

Research Article

Targeting Bcl6 in the TREX1 D18N murine model ameliorates autoimmunity by modulating T-follicular helper cells and germinal center B cells

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The Three Prime Repair EXonuclease I (TREX1) is critical for degrading post-apoptosis DNA. Mice expressing catalytically inactive TREX1 (TREX1 D18N) develop lupus-like autoimmunity due to chronic sensing of undegraded TREX1 DNA substrates, production of the inflammatory cytokines, and the inappropriate activation of innate and adaptive immunity. This study aimed to investigate Thelper (Th) dysregulation in the TREX1 D18N model system as a potential mechanism for lupus-like autoimmunity. Comparison of immune cells in secondary lymphoid organs, spleen and peripheral lymph nodes (LNs) between TREX1 D18N mice and the TREX1 null mice revealed that the TREX1 D18N mice exhibit a Th1 bias. Additionally, the T-follicular helper cells (Tfh) and the germinal center (GC) B cells were also elevated in the TREX1 D18N mice. Targeting Bcl6, a lineage-defining transcription factor for Tfh and GC B cells, with a commercially available Bcl6 inhibitor, FX1, attenuated Tfh, GC, and Th1 responses, and rescued TREX1 D18N mice from autoimmunity. The study presents Tfh and GC B-cell responses as potential targets in autoimmunity and that Bcl6 inhibitors may offer therapeutic approach in TREX1-associated or other lupus-like diseases.

Keywords: autoimmunity · Bcl6 · inflammation · Tfh · Th1 · TREX1



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Billions of cells die each day as part of normal homeostatic processes, and the genetic material of these cells are disman-

tled in a coordinated fashion. Defects in this process trigger inappropriate immune responses, which may promote autoimmunity, as indicated by mice with defects in apoptotic cell clearance developing systemic lupus erythematosus (SLE or lupus)-like autoimmune diseases [1–3]. Among the several DNA exonucleases involved in DNA metabolism, TREX1 is a major 3-prime to 5-prime exonuclease that can digest ss- and ds DNA [4–6].

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Several mutations contributing to a spectrum of human autoimmune disorders have been identified in this single-exon containing gene. Large deletions or frame shifts in the *TREX1* locus cause Aicardi-Goutières syndrome (AGS), a severe neuroinflammatory disease, while point mutations impacting catalytic activity cause familial chilblain lupus (FCL), an inflammatory skin disorder [7]. Additionally, certain *TREX1* mutations have been identified as common risk alleles for the development of SLE [8]. The *TREX1* D18N allele causes FCL in humans [9] and when translated into a murine model, mimics the disease phenotype and immune dysregulation observed in these patients [10].

Cytosolic DNA sensing and innate immune responses are vital for the maintenance of normal DNA metabolism and antiviral responses. The cGAS-STING pathway contributes to immune-activation with AIM2 inflammasome reported to be dispensable in *TREX1* null mice [11, 12]. While *TREX1* null animals represent a good model of the acutely aggressive AGS phenotype, mutations observed in lupus patients are not known to represent a complete deletion of the *TREX1* gene. Differences in the autoimmune phenotype and the role of various DNA sensors between global *TREX1* null (KO) and *TREX1* D18N animals remain to be fully explored.

Recent studies supports that there could either be a T-follicular helper cells (Tfh cells) transition into Th1 cells or Tfh cells can make more Th1-like cytokines in autoimmunity and other inflammatory conditions [13–16]. Tfh cells represent a specialized CD4 Th cell subset, which can be activated by conventional APCs. They are identified by the expression of surface molecules CXCR5 and PD-1 and the transcription factor Bcl6. It is known that antigen-activated T cells expressing CXCR5 localize at the T-cell/B-cell border, and some of these cells commit to Tfh lineage to provide help to activated B cells in the GC reaction. Overactivation of this process triggers autoimmune responses and production of high-affinity autoantibodies [17]. Whether such immune dysregulation occurs as a result of uncleared DNA is currently unknown.

The current study aimed to investigate immune dysregulation in the *TREX1* D18N mutant mouse model, relative to the more broadly studied *TREX1* null model and to explore therapeutic strategies to ameliorate autoimmunity. We present the first evidence of a Th1 bias in the *TREX1* D18N mutant mice, which correlates with a heightened Tfh–GC B-cell response. We successfully implement a therapeutic strategy of Bcl6 inhibition to target the Tfh–GC B-cell crosstalk and demonstrate the rescue of *TREX1* D18N mutant animals from autoimmunity. These results highlight the Tfh–GC B-cell response as a potential therapeutic target in autoimmunity and spontaneous lupus-like inflammatory diseases triggered by defective DNA clearance.

Results

Status of CD4 T-helper cells and T-effector memory in *TREX1* deficiency

Previous studies with 6- to 12-month-old *TREX1* D18N mice showed high mortality and immune activation [10]. To investi-

gate the early events that may contribute to autoimmunity in the *TREX1* mutant mice, we first investigated the status of CD4 Th cells and effector memory cells in the secondary lymphoid organs (spleen and pooled peripheral LNs) of 8- to 12-week-old *TREX1* D18N and *TREX1* null (KO) mice (Fig. 1). Both *TREX1* D18N and *TREX1* KO mice exhibited elevated numbers of activated (CD69⁺) CD4 Th cells in spleen and LNs. The numbers of CD44⁺CD62L^{lo} T-effector memory were also significantly higher in both *TREX1* D18N and *TREX1* KO mice, indicating an active T-cell dysregulation at the age of 8–12 weeks. Similarly, the proportions of the activated and effector memory cells within the CD4 T-cell population were also increased in the *TREX1* D18N and *TREX1* KO mice (Supporting information Figs. S1A and B, S8A and B).

