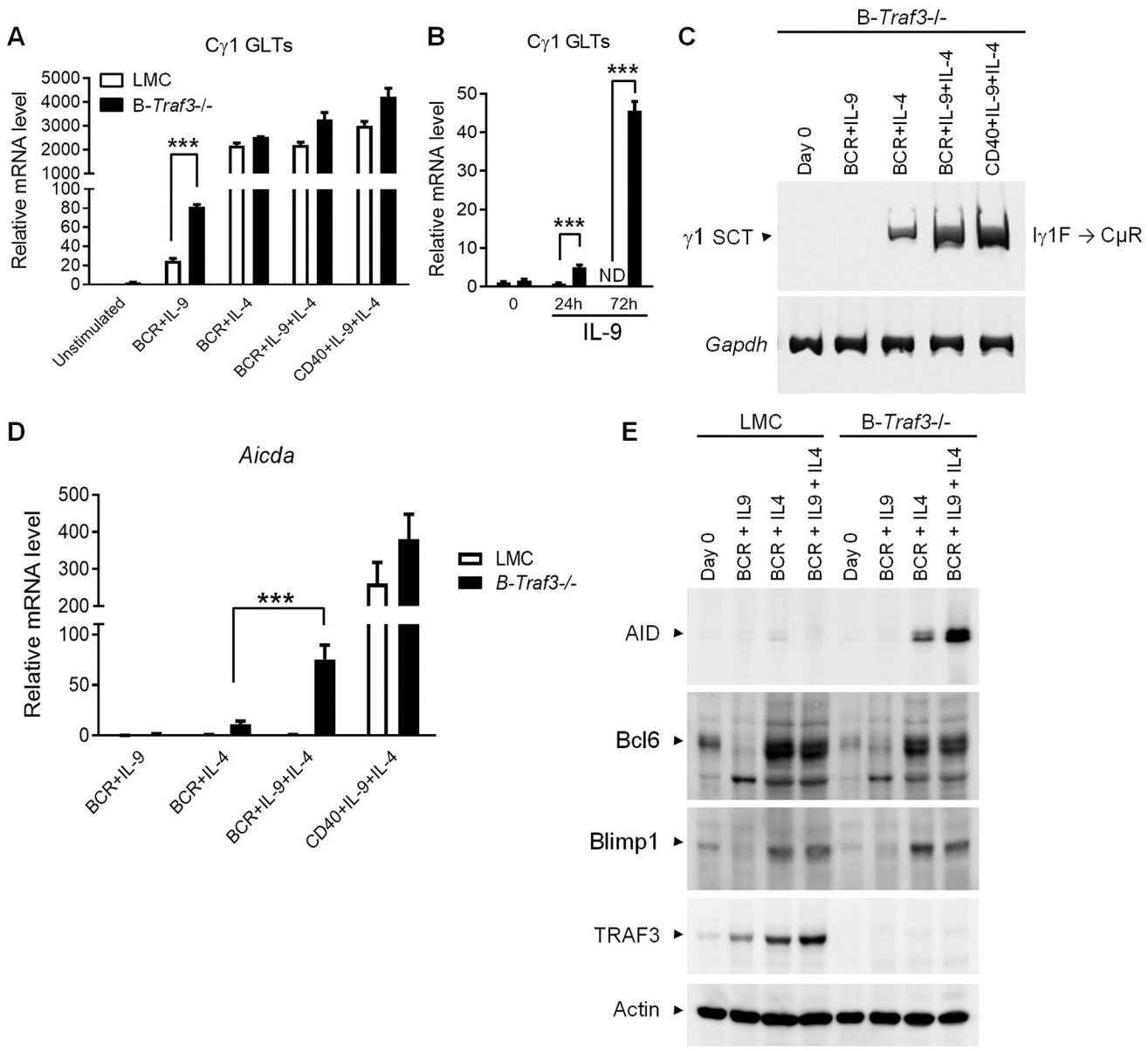

Figure 3. IL-9 together with anti-BCR plus IL-4 induced Cγ1 GLT, γ1 SCT and AID expression in *Traf3*$^{-/-}$ B cells.

