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TREX1 D18N mice fail to process erythroblast DNA resulting in inflammation and dysfunctional erythropoiesis

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Abstract

Anaemia is commonly observed in chronic inflammatory conditions, including systemic lupus erythematosus (SLE), where ~50% of patients display clinical signs of anaemia. Mutation at the aspartate residue 18 of the three prime repair exonuclease 1 (*TREX1*) gene causes a monogenic form of cutaneous lupus in humans and the genetically precise TREX1 D18N mice recapitulate a lupus-like disease. TREX1 degrades single- and double-stranded DNA (dsDNA), and the link between failed DNA degradation by nucleases, including nucleoside-diphosphate kinases (NM23H1/H2) and Deoxyribonuclease II (DNase II), and anaemia prompted our studies to investigate whether TREX1 dysfunction contributes to anaemia. Utilizing the TREX1 D18N mice we demonstrate that (1) TREX1 mutant mice develop normocytic normochromic anaemia and (2) TREX1 exonuclease participates in the degradation of DNA originating from erythroblast nuclei during definitive erythropoiesis. Gene expression, hematocrit, hemoglobin, immunohistochemistry (IHC) and flow cytometry were used to quantify dysfunctional erythropoiesis. An altered response to induced anaemia in the TREX1 D18N mice was determined through IHC, flow cytometry, and interferon-stimulated gene (ISG) expression analysis of the liver, spleen and erythroblastic islands (EBIs). IHC, flow cytometry, and ISG expression studies were performed *in vitro* to determine the role of TREX1 in the degradation of erythroblast DNA within EBIs. The TREX1 D18N mice exhibit altered erythropoiesis including a 20% reduction in hematocrit, 10–20 fold increased erythropoietic gene expression levels in the spleen and phenotypic signs of normocytic normochromic anaemia. Anaemia in TREX1 D18N mice is accompanied by increased erythropoietin (Epo), normal hepcidin levels and the TREX1 D18N mice display an inappropriate response to anaemic challenge. Enhanced ISG expression results from failed processing and subsequent sensing of undegraded erythroblast DNA in EBIs. TREX1 participates in the degradation of erythroblast DNA in the EBI and TREX1 D18N mice exhibit a normocytic normochromic anaemia.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Keywords

Exonuclease; anaemia; autoimmunity; lupus; erythropoiesis

Introduction

Anaemia, lupus, and DNA degradation

Anaemia is commonly observed in patients with chronic inflammatory conditions, such as infection, cancer and autoimmune disease [1–7]. In fact, more than 50% of patients with systemic lupus erythematosus (SLE), one of the most common autoimmune disorders, display clinical signs of anaemia [8,9]. Systemic inflammation is a hallmark of lupus and this chronic inflammatory state is thought to contribute to anaemia in these patients, although the specific mechanisms leading to anaemia in lupus are unclear [8–10]. Links between failed DNA degradation, anaemia and lupus are gleaned from the phenotypes of the Deoxyribonuclease II (*DNase II*) knockout, the nucleoside-diphosphate kinases NM23-H1/NDPK-A and NM23-H2/NDPK-B, (*NME1/NME2*) double knockout and the three prime repair exonuclease 1 (*TREX1*) knockout mice [11–18]. DNase II is a powerful non-cell autonomous DNA endonuclease and the DNase II knockout mice die perinatally due to severe anaemia [12]. DNase II knockout embryos exhibit numerous undegraded terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive DNA-containing bodies, especially in the liver, the site of definitive erythropoiesis [11]. In humans, DNase II mutations cause a severe autoimmune phenotype accompanied by anaemia that results from a combination of dysfunctional erythropoiesis and autoinflammation [19]. The *NME1/NME2* double knockout mice also die perinatally, displaying a hematological phenotype characterized by hypocellularity and increased nucleated erythroid precursors in the peripheral blood [15,16]. The NM23-H1 enzyme, encoded by *NME1*, is a cell-autonomous DNA endonuclease that nicks DNA to generate substrates for the TREX1 exonuclease [20,21].

Erythroblast DNA degradation is essential for postnatal erythropoiesis, as there are ~200 billion enucleated erythrocytes generated in humans each day [22]. Erythropoiesis primarily occurs in the yolk sac and liver during fetal development and the bone marrow (BM) in postnatal humans and mice [23]. During definitive erythropoiesis, erythroblasts proliferate and differentiate on central macrophages, and following the asymmetrical division of erythroblasts into reticulocytes and pyrenocytes, central macrophages quickly engulf pyrenocytes, while the reticulocytes are released into circulation [23]. Cellular changes that occur during erythroblast differentiation include decreased cell size, chromatin condensation, membrane remodeling, DNA degradation, and enucleation. Erythroblast nuclear condensation proceeds through a caspase 3-dependent mechanism leading to the formation of transient openings in the nuclear membrane [24]. Prior to enucleation, membrane proteins segregate, so the endoplasmic reticulum (ER) and nuclear proteins form the pyrenocyte, while the remaining cytoplasmic proteins form the reticulocyte [25]. Newly enucleated pyrenocytes present phosphatidylserine (PtdSer) on their outer membrane, recognized as an “eat-me” signal by macrophages through a MER proto-oncogene tyrosine kinase (MerTK)-protein S-dependent mechanism [23]. Following engulfment of