Increased cytokine production by CD4 T cells and Th1 bias in *TREX1* deficiency

To further delineate the mechanisms rendering *TREX1* mutant mice prone to autoimmunity, we performed intracellular cytokine analysis to investigate Th1 and Th2 phenotypes. CD4⁺ Th cells in the spleen and LNs of both *TREX1* D18N and *TREX1* KO mice displayed higher number and proportion of the IFN- γ producing Th1 cells as compared to WT littermates (Fig. 2A and Supporting information Fig. S2A). CD4 T cells from *TREX1* D18N mice also had high levels of cells producing the proinflammatory cytokine TNF- α , whereas the CD4 T cells from *TREX1* KO mice unexpectedly had significantly less cells producing TNF- α than WT littermates (Fig. 2B and Supporting information Fig. S2B). Differences in TNF- α production between *TREX1* D18N and *TREX1* KO cells is particularly interesting and require further investigation. Splenic CD4⁺ T cells producing the Th2 cytokines IL-4 and IL-5 were also higher in the *TREX1* D18N and *TREX1* KO mice compared to the WT littermates (Fig. 2C and D and Supporting information Fig. S2C and D). Interestingly, CD4 T cells from the LNs of *TREX1* D18N or *TREX1* KO animals did not show elevated levels of IL-4 producing CD4 T cells as compared to the WT littermates, although the number and proportion of IL-5 producing cells were higher in the LNs of *TREX1* D18N mice (Fig. 2D and Supporting information Fig. S2D).

Calculation of the ratio of the absolute numbers of CD4⁺ T cells producing IFN- γ over IL-4 revealed a significantly higher IFN- γ /IL-4 ratio in the *TREX1* D18N mice, suggestive of a Th1 bias. *TREX1* KO mice also showed a similar trend, though it did not reach statistical significance (Fig. 2E). The ratio of the percentages of IFN- γ and IL-4 producing cells produced the same results, because the absolute numbers were calculated based on the percentages. To confirm the Th1 bias in the *TREX1* D18N mice, we established an in vitro assay by employing BM-derived macrophages (BMDM), treated with apoptotic cells, to delineate the role of defective *TREX1* in inducing a dysregulated T helper response extrinsically in WT T cells. The rationale behind this experiment was that postengulfment, apoptotic cells would represent a source of DNA that would be degraded in WT BMDM and not in *TREX1* D18N BMDM, thus, inducing proinflammatory

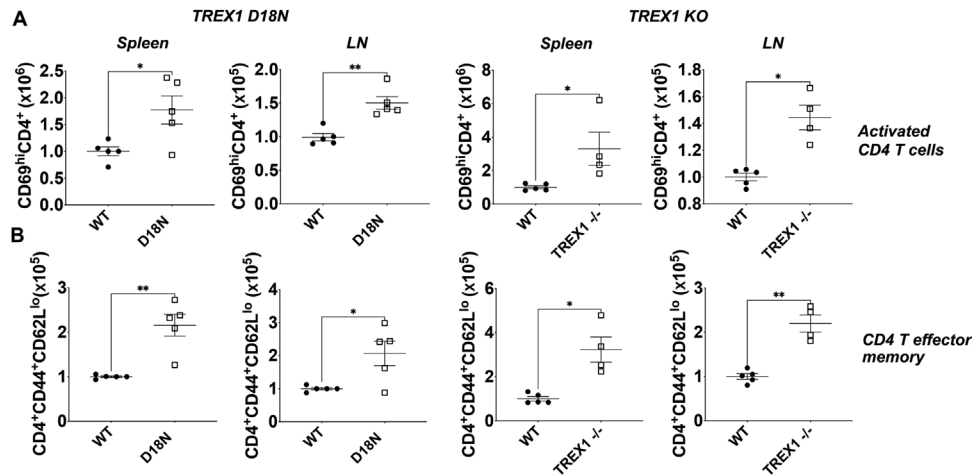


Figure 1. Elevated levels of CD4⁺ T helper cells and T effector memory (T_{em}) in TREX1 deficiency. Spleen and pooled peripheral lymph nodes (cervical, axillary, brachial, and inguinal lymph nodes) were obtained from female (8–12 weeks old) TREX1 D18N and TREX1 knockout mice and levels of (A) activated CD4⁺ T cells (CD4⁺CD69^{hi}) and (B) T_{em} status (CD4⁺CD44⁺CD62L^{lo}) were analyzed by flow cytometry. Absolute number data are presented as mean \pm SEM relative to WT mice (normalized absolute numbers) and a total of four to five mice per group over three independent experiments, with the dots representing individual mice. * $p < 0.01$, ** $p < 0.001$ as calculated by two-tailed unpaired Student's t-test and Mann-Whitney test. Also see Supporting information Fig. S1 for data in percentages.