Splenic B cells were purified from gender-matched, young adult LMC and B-*Traf3*$^{-/-}$ mice. Cells were cultured *ex vivo* in the absence or presence of 10 ng/ml IL-9, 5 μg/ml anti-BCR (BCR), 10 ng/ml IL-4, or 2 μg/ml anti-CD40 (CD40), alone or in various combinations as indicated in the figure. (A and B) Quantitative real-time PCR data of Cγ1 GLT (mean ± SEM, n=6/group). Total cellular RNA was extracted at day 2 (A) or at day 1 and day 3 (B) after culture. (C) Representative gel images of γ1 SCT and the housekeeping gene *Gapdh* analyzed by reverse transcription PCR. Total cellular RNA was extracted at day 3 after culture. Similar results were obtained from 2 additional experiments. (D) Quantitative real-time PCR data of *Aicda* transcript (mean ± SD, n=6/group). Total cellular RNA was extracted at day 2 after culture. ***, highly significantly different ($p < 0.001$) as determined

by ANOVA (A, B and D). (E) Total protein lysates were prepared from B cells directly *ex vivo* (day 0) or at day 3 after culture. Proteins were separated by SDS-PAGE and then immunoblotted for AID, Bcl-6, and Blimp-1 followed by TRAF3 and actin. Western blots shown are representative of 3 independent experiments.

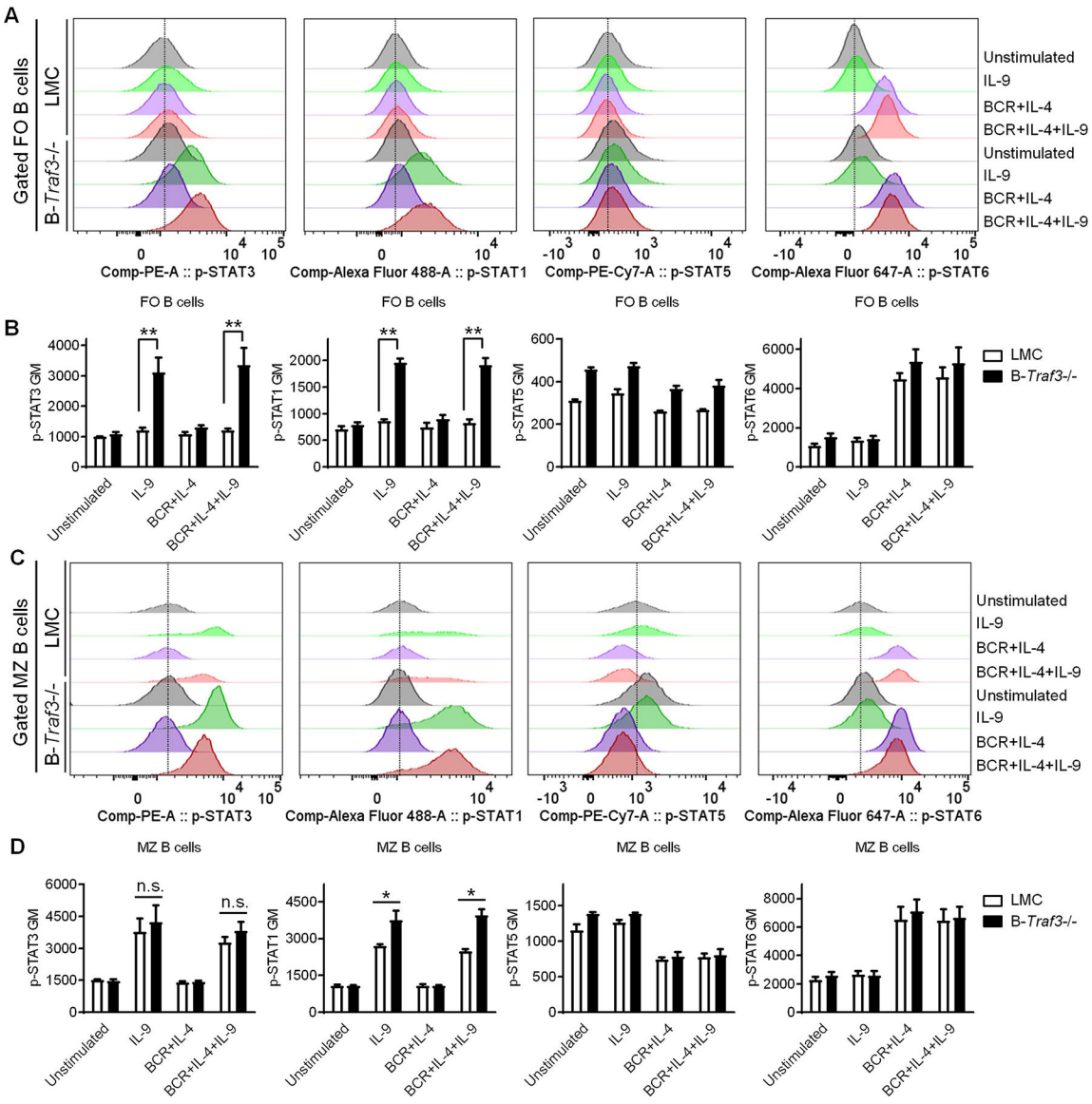

Figure 4. IL-9 induced the phosphorylation of STAT3 and STAT1 in *Traf3*$^{-/-}$ B cells.