pyrenocytes, DNase II further degrades fragmented pyrenocyte DNA within macrophages [23,26]. The nuclease(s) that initiate cell-autonomous erythroblast genomic DNA degradation have yet to be identified, although it has been shown that caspase-activated DNase (CAD) is not involved [16,27]. The nucleases NM23-H1, apurinic/apyrimidinic endonuclease 1 (APE1) and TREX1, localize to the ER in the perinuclear space and might gain access to erythroblast nuclear DNA during the generation of transient openings in the nuclear membrane or membrane remodeling that segregates the ER to the pyrenocyte [16,24,27,28]. Endonucleolytic activities associated with the NM23-H1 and APE1 generate nicked double-stranded DNA (dsDNA) that is further degraded by the TREX1 exonuclease [20,21,28,29].

TREX1, lupus and DNA sensing

TREX1 is a 3' to 5' DNA exonuclease that is structurally designed to degrade nicked duplex DNA [13,21,30,31]. More than sixty TREX1 mutant alleles identified in humans cause a spectrum of DNA-mediated autoimmune diseases, including Aicardi-Goutières syndrome (AGS), Retinal Vasculopathy and Cerebral Leukodystrophy (RVCL), and Familial Chilblain Lupus (FCL) [32–35] and are associated with SLE [18,36–38]. The failure to process self-DNA has long been associated with lupus [17], and mutations in the aspartate residue at position 18 of the TREX1 protein are reported in the majority of families with TREX1-associated chilblains lupus [33] and in patients with AGS [39,40]. The genetically precise TREX1 D18N mouse recapitulates a lupus-like phenotype, providing an excellent model to investigate autoimmune diseases related to failed DNA polynucleotide processing [13]. Homozygous TREX1 D18N mice have decreased life spans, splenomegaly, vasculitis, glomerulonephritis and congestive heart failure associated with an aberrant immune response to self-DNA [13]. However, the source of the disease-driving DNA polynucleotides in TREX1 D18N mice remains unclear. Our biochemical studies indicate the TREX1 exonuclease acts on nicked dsDNA and others have proposed TREX1 degrades ssDNA fragments from different sources [14,41]. TREX1 recognizes and rapidly degrades dsDNA nicked by endonucleases, indicating that failed processing of genomic dsDNA in TREX1 mutants might be a source of self-DNA antigen [13,20]. This is further supported by the presence of elevated levels of serum anti-dsDNA antibodies in lupus patients and in the TREX1 D18N mouse [13].

Failed DNA degradation by the TREX1 and DNase II nucleases initiates aberrant DNA signaling leading to systemic autoinflammation [11,13,17]. TREX1 dysfunction in humans and mice causes varied phenotypes dependent upon the TREX1 mutation [13,14,42,43]. The TREX1 KO mouse dies of inflammatory myocarditis caused by the sensing of undegraded DNA polynucleotides by the cytosolic cyclic GMP-AMP synthase (cGAS). Activated cGAS generates cGAMP that binds to the stimulator of interferon (IFN) genes (STING) leading to TANK-binding kinase 1 (TBK1) activation, interferon regulatory factor 3 (IRF3) phosphorylation and type I IFN gene expression [17,41,44,45]. The lethal phenotype observed in DNase II-deficient mice is also mediated through STING [46], and there is accumulating evidence of crosstalk between several DNA sensing pathways [41,44,45]. Importantly, TREX1-mediated disease in humans and mice is allele-dependent and will

likely require precise genetic models to elucidate the pathways linking TREX1 dysfunction and autoinflammation [13,42,43].

The underlying causes of anaemia in lupus are unknown, presenting a barrier to the development of new treatments. Currently, lupus patients exhibiting anaemia are treated with erythropoiesis-stimulating agents or immunosuppressants that correct anaemia in only 50% of patients, frequently with transient response [8,47]. Here, we demonstrate that the TREX1 D18N mice display a distinct anaemia phenotype associated with failed TREX1 degradation of erythroblast dsDNA during definitive erythropoiesis leading to interferon-stimulated gene (ISG) expression. The TREX1 D18N model will provide an excellent tool to identify novel therapies to treat anaemia and autoinflammation in lupus.