signals, leading to the observed T-cell polarization. The BMDM were cocultured for 16 h with apoptotic WT thymocytes as described in the Material and Methods. Apoptosis induction was confirmed by flow cytometry analysis by employing Annexin V and 7AAD staining (Supporting information Fig. S5). Subsequently, naive CD4⁺ T cells from WT mice were added and the ability of the BMDM to dictate the Th1 and Th2 differentiation was investigated. Both WT and D18N BMDM induced similar IFN- γ production in the responder WT T cells, whereas in the presence of TREX1 D18N BMDM, IL-4 production in the responder CD4 T cells was significantly attenuated (Fig. 2F). Calculation of the ratio of IFN- γ /IL-4 recapitulated the observed in vivo Th1 bias of the TREX1 D18N mice. It should be taken into account that in this assay we only looked at WT cells as T-effector cells. Normalization on Th2 shows Th1 elevation. We therefore only see an extrinsic effect. The BMDM experiments suggest that macrophages from the D18N mutant mice, induce less anti-inflammatory cytokine IL-4 without affecting the IFN- γ production, which may contribute to the Th1 bias as observed in vivo. We also investigated the levels of Th17 cells and observed a marginal elevation of IL-17 producing cells in spleen of TREX1 D18N mice that was close to significance, however, no significant differences were observed in the LNs and in FX1-treated animals (See Fig. 4 below and Supporting information Fig. S10). There was also a marginal elevation of *ROR γ* transcript levels in the spleens without reaching statistical significance (Supporting information Fig. S10C).

Elevated T-follicular helper cell and activated B-cell responses in TREX1 deficiency

We next sought to delineate the mechanism leading to the observed skewing of the CD4 T-cell compartment toward the Th1

lineage. Tfh can facilitate autoimmunity by helping autoreactive B cells to undergo maturation and produce autoantibodies [18–20]. Since common transcriptional features between Th1 and Tfh generating cells has been reported in autoimmunity [21–23], we hypothesized that the Th1 bias observed in TREX1 D18N animals may coincide with an increased and potentially proautoimmune Tfh response. Indeed, elevated levels of CXCR5⁺PD1⁺CD4⁺ Tfh cells in the TREX1 D18N and TREX1 KO mice were observed consistent with this hypothesis. Correspondingly, using gating strategy based on earlier studies to measure B-cell responses [24], we also observed elevated levels of GL7⁺CXCR5⁺B220⁺ cells in the secondary lymphoid organs of the both TREX1 KO and TREX1 D18N mice as compared to WT littermates (Fig. 3A and B, Supporting information Figs. S3A–B and 9A–B), suggesting an exacerbated GC reaction. Indeed, the TREX1 D18N mice displayed greater GC B cells, measured as GL7^{hi}Fas⁺ cells in the B220 gated cells (Supporting information Fig. S6A), which correlated well with the increased GL7⁺CXCR5⁺B220⁺ cells in the TREX1 mutant mice. Thus, TREX1 D18N mice exhibit a simultaneous dysregulation of Th1 as well as Tfh-/B-cell responses, which may drive the autoimmune phenotype.

Pharmacologic targeting of Bcl6 ameliorates T-cell dysregulation in TREX1 D18N mice

Bcl6 is an important transcription factor known for its role in the differentiation of Tfh and GC B-cells [18, 25–27]. Bcl6 is also highly expressed in B-cell lymphoma and acts as a survival factor. Targeting of Bcl6 has been tested in experimental studies for the treatment of B-cell lymphoma [28–31]. We hypothesized that Bcl6 may be targeted to intervene with the Tfh and GC B cells that may also intervene with the Th1 bias as a potential