Splenic B cells were purified from gender-matched, young adult LMC and B-*Traf3*$^{-/-}$ mice. Cells were starved in serum-free mouse B cell culture medium for 2 h, and then incubated with PBS (Unstimulated) or stimulated with 10 ng/ml IL-9 or 5 μg/ml anti-BCR (BCR) plus 10 ng/ml IL-4, alone or in combination as indicated in the figure, at 37°C for 15 minutes. Cells were subsequently kept at 4°C and stained with surface markers of mature B cell subsets followed by fixation, permeabilization and intracellular staining of phosphorylated STAT3 (p-STAT3), p-STAT1, p-STAT5, or p-STAT6. (**A**) FACS histograms comparing each specific p-STAT staining intensity in gated FO B cells (CD3-B220+CD21^{int}CD23^{hi}). (**B**) Graphical results of the geometric mean (GM) of each specific p-STAT staining intensity in gated FO B cells as determined by FACS (mean ± SEM, n=4/group). (**C**) FACS histograms comparing each specific p-STAT staining intensity in gated MZ B cells (CD3-B220+CD21^{hi}CD23^{int}). (**D**) Graphical results of the geometric mean (GM) of each specific

p-STAT staining intensity in gated MZ B cells as determined by FACS (mean ± SEM, n=4/group). FACS profiles shown (A and C) are representative of 4 independent experiments. n.s., not significantly different ($p > 0.05$); *, significantly different ($p < 0.05$); **, very significantly different ($p < 0.01$) as determined by ANOVA (B and D).