Materials and methods

Mice, cell isolation, and culture

All animal experiments were conducted in accordance with approved guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest Baptist Medical Center. TREX1 D18N mutant mice on 129S6/SvEvTac background were genotyped as previously described [13]. Homozygous TREX1 D18N mice between 7 and 13 weeks were utilized. Anaemia was induced through daily intraperitoneal (IP) injections of 40 mg/kg phenylhydrazine (PHZ, Sigma-Aldrich, MO) for 4 days or a single 0.3 mL bleed. Isolation of erythroblastic islands (EBIs) from spleens was performed as previously described [23]. Briefly, spleens were isolated and minced in sterile conditions. Extracellular matrix (ECM) was digested with 0.075% collagenase D (Worthington Biochemicals, NJ) in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Thermo Fisher Scientific, MA) at 100 rpm for 1 hour at 37 °C. Digested spleens were 70 µm filtered into RPMI 1640 containing 30% fetal bovine serum (FBS, HyClone Laboratories, UT). Cells were sedimented for 1 hour at room temperature (RT) and washed twice with RPMI (100 × g for 10 minutes at RT). Enriched EBIs were either analyzed with flow cytometry or allowed to attach for 90 minutes at 37 °C in Dulbecco's Modified Eagle medium (DMEM, Thermo Fisher Scientific, MA) containing 10% FBS. Following attachment, cells were either lysed for ribonucleic acid (RNA) analysis, fixed for staining or incubated in Iscove Modified Dulbecco's medium (IMDM, Thermo Fisher Scientific, MA) containing 15% FBS, 1% methylcellulose, 3 U/mL murine erythropoietin (mEpo), and 200 µg/mL transferrin to facilitate erythrocyte differentiation.

Quantitative polymerase chain reaction (qPCR) and RNA sequencing (RNAseq)

Cells were lysed, and total RNA was isolated from samples utilizing the RNeasy Plus Mini kit (Qiagen, MD). RNA was subjected to RNAseq analysis (University of Texas Southwestern Medical Center Genomics and Microarray Core, TX) and gene enrichment analysis [48] or reverse transcribed into complementary DNA (cDNA) with the ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, MA) and qPCR performed using the TaqMan assays listed in Table 1 (Thermo Fisher Scientific, MA) using standard settings on 7500 Real-Time PCR System (Applied Biosystems, CA). For RNAseq, RNA quality was determined using an Agilent 2100 Bioanalyzer and samples with a RIN

Score >8 were used. RNA concentrations were determined with the Qubit fluorometer and 4 µg of total DNase treated RNA were used for cDNA library generation using TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina, CA). mRNA was purified, fragmented, cDNA synthesized, A-tailed and indexed adapters ligated, PCR amplified, purified with Ampure XP beads, and revalidated using the Agilent 2100 Bioanalyzer. Samples were normalized, pooled, and run on the Illumina HiSeq 2500 using SBS v3 reagents. DAVID Bioinformatics Resources 6.8 tool was used to perform functional annotation (GOTERM_BP_FAT) [48].

Blood and urine analysis (reticulocytes, hemoglobin, MCV, MCH, MCHC, and proteinuria)

Blood samples were collected in 5 mM ethylenediaminetetra-acetic acid (EDTA). Reticulocytes were stained with Richard-Allen Scientific Reticulocyte Stain Solution (Thermo Scientific, MA) and microphotographs captured at 400 × magnification to visualize at least 1000 red blood cells (RBCs). Blood hemoglobin levels were determined using the Hemoglobin Reagent Set (Pointe Scientific Inc., MI) and absorbances measured at 540 nm. Protein levels in urine were determined using Uristix (Siemens, NY). Hematocrit levels were measured in heparinized micro-hematocrit capillary tubes (Thermo Fisher Scientific, MA) spun for 3 minutes in a hematocrit centrifuge. Blood samples were sent to IDEXX Laboratories, Inc. (ME) for analysis of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), hematocrit, hemoglobin, and RBC counts. For analysis of spherocytes and hemolysis, peripheral blood smears of EDTA treated blood were prepared, fixed in methanol, stained with Wright-Giemsa stain (Sigma-Aldrich, MO), mounted in Permount (Thermo Fisher Scientific, MA) and reviewed by an expert hematologist.