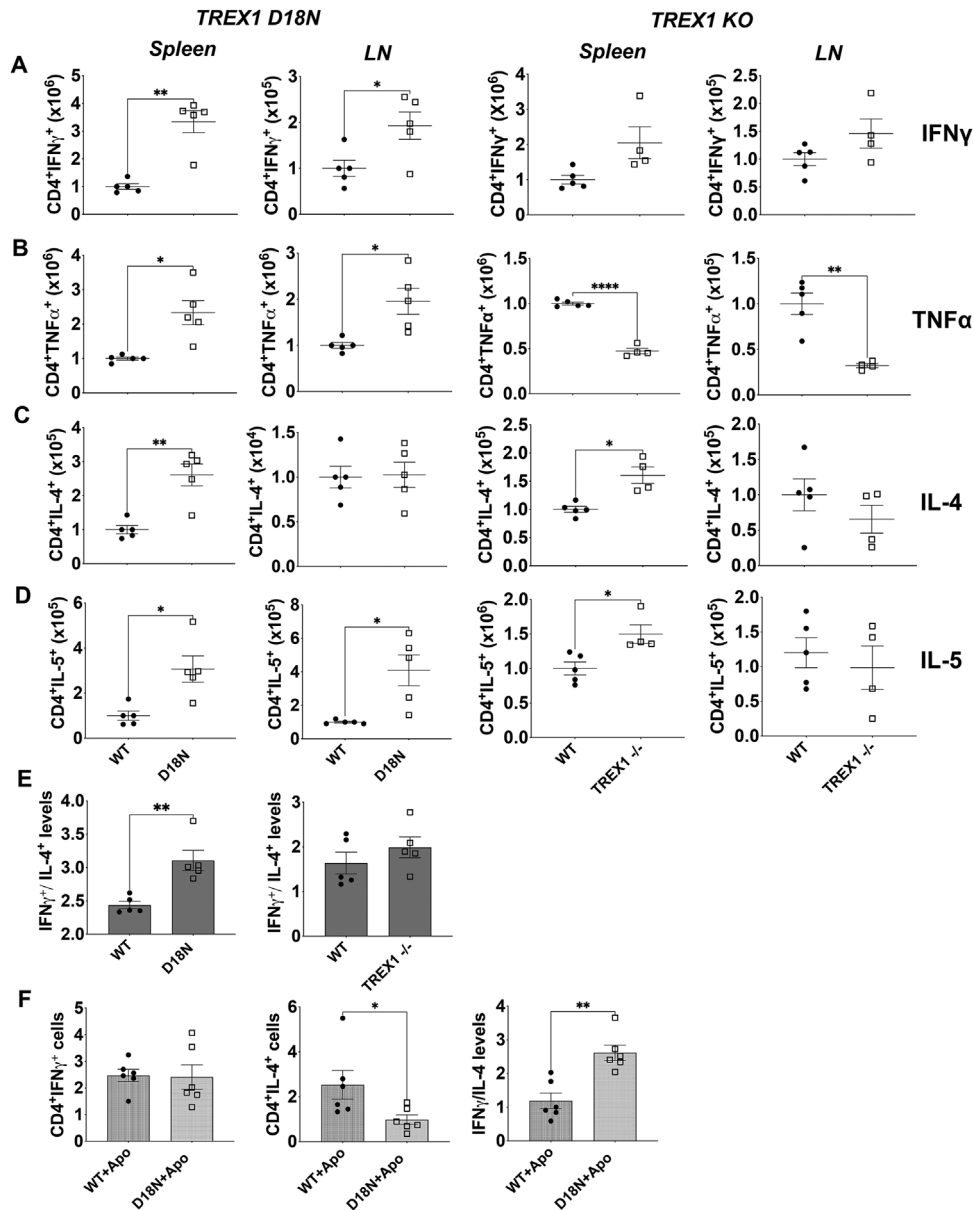


Figure 2. Status of T-helper cytokines in TREX1 deficiency. Spleen and lymph nodes (LN) from female TREX1 D18N and TREX1 knockout mice were stimulated with PMA/Ionomycin for 5 h and levels of Th1 cytokines IFN- γ (A), TNF- α (B), and Th2 cytokines IL-4 (C) and IL-5 (D) were analyzed by flow cytometry. (E) The ratio of IFN- γ to IL-4 producing CD4 T cells indicated a significant Th1 bias as shown in the spleen of TREX1 D18N mice, but did not reach statistical significance in TREX1 KO animals. (F) In vitro T-cell stimulation assay using BM-derived macrophages as described in the Materials and Methods showed that TREX1 D18N macrophages induced similar levels of %IFN- γ producing T cells, but much fewer %IL-4 producing cells. A ratio of IFN- γ /IL-4 confirmed the greater Th1 bias induced by the TREX1 D18N macrophages. Data presented as mean \pm SEM relative to WT mice (normalized absolute numbers) and a total of four to five mice per group over three independent experiments, with the dots representing individual mice, and three independent experiments for in vitro assays. * p < 0.01, ** p < 0.001 as calculated by two-tailed unpaired Student's t -test. Also see Supporting information Fig. S2 for data in percentages.

treatment for TREX1 D18N-associated autoimmunity. To test this hypothesis, we used the commercially available Bcl6 inhibitor FX1, which was shown to phenocopy Bcl6 deletion in mice by inhibiting GC formation in the anti-sheep RBC murine model [29] and also suppressed GC-diffuse large B-cell lymphomas (DLBCL) [29]. We treated TREX1 D18N mice with FX1 for 8 days followed by detailed investigation on day 10. The levels of activated CD4 T helper cells were significantly reduced in

the FX1-treated TREX1 D18N mice as compared to the vehicle controls down to the levels of WT controls in LNs, with a similar trend in spleens as well (Fig. 4A and Supporting information Fig. S4A). FX1-treated TREX1 D18N mice also demonstrated a significant attenuation of the Th1 response in the LNs and showed a similar trend in the spleen, as measured by the reduced ability of the CD4 T cells to produce IFN- γ (Fig. 4B and Supporting information Fig. S4B). The TREX1 D18N mice treated with FX1

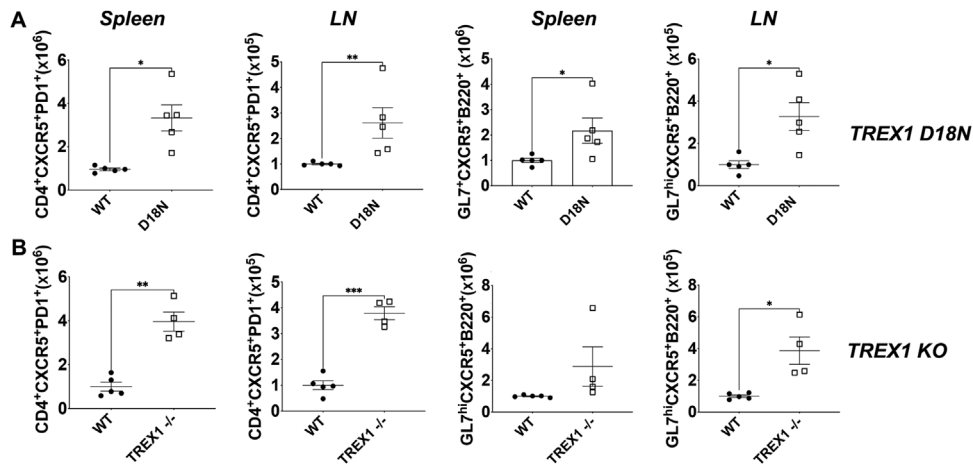


Figure 3. Elevated T-follicular helper (Tfh) cell response and germinal center reaction in TREX1 deficiency. Levels of Tfh cells (CD4⁺CXCR5⁺PD1⁺) and GL7^{hi}CXCR5⁺B220⁺ B-cells in (A) TREX1 D18N and (B) TREX1 KO female mice analyzed by flow cytometry. Data presented as mean ± SEM relative to WT mice (normalized absolute numbers) and a total of four to five mice per group over three independent experiments, with the dots representing individual mice. **p* < 0.01, ***p* < 0.001, ****p* < 0.0001 as calculated by two-tailed unpaired Student's *t*-test. Also see Supporting information Fig. S3 for data in percentages.