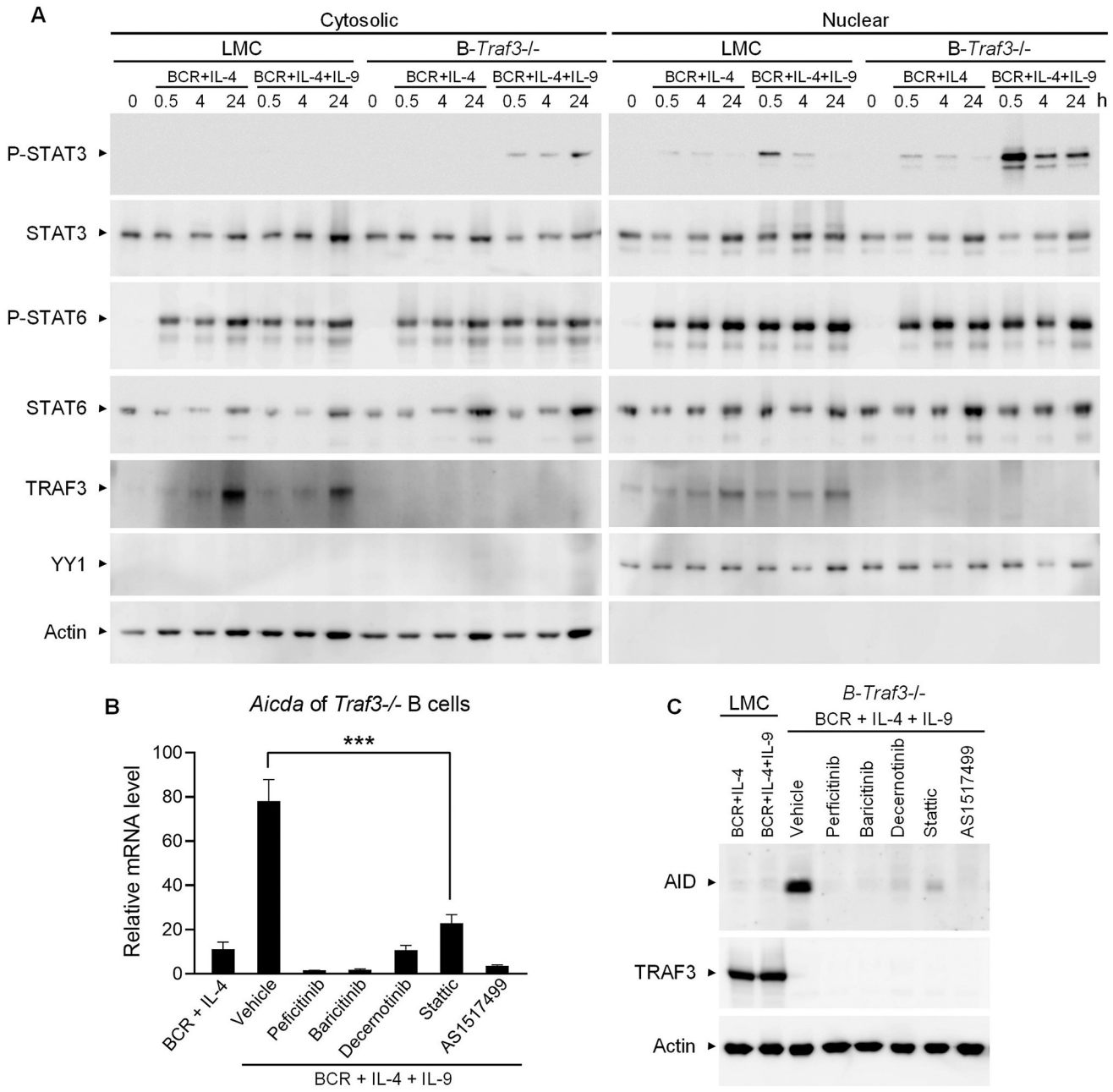

Figure 5. Activation of STAT3 and STAT6 as well as their roles in AID up-regulation induced by IL-9 together with anti-BCR plus IL-4 in *Traf3*$^{-/-}$ B cells.

Splenic B cells were purified from gender-matched, young adult LMC and B-*Traf3*$^{-/-}$ mice. (**A**) Differential activation of STAT3 and STAT6 in LMC and *Traf3*$^{-/-}$ B cells by IL-9 *versus* IL-4 in the presence of anti-BCR. Cells were analyzed directly *ex vivo* (0 h) or cultured at 37°C in the presence of 5 μg/ml anti-BCR (BCR) plus 10 ng/ml IL-4 with or without 10 ng/ml IL-9. Cytoplasmic and nuclear extracts were prepared at different time points after treatment (0, 0.5, 4, and 24 h). Proteins were separated by SDS-PAGE and then immunoblotted for phosphorylated (P-) or total STAT3 and STAT6 followed by TRAF3, YY1 and actin. (**B** and **C**) JAK and STAT inhibitors blocked the up-regulation of AID

induced by IL-9 together with anti-BCR plus IL-4. Cells were stimulated with 5 μg/ml anti-BCR (BCR) plus 10 ng/ml IL-4 with or without 10 ng/ml IL-9 in the absence (Vehicle) or presence of 0.5 μM of a specific inhibitor, including Peficitinib, Baricitinib, Decernotinib, Stattic or AS1517499. Total cellular RNA was extracted at day 2 and total protein lysates were prepared at day 3 after culture. (**B**) Quantitative real-time PCR data of the *Aicda* transcript expressed in *Traf3*$^{-/-}$ B cells (mean ± SD, n=6/group). ***, highly significantly different ($p < 0.001$) as determined by ANOVA. (**C**) Total protein lysates were separated by SDS-PAGE and then immunoblotted for AID followed by TRAF3 and actin. Western blots shown (A and C) are representative of 3 independent experiments.