Tissue processing, histology, and immunofluorescence

Tissue samples were fixed overnight in 4% paraformaldehyde (PFA) at RT, dehydrated, processed and embedded in paraffin. The 5 µm sections were placed on slides, incubated at 55 °C, deparaffinized, and rehydrated. Hematoxylin and eosin (H&E) or Modified Perls' Prussian Blue Iron staining (Leica Biosystems Richmond, Inc.) was performed on sections as previously described [25,49]. For fluorescence microscopic analysis of isolated EBIs cultured in 8-well chamber slides, samples were fixed with 4% PFA at RT for 10 minutes followed by TUNEL using the Click-iT® TUNEL Alexa Fluor®^R 647 imaging assay and antibody staining (Thermo Fisher Scientific, MA). Briefly, cells were permeabilized with 0.25% Triton-X 100, TUNEL reaction performed at 37 °C followed by antibody staining. Samples were incubated in primary antibodies, including Ter119 (1:100, Abcam, MA), F4/80 (1:100, Abcam, MA) and CD71 (1:100, Abcam, MA), diluted in PBS containing 3% BSA and 0.1% Triton-X 100 overnight at 4 °C followed by appropriate secondary for 1 hour at RT. Slides were mounted with fluoroshield mounting medium with DAPI (Abcam, MA). Images were acquired using a Nikon Eclipse TE300 fluorescent microscope (Nikon, Tokyo, Japan) with a QImaging Retiga EX camera (QImaging, BC, Canada) and analyzed using ImageJ (National Institutes of Health, MA). Pyrenocytes/EBI were quantified in 5 randomly selected fields per sample from at least 4 independent cultures.

Flow cytometry

Spleen, BM, enriched EBIs, or EDTA treated blood samples were isolated, single cell suspensions generated by filtering through 70 μm mesh, and 1×10^6 cells were stained with Zombie Aqua™ Fixable Viability dye (BioLegend, CA) for 15 minutes at room temperature, washed twice with FACS buffer (PBS +2%FBS) and fixed with 1% PFA for 1 hour on ice. Cells were washed with FACS buffer and pellets resuspended in PE anti-mouse CD71 (BioLegend, CA, 1:200), APC anti-mouse Ter119 (BioLegend, CA, 1:200) and FITC anti-mouse F4/80 (BioLegend, CA, 1:200) for 30 minutes on ice protected from light. Cells were washed twice in FACS buffer, resuspended in ethanol at -20°C for 1 hour and stained with Hoechst 33342 Solution (Thermo Fisher Scientific, MA, 1:1000) for 15 minutes before flow cytometry analysis was performed using a BD LSRFortessa™ X-20 (BD Biosciences, CA). Results were analyzed using FlowJo® v10.4.2 (FlowJo, LLC, CA).

Enzyme-linked immunosorbent assays (ELISA)

ELISAs to assess total iron binding capacity (TIBC) and ferritin levels in serum samples collected from WT and D18N mice were performed according to manufacturer's recommendations (MyBioSource, Inc., CA). Briefly, serum samples were diluted 1:1 or 1:40 in sample diluents for TIBC and ferritin, respectively. Samples or standards 100 μL of were added to precoated wells and incubated for one hour. Plates were washed three times with wash buffer and 100 μL of appropriate horseradish peroxidase (HRP) conjugated antibody added to each well. Plates were washed as before and 100 μL of 3,3',5,5'-Tetramethylbenzidine (TMB) added for 10 minutes. Reactions were stopped with 100 μL of 0.3 molar sulfuric acid and absorbance read at 450 nm using a POLARstar® Omega plate reader (BMG Labtech, NC).

Statistical analysis

All data are expressed as mean $\pm$ SEM of at least 2 independent experiments consisting of 4 animals in each group unless otherwise specified. Relative qPCR data are presented as relative quantization (RQ) values, indicating fold difference compared to control WT samples, unless otherwise stated. Values were analyzed using GraphPad software (Prism, Graphpad Software, Inc., La Jolla, CA) and two-way ANOVA and Bonferroni *post-hoc* tests were used to determine significant differences ($p < .05$) between groups unless otherwise specified.

Results

Dysregulated erythropoiesis, spontaneous normocytic normochromic anaemia and enlarged spleen in TREX1 D18N mice

Defective erythropoiesis and anaemia are common conditions observed in patients with autoimmune disorders, including SLE [8]. DNase II deficiency in humans [19] and mice [12] provides a strong link between the autoimmune phenotype, anaemia, and dysfunctional erythropoiesis. The TREX1 D18N mutation causes a lupus-like disease in humans and mice [13], and dysregulated erythropoiesis is revealed by analyses of gene expression and circulating erythroblasts (Figure 1). The GATA Binding Protein 1 (GATA1) is a global