exhibited a noticeable trend toward attenuation of Tfh and GC B-cell responses, with the most prominent effects being observed in splenic GC B cells (Fig. 4C and D and Supporting information Fig. S4C and D). Histological analysis of spleens showed that

TREX1 D18N GCs are significantly enlarged relative to WT mice (Fig. 4E). Such histological analysis using H&E staining for investigation of GC changes have also been utilized by others [32] [https://www.arppress.org/v/vspfiles/assets/images/Chapter-

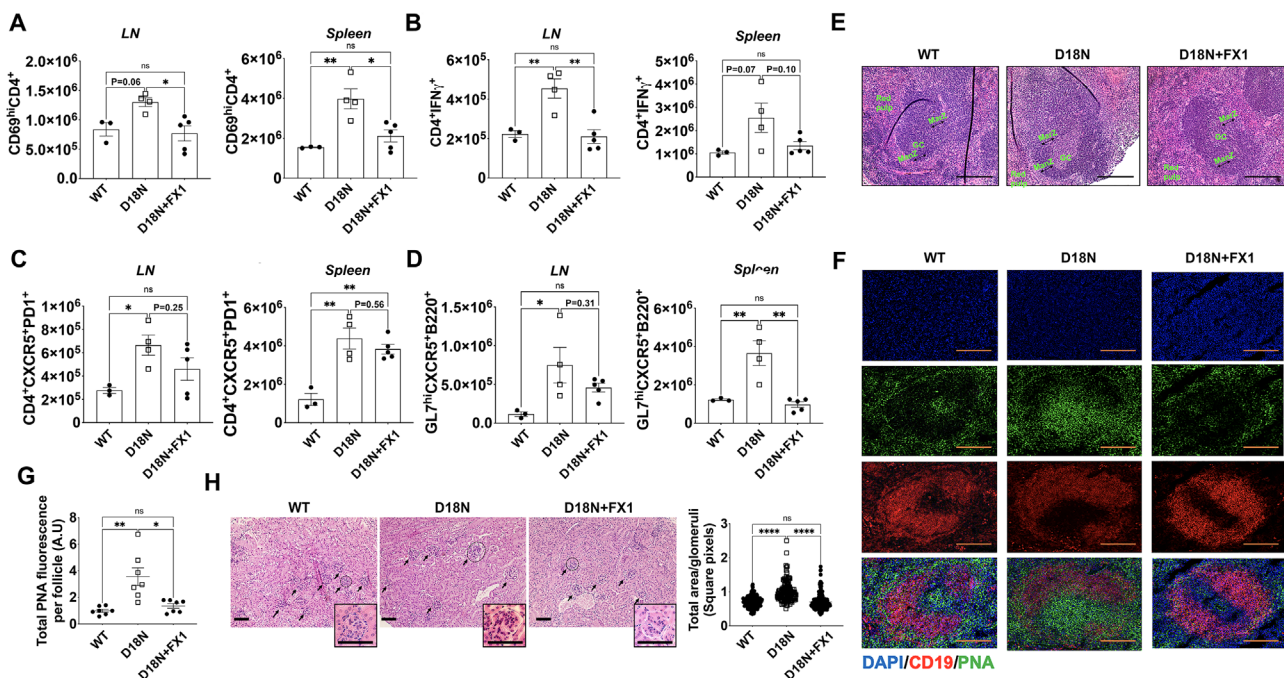


Figure 4. Bcl6 inhibition rescues from autoimmunity in TREX1 D18N mutant mice. Mice were treated as described in Materials and Methods and analyzed by flow cytometry for (A) Activated (CD69⁺) CD4⁺; (B) IFN- γ producing CD4⁺ T cells; (C) CD4⁺CXCR5⁺PD1⁺ T follicular helper cells; and (D) GL7^{hi}CXCR5⁺B220⁺ B cells; (E) Representative H&E stained sections of the spleens. Spleens from WT, D18N mutant DMSO control, and D18N mutant FX1-treated animals were stained with H&E and screened for germinal centers (GC), Mantle zone (ManZ), Marginal zone (MarZ), and Red pulp, Scale bars 50 μ m. (F) Immunofluorescence for PNA/CD19/DAPI, Scale bars 50 μ m and (G) Quantification of total PNA fluorescence per splenic follicle by ImageJ. (H) Representative H & E stained sections of the kidneys and quantification of total area per glomeruli by ImageJ, Scale bars 50 μ m (Insert shows glomeruli). Arrows point to individual glomeruli. Data are shown as mean ± SEM and show a total of five mice per group over two independent experiments and shown as relative to D18N animals. **p* < 0.01, ***p* < 0.001, ****p* < 0.0001 as calculated by multiple comparison by one-way ANOVA. Also see Supporting information Fig. S4 for data in percentages and GL-7 staining for GC B cells.