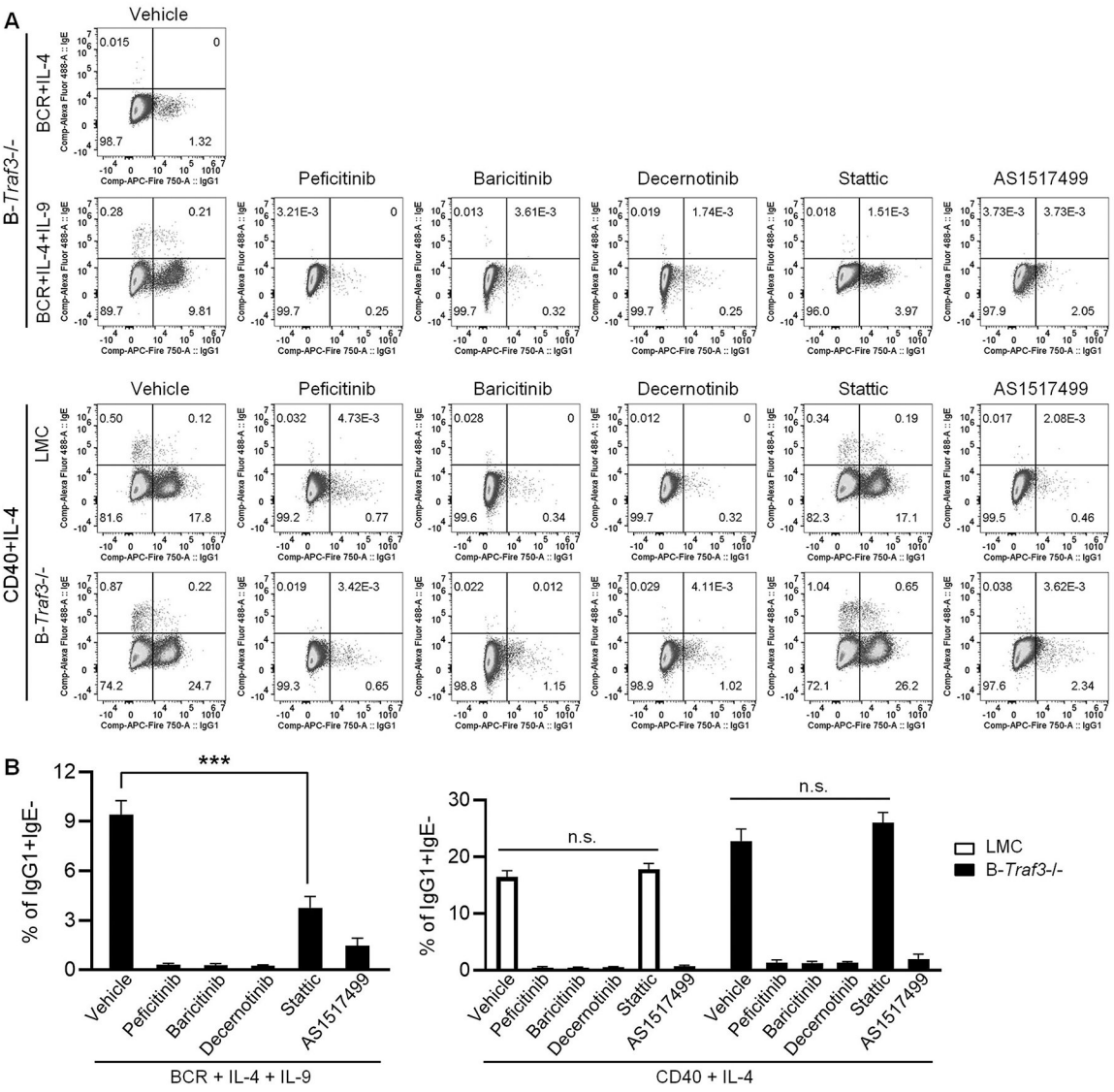

Figure 6. Effects of JAK or STAT inhibitors on IgG1 CSR induced by anti-BCR + IL-4 + IL-9 or anti-CD40 + IL-4.

Splenic B cells were purified from gender-matched, young adult LMC and B-*Traf3*$^{-/-}$ mice. Cells were stimulated with 5 μg/ml anti-BCR (BCR) plus 10 ng/ml IL-4 with or without 10 ng/ml IL-9, or 2 μg/ml anti-CD40 (CD40) plus 10 ng/ml IL-4, in the absence (Vehicle) or presence of 0.5 μM of a specific inhibitor, including Peficitinib, Baricitinib, Decernotinib, Stattic or AS1517499 as indicated in the figure. At day 4 after culture, cells were treated with Golgi-Plug for 5 hours before intracellular staining of IgG1 and IgE for FACS. (**A**) FACS profiles of IgG1 *versus* IgE staining. Results shown are representative of 4 independent experiments. (**B**) Graphical results of the percentage of IgG1+IgE- B cells as determined by FACS (mean ± SEM, n=4/group). n.s., not significantly different ($p > 0.05$); ***, highly significantly different ($p < 0.001$) as determined by ANOVA.