regulator of erythroid genes, including Hbb-b1, Hbb-b2 and Abcb10 [50], and increased expression of numerous GATA1 regulated erythroid differentiation genes is indicated by RNAseq analysis of spleen tissues from 3 week old TREX1 D18N mice (Figure 1(A)). Furthermore, gene enrichment analysis of RNAseq data indicated an ~9-fold enrichment in erythrocyte differentiation (GO:0030218) and erythrocyte homeostasis (GO:0034101) pathways in the spleen of TREX1 D18N mice compared to WT (Figure 1(B)). A similar enrichment of erythropoiesis genes was observed in whole blood samples of DNase II-deficient patients [19]. Increased expression levels of the erythroid differentiation genes, Hbb-b2 (13-fold, Figure 1(C)), Hbb-b1 (13-fold), and Abcb10 (16-fold), was verified by qPCR analysis in the spleens of 7–13-week-old TREX1 D18N mice compared to WT. Additionally, increased expression of Hbb-b2 (6-fold, Figure 1(C)) and Hbb-b1 (5-fold) are observed in the liver of TREX1 D18N mice. In contrast, there were no differences in erythroid differentiation gene expression in the BM of TREX1 D18N compared to WT mice indicating fully active erythropoiesis in the BM compartments of both TREX1 D18N and WT mice (Figure 1(C)). Flow cytometry analysis of whole blood indicated a 2.4-fold increase in the percentage of circulating erythroblasts in TREX1 D18N mice compared to WT (Figure 1(D)). Increased engulfment of RBCs was also indicated by the substantial number of hemosiderin containing Kupffer cells in the liver of TREX1 D18N mice that were not detected in WT mice (Figure 1(E)). Together, these results demonstrate defective erythropoiesis in the exonuclease deficient TREX1 D18N mice, similar to that detected in DNase II mutant humans [19].

Dysregulated erythropoiesis is linked to a unique anaemia phenotype in the TREX1 D18N mice (Figure 2). Hematocrit levels in TREX1 D18N mice are nearly 20% lower than WT mice (42 ± 1.8 versus 52 ± 0.63 , Figure 2(A)), while blood hemoglobin levels are within the normal range of 14.3 ± 2.64 g/dL for TREX1 D18N and WT mice (Figure 2(B)). MCV, MCH, and MCHC are also within the normal range indicating normocytic normochromic anaemia (Figure 2(C)). The percentage of reticulocytes is 3-fold higher (Figure 2(D)) and Epo gene expression levels are 3-fold higher in kidney tissue (Figure 2(E)) in TREX1 D18N mice compared to WT. Collectively, the decreased baseline hematocrit, increased levels of reticulocytes and higher Epo gene expression levels indicate mild anaemia and dysregulated erythropoiesis in the TREX1 D18N mice. Anaemia associated with normal hemoglobin, MCV, MCH and MCHC levels mimic normocytic normochromic anaemia observed in some humans with anaemia of inflammation (AI) [51]. However, IL-6 gene expression was not detected in the BM of TREX1 WT or D18N mice and there is no difference in hepcidin expression in the liver (Figure 2(F)), indicating a distinct non-iron restricted anaemia in TREX1 D18N mice. Further, no significant differences in TIBC or ferritin levels were observed between TREX1 WT and D18N mice (Supplemental Figure 2(A,B)). The TREX1 D18N mice exhibit splenomegaly, increased red pulp cellularity and Ter119 expression (Figure 2(G)) providing further evidence of a modified erythropoiesis pathway. However, there are no detectable differences in the overall percentages of erythroblast populations (ProE, EryA, EryB, and EryC) in the BM or spleens in TREX1 D18N compared to WT mice (Figure 2(H)). No increases in spherocytes or obvious signs of hemolysis were observed in peripheral blood smears of TREX1 D18N mice, indicating that the anaemia phenotype was not caused by a hemolytic process (Supplemental Figure 2(C)).

Impaired processing of erythroblast DNA in TREX1 D18N erythroblastic islands and increased ISG expression

Since no difference in erythroblast levels were observed, we next investigated whether changes in DNA processing during definitive erythropoiesis within the EBI of TREX1 D18N mice were present, that could contribute to anaemia and dysregulated erythropoiesis. The persistent sensing of self-DNA leading to a type I IFN-dependent immune response causes the autoimmune phenotype in mice and humans with deficient TREX1 exonuclease activity [13]. DNA degradation during definitive erythropoiesis occurs in the EBI and TREX1 degrades nicked dsDNA [13]. To test whether degradation of erythroblast nuclear DNA is altered by the TREX1 D18N mutation, we isolated EBIs from the spleen and confirmed that cell clusters containing 2 or more cells were F4/80-CD71 double positive using fluorescence microscopy (Figure 3(A), top panel) [23]. Additionally, the pyrenocytes in cultured EBIs were shown to be TUNEL positive (Figure 3(A), bottom panel). Next, the number of unprocessed pyrenocytes associated with isolated EBI central macrophages was quantified, demonstrating that the number of pyrenocytes per EBI is higher immediately after isolation and higher levels persist over time in culture (Figure 3(B)). The DNA content in freshly isolated EBIs was also measured using flow cytometry demonstrating a 1.8-fold increase in Hoechst MFI in TREX1 D18N EBIs compared to WT (Figure 3(C)). The levels of four representative ISGs were measured in cultured EBIs to determine if the failed processing of erythroblast DNA contributed to immune activation. Upon isolation, the TREX1 D18N EBIs express significantly higher levels of all four ISGs compared to those from WT mice (Figure 3(D), control, $**p < .01$). When the EBIs are maintained in erythrocyte differentiation conditions *in vitro*, the ISG expression remained at higher levels in TREX1 D18N EBIs (Figure 3(D), solid line) compared to ISG expression in the WT islands. In contrast, the ISG expression levels in the WT islands decreased over time to nearly undetectable levels (Figure 3(D), dashed line). Thus, the TREX1 D18N EBIs express higher levels of ISGs compared to WT islands and the elevated ISG expression levels persist for a greater period of time (Figure 3(D)). These results reveal the presence of a robust and sustained activated DNA-sensing pathway in EBIs isolated from TREX1 D18N mice, which correlates with the presence of increased levels of unprocessed pyrenocyte DNA.