1T25LymphNodes.pdf; Figs. 1–6]. As anticipated, the GC size was markedly reduced in TREX1 D18N mice treated with FX1. This finding was further confirmed by immunofluorescence analysis with peanut agglutinin (PNA) staining, which similarly demonstrated a reduction in FX1-treated TREX1 D18N mice (Fig. 4F). We also performed immunofluorescence analysis with GL7 staining, which displayed a similar trend of a reduction of GC size in FX1-treated TREX1 D18N mice (Supporting information Fig. S4E). We scored multiple splenic follicles from multiple mice for total PNA and GL7 fluorescence that further confirmed a reduction of GC (Fig. 4G and Supporting information Fig. S4F). We also investigated the transcript levels of *Tbet* and *Gata3* and observed significantly higher levels of *Gata3* in the spleens of FX1-treated TREX1 D18N mice. The ratios of *Tbet:Gata3* further confirmed the observed inhibitory effects on the Th1 responses in FX1-treated mice (Supporting information Fig. S6B).

FX1-mediated targeting of Bcl6 rescues TREX1 D18N mice from autoimmunity

To determine the effects of the Tfh-GC inhibition by FX1 on autoimmunity in TREX1 D18N mutant mice, we performed immunofluorescence analysis of the immune complex deposition in the kidneys. Although the TREX1 D18N mutant mice do not develop glomerulonephritis-related loss of renal function at this age, they demonstrate strong immune-complex deposition in glomeruli and tubulointerstitium [10]. Analysis of immune-complex deposition in the kidneys revealed that there was an apparent reduction of IgG deposits in FX1-treated mice in comparison to vehicle control in the glomerular and extra-glomerular regions in the kidneys (Supporting information Fig. S7A). We observed a significant reduction in the glomerular hypertrophy upon FX1 treatment as compared to the vehicle-treated TREX1 D18N mice (Supporting information Fig. S7A). Morphometric analysis of the size of glomeruli using the H & E stained sections confirmed that compared to the WT mice, the glomeruli in the TREX1 D18N mutant mice are significantly enlarged and that FX1 treatment of TREX1 D18N mice restored the size of the glomeruli in similar to WT mice (Fig. 4H). However, no other pathological changes were obvious in the kidneys. We also investigated the levels of Blood Urea Nitrogen in these mice to measure renal function and observed that the levels were not affected (Supporting information Fig. S7D). The TREX1 D18N mice, thus, does not exhibit overt renal pathology, but display glomerular hypertrophy, which was inhibited by FX1 treatment (Fig. 4H and Supporting information Fig. S7A). The data are suggestive of not only immunological changes systemically, but also restoration of organ-specific autoimmunity as indicated by glomerular integrity upon FX1 treatment (Fig. 4H). We also did not observe any obvious adverse effects at the dose of FX1 utilized in the TREX1 D18N mice.

Next, we used a Western blot assay to evaluate the autoantibodies in plasma samples of WT, TREX1 D18N mutant mice treated or untreated with FX1 by measuring reactivity to murine B-cell lymphoma WEHI-231 cell extract. Compared to the WT

littermates, the plasma of 100% of TREX1 D18N mice showed higher reactivity to WEHI-231 cell extract. On the other hand, the plasma reactivity in 60% of FX1-treated animals was similar to the WT mice and lower than the control TREX1 D18N mice, indicating a rescue from autoimmunity (Supporting information Fig. S7B). We then investigated the plasma samples for anti-dsDNA antibody levels, as a surrogate marker for autoantibody production and observed reduced levels in the FX1-treated mice. Although there is a noticeable decline in the anti-dsDNA antibody levels, the difference however did not reach statistical significance (Supporting information Fig. S7C). We further compared the plasma samples from the FX1-treated mice with WT and vehicle-treated samples using a novel autoantibody array. Heat map and clustering analysis of the antibody array confirms differential autoantibody patterns in FX1-treated and untreated TREX1 D18N mice. However, it also indicates the large variability in the autoantibody profiles of WT as well as TREX1 D18N mutant mice (Supporting information Fig. S7E). Nevertheless, three out five FX1-treated mice showed a strong reduction in the levels of autoantibodies especially against the cluster of autoantigens containing dsDNA, ssDNA, genomic DNA, ssRNA, Histone H4 along with several others that include matrix-associated proteins collagen I and II, proteoglycan, chondroitin sulfate, heparin sulfate, sphingomyelin, and laminin in the bottom of the heat map. Overall, these data demonstrate that Bcl6 inhibition in TREX1-deficient mice induced a trend for the reduction in the autoantibody response, which could contribute to a rescue from the autoimmune phenotype observed in the TREX1 D18N mice. Furthermore, FX1-treated mice exhibited a significant decline in helper T cell dysregulation, indicating a kinetic disconnect of the Th1/Tfh immune response with the circulating autoantibody levels. These data indicate that future long-term studies using Bcl6 inhibition for the treatment of lupus-like autoimmune diseases are warranted.

Discussion

Current treatment options available for autoimmune diseases, such as lupus nephritis, involves steroids and immunosuppressants that have serious side-effects [33–37]. TREX1 is a key cytoplasmic exonuclease which protects the cell from self-DNA-induced immune activation [4–6]. The TREX1 D18N mutation found in human patients was translated into a murine model, and these mice exhibit spontaneous lupus-like features [10]. Both TREX1 D18N and TREX1 KO mice exhibited an active disease state at 8 to 12 weeks of age as indicated by elevated levels of CD4 T helper cells and elevated T effector memory status (Fig. 1).