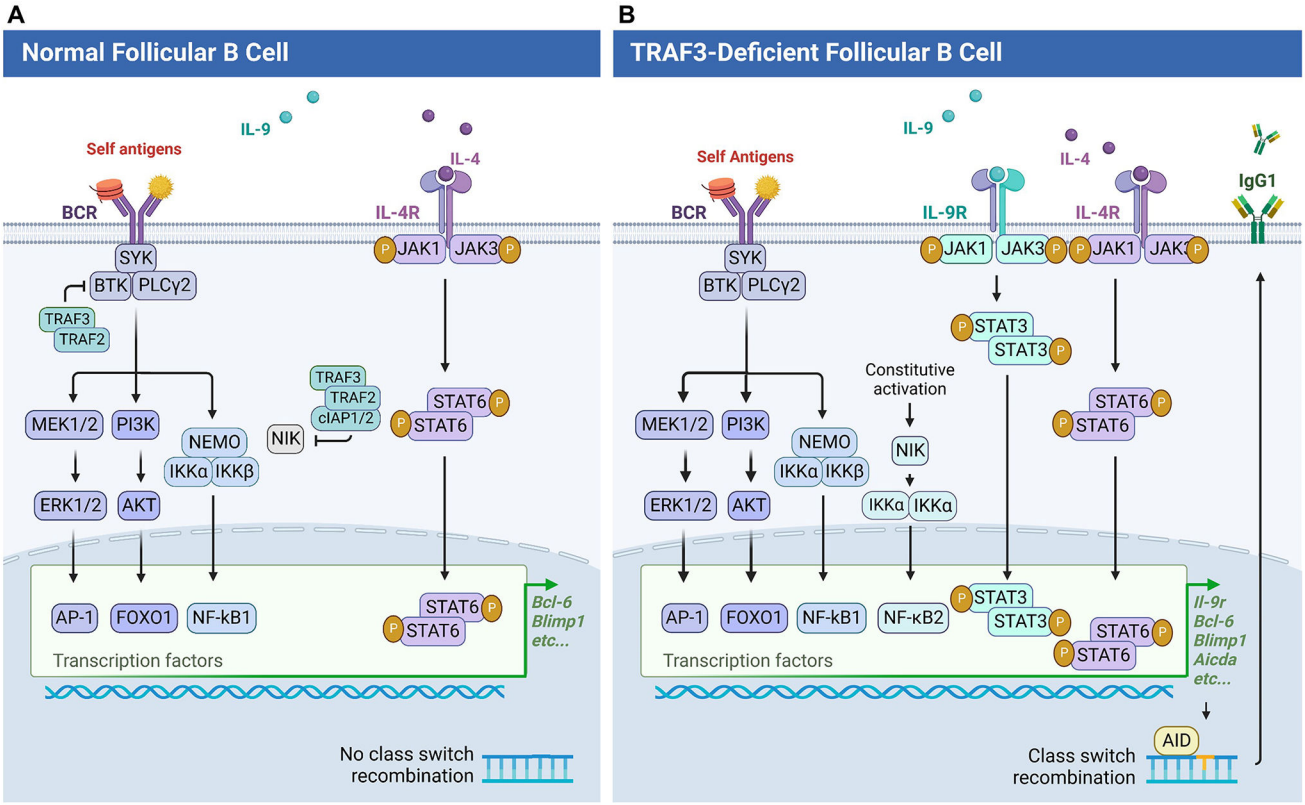

Figure 7. Signaling mechanisms of B cell activation and CSR induced by self antigens in the absence of intracellular TRAF3.

(**A**) Engagement of normal FO B cells by self antigens could not induce full activation and CSR due to lack of membrane-bound CD40L and secreted IL-21 provided by T_{FH} cells, even though inflammatory cytokines IL-4 and IL-9 may be present in the microenvironment. BCR signaling induces the activation of NF-κB1, AP-1, and Akt. IL-4 signaling induces STAT6 activation. Normal FO B cells do not express IL-9R and therefore are irresponsive to IL-9. Without CD40L-mediated NF-κB2 activation and IL-21-induced STAT3 activation, AID proteins are not sufficiently produced and thus CSR cannot be induced by self antigens in normal FO B cells. (**B**) In the absence of intracellular TRAF3, full activation and CSR of FO B cells can be induced by self antigens as long as they can engage the BCRs and the necessary cytokines IL-4 and IL-9 are present in the microenvironment. *Traf3* deficiency leads to constitutive NF-κB2 activation and highly up-regulated IL-9R expression in FO B cells. With constitutive NF-κB2 activation and IL-9-IL-9R-mediated STAT3 activation together with self antigen-BCR-induced NF-κB1 activation and IL-4-IL-4R-mediated STAT6 activation, the concerted action of NF-κB1, NF-κB2, STAT3 and STAT6 robustly induces the expression of AID, which mediates CSR to IgG1 in *Traf3*$^{-/-}$ FO B cells, thereby increasing the risks of autoimmunity. This figure was created with BioRender.com.