Defective response to anaemic challenge in the TREX1 D18N mice

Patients with lupus are often hyporesponsive to Epo leading to deficient activation of erythropoiesis in response to further anaemic challenges, such as hemorrhage [8]. This might explain why Epo administration in SLE patients is commonly ineffective [8]. Likewise, the failed processing of pyrenocyte DNA during erythropoiesis by the TREX1 D18N mutation would likely result in a defective response to anaemic challenge. To test this idea, TREX1 D18N mice were challenged with PHZ, a potent hemolytic agent, or by submandibular bleeding, and erythropoietic response assessed. Prior to PHZ treatment, TREX1 D18N mice exhibit larger spleens than WT mice (Figure 2(G)) and display a 4-fold higher percentage of reticulocytes in their peripheral blood (Figure 2(D)). Following PHZ treatment, 26% of the TREX1 D18N mice die compared to less than 6% of the WT mice (Figure 4(A)), indicating TREX1 D18N mice are more sensitive to anaemic challenge. In response to PHZ, WT mice demonstrate an earlier increase in spleen weight, compared to TREX1 D18N mice, and by day 10 the spleen weight in WT mice normalizes back to

baseline levels (Figure 4(B)). The percentage of reticulocytes compared to total RBCs in WT mice dramatically increases in response to anaemic challenge, whereas the levels of reticulocytes in TREX1 D18N mice display a milder increase, with significantly fewer reticulocytes on day 5 compared to WT mice (Figure 4(C), *** $p < .001$). Urinalysis demonstrates proteinuria (>2000mg/dL) in TREX1 D18N mice 1 day after PHZ injections that corresponds to hematuria (data not shown). A dramatic reduction in hematocrit, RBC counts and hemoglobin occurred following PHZ injections in TREX1 D18N and WT mice (Figure 4(D-F)). Hematocrit, RBC counts, and hemoglobin levels rebounded promptly in WT mice, to normal levels by day 10 (Figure 4(D-F), solid lines). Significantly lower hematocrit, RBC counts, and hemoglobin levels were observed in TREX1 D18N mice at days 5 and 10 following PHZ injections compared to WT (Figure 4(D-F)). These results indicate TREX1 D18N mice exhibit an altered response to anaemic challenge that diminishes their ability to recover from anaemic insults.

Anaemia in humans and mice is accompanied by extramedullary hematopoiesis in the spleen and liver [23,26]. Hematopoietic foci containing EBIs are detected in the liver of TREX1 D18N mice but not in untreated WT mice (Figure 4(G), untreated controls). At 1 day after PHZ injections, TREX1 D18N mice have greater numbers of hematopoietic foci (~5/field) compared to WT mice (~1/field) (Figure 4(G), d1)). Interestingly, the number of EBIs in the liver of WT mice 1 day after PHZ injections is comparable to untreated TREX1 D18N mice, consistent with the finding of mild anaemia in untreated TREX1 D18N mice. At 5 days after PHZ injections, hematopoietic foci are present at elevated levels (>15/field) in the livers of both TREX1 D18N and WT mice (Figure 4(G), d5)). Higher numbers of EBIs in the livers of TREX1 D18N mice are observed at days 1 and 10 after PHZ injections compared to WT mice (Figure 4(G)), likely due to unresolved anaemia. Together, these results demonstrate a deficient response to hemolytic anaemic challenge. Bleeding mice and analyzing erythroblast populations in the BM and spleen confirmed the deficient response of TREX1 D18N mice to anaemic challenge. Prior to bleeding, TREX1 D18N and WT mice show no significant differences in ProE, EryA, EryB or EryC percentages in the BM (data not shown) or spleen (Figure 2(H)). However, 3 days after 0.3 mL bleeds, WT mice show a 2-fold increase in the percentage of EryA in the BM (data not shown) and 4-fold and 2-fold increases in EryA and B in the spleen, respectively (Figure 4(H)). Hematotoxicity genes Ahsp, Hmox1, and Hbb-b1 are expressed at higher levels in the liver and spleen in response to anaemic insults [52]. Liver samples from untreated TREX1 D18N mice display elevated levels of hematotoxicity genes compared to WT mice (Figure 5(A)). After subjecting mice to anaemic challenge with PHZ, the expression of hematotoxicity genes in the livers of WT mice are increased to the similarly high expression levels observed in the TREX1 D18N mice at days 1 and 5 after treatment (Figure 5(B,C)). However, by 10 days after PHZ administration, the expression levels of Ahsp and Hmox1 in WT mice are reduced while the higher levels of expression in TREX1 D18N liver persist (Figure 5(D)). A similar pattern of hematotoxicity gene expression is observed in the spleen, with higher basal expression levels of Hbb-b2, Abcb10 and Alas2 in untreated TREX1 D18N mice compared to WT (data not shown). These results indicate that untreated TREX1 D18N mice exhibit increased levels of hematotoxic stress that is comparable to WT mice subjected to PHZ-mediated anaemic challenge.