Dysregulated cytokine production is central to autoimmunity, with alterations in the balance between Th1 and Th2 signaling strongly implicated in the loss of self-tolerance [38–40]. Although both Th1 and Th2 cytokine production was elevated in the secondary lymphoid organs of TREX1-deficient mice (Fig. 2), analysis of the Th1/Th2 ratio revealed an Th1 bias in TREX1 D18N mice (Fig. 2E), though not as robustly in the TREX1 KO mice. TNF- α production was also elevated in the TREX1 D18N

mutant mice but was surprisingly downregulated in TREX1 KO mice, an observation warranting further investigation. These data demonstrate that TREX1 D18N animals mimic the Th1 bias commonly observed in SLE patients [41–43].

Tfh dysregulation can facilitate proautoimmune GC responses observed in SLE patients [15, 20, 44]. Cells generating both Th1 and Tfh cells have been postulated to have features of both subsets, with possible interconversion [13–16]. The transcription factor Bcl6 participates in the differentiation and maintenance of both Tfh and GC B-cells [25–27]. Targeting Bcl6, therefore, represents an attractive strategy for ameliorating autoimmune disease involving Tfh-GC B-cell dysregulation and abnormal B-cell activation. The Bcl6 inhibitor FX1 exhibits therapeutic efficacy in targeting B-cell lymphoma, and was shown to reduce Tfh and GC B-responses in sheep RBC immunization model [29], making it a promising candidate for attenuation of the TREX1 D18N autoimmune phenotype.

As evident from Fig. 4A and B, activated CD4⁺ T cells and IFN- γ producing cells in LNs were significantly reduced in FX1-treated mice with spleen samples displaying a trend toward reduction. This may either be due to a reduction in the IFN- γ producing Tfh cells or a direct inhibition of activated T cells, because of a direct effect of FX1 on CD69, a target of Bcl6 [29]. FX1-treated TREX1 D18N mice exhibited a trend toward reduction of Tfh levels and GC B cells in the secondary lymphoid organs, with the levels in spleen approaching the WT levels (Fig. 4C and D). This was further confirmed with histological analysis with H&E staining as well as immunofluorescence, revealing effects on germinal centers with FX1 treatments (Fig. 4E–G). A variety of autoantibodies have been reported with antigenic locations in nuclear, cytoplasmic, or mitochondrial, and extracellular in SLE [45]. Three mechanisms have been postulated as being at the forefront of autoantibody production: (1) cell death which leads to the release of intracellular components; (2) neoantigens that are produced as a result of modification of self-antigens to trigger B-cell autoreactive responses; and (3) responses toward foreign antigens [45]. Treatment with FX1 induced a discernible effect on the circulating autoantibody levels in the WEHI-231 B-cell lymphoma Western blot assay, anti-dsDNA antibody assay, as well as the multiplex autoantibody profiling (Supporting information Fig. S4).

Interestingly, these effects coincided with the reduction of a generalized immune complex deposition in the kidneys of FX1-treated mice, and with significant indications of preserved glomerular integrity and rescue from autoimmunity (Fig. 4H). Mild reduction of the autoimmune readouts were expected, as FX1 treatment was only given short-term. Long-term treatment was not possible due to the intraperitoneal route of drug administration, and better delivery systems need to be developed. Notably, FX1 was recently shown to alter Tfh-GC responses to sheep RBC injection and lymphoid hyperplasia in mice as well as a short-term nonhuman primate study [46, 47]. However, if approaches such as these were to be employed for treatment options in autoimmune diseases, studies would need to be conducted to establish their safety and interference with the host-immunity to infectious diseases or response to immunizations.

Investigations into an effect of such therapies on myeloid, T cell, B cell, or mesangial compartments are particularly interesting and are in prospects.

Nevertheless, these data demonstrate that Bcl6-targeting with FX1 may not just inhibit the Tfh/GC B-cell axis, but more critically translate into a reduced Th1 response in the secondary lymphoid organs and ultimately to the rescue of TREX1 D18N mutant mice from end-organ autoimmunity. These findings further our understanding of autoimmune pathogenesis caused by failed DNA clearance and may have relevance for the treatment of autoimmunity in human TREX1 deficiency and other lupus-like diseases.

Materials and methods

Animals

TREX1^{D18N/D18N} (TREX1 D18N) mutant mice were generated on a 129S6/SvEvTac background using an allelic replacement strategy as described before [10]. The mice were backcrossed 10 generations with C57BL/6J (B6) mice. Transmission of the TREX1 D18N allele was confirmed by tail-DNA sequencing. All experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the University of Virginia and Wake Forest Baptist Medical Center. TREX-null (KO) mice revived from cryopreserved embryos were obtained from KOMP Repository, UC Davis. Eight to twelve weeks old female mice were used in this study.

Flow cytometry and intracellular cytokine analysis

Flow cytometry analysis was performed as described previously [48, 49]. Antibodies used for analysis are listed in Supporting information Table S1. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (BD Biosciences) using the gating strategies shown in Supporting information Figs. 1 and 2. Data are presented as absolute number relative to WT mice and normalized to treatment controls for in vitro analysis.