The spleens of untreated TREX1 D18N mice exhibit expanded lymphoid follicles and increased germinal centers (Figure 6(A,B), controls), in line with our previous observations [13]. In WT mice, splenic follicular architecture is maintained at 1 day after anaemic challenge, although an expected increase in cellularity of the red pulp is observed (Figure 6(A,B), d1 top panel, dotted white lines). The peak of extramedullary hematopoiesis after anaemic challenge with PHZ in mice occurs at 5 days [23], and is observed here as a loss of follicular architecture at this time in WT mice and the reappearance of follicles by day 10 (Figure 6(A,B), top panels). In contrast, anaemic challenge in TREX1 D18N mice induces a complete loss of splenic follicular architecture by day 1, indicating rapid immune activation (Figure 6(A,B), bottom panel). The splenic follicular architecture in TREX1 D18N mice remains disorganized for the entire 10 days after anaemic challenge (Figure 6(A,B), bottom panels). These results demonstrate a clear alteration in response and recovery to anaemic challenge in the TREX1 D18N mice. The ISG levels in the spleen following anaemic challenge were measured to confirm our *in vitro* results demonstrating a correlation between persistent erythroblast DNA in TREX1 D18N EBIs and immune activation. Significantly higher ISG expression levels are observed in TREX1 D18N spleens at all time points tested before and after PHZ injections (Figure 6(C), solid line, $*p < .05$). The highest ISG levels are mostly observed at day 5 following anaemic challenge in the spleen of TREX1 D18N mice (Figure 6(C)), and either day 1 or day 10 after PHZ injections in the liver of TREX1 D18N mice (data not shown). Together, the higher levels of ISG expression in the spleen correlate with the persistently higher levels of erythropoiesis in the TREX1 D18N mice supporting the link between erythrocyte DNA processing and type I IFN activity. The exonuclease deficient TREX1 D18N mice exhibit an altered response to anaemic challenge that impairs their ability to recover.

Discussion

Anaemia is commonly observed in patients with chronic inflammatory conditions, including SLE, but the mechanisms contributing to anaemia are not well understood [2,9]. Mice deficient in the nucleases DNase II and NM23 die perinatally from severe anaemia, indicating the central importance of DNA degradation in erythropoiesis [11,15]. Mutations in the *TREX1* gene, encoding a cytosolic DNA exonuclease, cause FCL, AGS, and RVCL in humans, and the TREX1 D18N mutation causes a lupus-like disease in mice [13,42]. Our published data supports the idea that TREX1 functions to degrade dsDNA to limit autoimmune response to self-DNA [13]. Here we demonstrate that the TREX1 D18N mice exhibit a distinct form of spontaneous anaemia characterized by normocytic normochromic anaemia, increased erythropoietic and hematotoxic gene expression, and an inappropriately exaggerated inflammatory response to anaemic challenge. Our results show increased circulating erythroblasts and heightened Epo and erythropoiesis gene expression in the TREX1 D18N mouse, likely representing an attempt to minimize the anaemic condition. This anaemia phenotype resembles patients with DNase II mutations [19]. In addition, this study indicates that the inability of TREX1 D18N mice to efficiently process erythroblast DNA results in increased expression levels of ISGs during terminal erythropoiesis. Together, these data suggest that the TREX1 D18N enzyme fails to degrade erythroblast dsDNA,

altering erythropoiesis, resulting in the sensing of pyrenocyte dsDNA, and contributing to the autoimmune phenotype.