Generation of apoptotic cells and BM-derived myeloid cells (BMDM) for in vitro experiments

Thymii from WT female B6 mice were crushed in RPMI complete media and the cell suspension was filtered using a 70-micron cell strainer. A total of 5×10^6 cells/mL were incubated in a 10 cm bacterial dish with 5 μ M dexamethasone for 6 h in a 37°C and 5% CO₂ incubator. The cells were then washed 3 \times with 15 mL DPBS before using for the experiment. Cells were stained with FITC-labeled Annexin-V and 7AAD for flow cytometry to confirm apoptotic cell generation. BMDM have been used to study T-cell stimulation [50] and were generated as before [51]. Briefly, single-cell suspension of BM, flushed from the femurs and tibias of mice, was

cultured in bacteriological petri plates in 10 mL RPMI complete media (RPMI containing 2 mM L-glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin (all from Life Technologies), and 10% fetal bovine serum (Sigma) supplemented with 15% L929 conditioned media [52] initially and 50% media replacement every 3 days. The L929 conditioned media was prepared by culturing the murine L929 cells to confluency in complete DMEM for 7 days, followed by filtering through 0.22 µm and stored at –20°C in aliquots.

Bcl6 inhibitor treatments

Bcl6 inhibitor FX1 (Sigma) was dissolved in DMSO (Sigma) at 25 mg/mL concentration and stored as aliquots at –20° C. Aliquots were thawed and diluted to 300 µL in USP-grade sterile saline before intraperitoneal injections in TREX1 D18N mice at 40 mg/kg body weight. An equal volume of DMSO similarly diluted in saline was used as placebo controls. Treatments involved daily injection for 8 days and the mice were euthanized on day 10.

Immunofluorescence and microscopy

Kidneys frozen in liquid nitrogen were embedded in Optimal Cutting Temperature compound (Ted Pella Inc.). Tissue was cryosectioned (5 µm) and permeabilized with cold acetone for 20 min at room temperature. The slides were washed with PBS and blocked with 2% horse serum prepared in 3% BSA/PBS for 20 min at room temperature. The sections were stained for 30 min with 1:200 diluted horse anti-mouse IgG-Fluorescein (Vector Laboratories) followed by extensive washing in PBS containing 0.1% Tween 20 and mounting using ProLong Diamond Antifade (Thermo Fisher Scientific) containing DAPI counterstain. Images were acquired using Axiovert 200 microscopy system with Apotome imaging and Axiovision software (Carl Zeiss Microscopy, LLC). For GC studies, spleen sections were rehydrated for 30 min with PBS and blocked for 1 h with 10% BSA/PBS at room temperature. The sections were stained for 1 h with 1:100 diluted PNA-Fluorescein (Vector Laboratories) or anti-GL7-Alexafluor 488 and washed three times with PBS, followed by incubation with 1:50 diluted PE-anti-mouse CD19 antibody (Thermo Fisher Scientific) at room temperature for 1 h. The slides were washed and mounted using ProLong Diamond Antifade (Thermo Fisher Scientific) containing DAPI. Germinal centers were mapped using the selection tool and quantified using ImageJ (NIH). H & E stained spleen sections were also imaged for GC analyses.

Anti-dsDNA assay

Serum samples collected at necropsy were analyzed for anti-dsDNA antibodies by ELISA as described earlier [49, 53]. Briefly, pGEM-3zf plasmid DNA (Promega) was digested with S1-nuclease

(Thermo Fisher Scientific) and biotinylated using Photoprobe Biotin (Vector Labs) following the manufacturer's instructions. Biotinylated dsDNA (1 ng/mL) was used for the assay.

SDS-PAGE and immunoblot analysis

B-cell lymphoma WEHI-231 cell extract prepared by repeated freeze thawing was quantified for protein content with bicinchoninic acid protein assay kit (Pierce Chemical). Total 150 µg of protein extract was loaded onto a preparative 10% SDS-PAGE gel and transferred to Immun-Blot PVDF Membrane (Bio-Rad). After blocking the membrane in 3% BSA for 1 h, thin strips were cut and probed with 1:50 dilution of plasma samples overnight at 4°C. The strips were washed with PBS containing 0.1% Tween 20 and were incubated in 1:5000 IRDye 680RD anti-Mouse IgG (H+L) secondary antibody (LI-COR Biosciences) for 1 h at room temperature. The strips were aligned and imaged using Odyssey Fc Imaging System (LI-COR Biosciences).

Multiplex autoantibody profiling

Blood was collected at euthanasia by retro-orbital bleeding using heparinized capillary tubes (Fisher Scientific). Plasma obtained by centrifugation was used for the multiplex autoantibody profiling at UT Southwestern Medical Center Genomics and Microarray core facility (<https://microarray.swmed.edu/products/product/autoantigen-microarray-service-igg-igm/>).

Statistical analysis

All statistical analyses were performed using Prism 8 software (GraphPad). Representative data from three or more independent experiments are shown as mean ± SEM. For comparison of more than two groups, one-way ANOVA or Kruskal–Wallis test was used. For statistical analysis testing of two groups, two-tailed unpaired Student's *t*-test or Mann-Whitney U test was used.

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Conflict of interest: RS is an inventor for US patents 6897041 and 9840545 licensed to Slate Bio Inc., holds equity and is a consultant for Slate Bio Inc., unrelated to this work. The research was conducted in the absence of any commercial or financial relationships that could pose a potential conflict of interest.

Author contribution: RS, RV, and FWP conceived the project. RS, JMG, and FWP provided overall supervision. RV conducted the majority of the experiments. RV, VS, MD, SRS, and RS collected and analyzed data, and wrote the manuscript. NY helped with the autoantibody multiplex array. SEH, RV, and SM managed the mouse colony.

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Abbreviations: AGS: Aicardi-Goutières syndrome · BMDM: BM-derived macrophages · FCL: familial chilblain lupus · PNA: peanut agglutinin · SLE: systemic lupus erythematosus · Tfh cells: T-follicular helper cells

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