The distinct normocytic normochromic anaemia phenotype observed in TREX1 D18N mice is characterized by reduced hematocrit, normal hemoglobin and MCV, increased reticulocytes, Epo and circulating erythroblasts and heightened erythropoiesis and ISG gene signatures. Erythropoiesis in patients presenting with severe autoimmune disease due to DNase II mutations display a similar anaemic phenotype [15]. Anaemia is commonly observed in patients with autoimmune diseases, including SLE [9] and TREX1-associated autoimmune diseases [3,53], however, the anaemia phenotype in these patients is not well defined. Anaemia in the TREX1 D18N mice does not appear to be iron-restricted [54] as elevated levels of IL-6 in the BM or hepcidin in the liver were not detected. Thus, further investigations are warranted into possible anaemic conditions in autoimmune patients with TREX1 dysfunction. Additionally, the TREX1 D18N mice should prove helpful to uncover the precise mechanisms leading to anaemia associated with nuclease dysfunction.

The requirement for DNA degradation in erythropoiesis is highlighted by the severe anaemia and perinatal lethality observed in *DNase II* knockout and *NME1/NME2* double knockout mice [11-16]. DNase II knockout embryos exhibit numerous TUNEL-positive DNA-containing bodies, especially at the sites of definitive erythropoiesis in the liver [11]. The TUNEL-positive DNA indicates cleavage by an endonuclease other than DNase II, since DNase II cleavage generates TUNEL-negative 3' phosphate ends. In the absence of the central macrophage, monocultures of hematopoietic stem cells differentiate into erythrocytes in a pathway that includes DNA fragmentation by a cell-autonomous endonuclease that generates 3'-OH DNA ends, as indicated by TUNEL positivity and large DNA fragments [11,27]. These data indicate that an unidentified cell-autonomous endonuclease(s) functions to initiate erythroblast DNA processing prior to macrophage engulfment and further degradation by DNase II. Our data suggest that TREX1 processes erythroblast dsDNA that has been endonucleolytically cleaved during erythropoiesis. Higher levels of DNA are observed in TREX1 D18N EBIs and pyrenocyte clearance is less efficient in TREX1 D18N EBIs compared to WT (Figure 3). The TREX1 D18N spleen, liver and isolated EBIs exhibit increased ISG levels further exacerbated by anaemic challenge. Thus, we propose that TREX1 functions to process erythroblast dsDNA that has been nicked to generate 3'-OH DNA ends during erythropoiesis, and that dysfunction of TREX1 results in the accumulation of pyrenocyte DNA within central macrophages, persistent DNA sensing, and activation of type I-IFN signaling. This concept is in agreement with our published biochemical and structural data indicating that TREX1 degrades nicked duplex DNA [13,21,29,31]. In addition, the cGAS cytosolic DNA sensor that drives autoimmune disease in TREX1 deficient mice is activated by dsDNA [44,55]. Thus, the ~200 billion pyrenocytes generated daily in humans may represent a substantial source of immune-activating DNA polynucleotide in the context of TREX1 and other nuclease deficiencies. Anaemia in TREX1 D18N mice is exacerbated by PHZ injections and bleeding. It is possible that alternative methods to enhance anaemia, such as splenectomy or infections that affect RBC cycle, may provide additional insights into the pathogenesis of anaemia in this model.

The anaemia phenotype detected in the TREX1 D18N mouse indicates a novel role for TREX1 in degrading dsDNA originating from erythroblasts in the EBI during the terminal stages of definitive erythropoiesis to prevent autoinflammation. We propose that perinuclear located NM23-H1 and TREX1, and perhaps other endonucleases, gain access to genomic DNA by a caspase 3-dependent mechanism that generates openings in the erythroblast nuclear membrane [24] or during membrane remodeling to position ER-associated proteins in the pyrenocyte [20,25]. The endonucleases generate 3'-OH nicks in the dsDNA that are further degraded by the TREX1 exonuclease [20,21]. DNA processing by endonucleases and TREX1 exonuclease likely occur during the later stages of definitive erythropoiesis, helping to explain how widespread and dynamic transcription and translation continues up until erythroblast enucleation [56]. The exonuclease deficient TREX1 D18N mouse exhibits spontaneous lupus-like and normocytic normochromic anaemia phenotypes providing a genetically precise model for studies to determine the specific mechanism(s) of dysfunctional erythropoiesis leading to persistent DNA sensing in central macrophages. These studies should provide additional insights into anaemia associated with autoinflammation in lupus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Dysregulated erythropoiesis in TREX1 D18N mutant mice. RNAseq was performed on spleen samples collected from 3-week-old TREX1 WT [1–3] and TREX1 D18N [4–6] mice. (A) Heatmap of GATA1 regulated erythroid differentiation genes (data are displayed as row z-score) and (B) pathway analysis. (C) qPCR analysis of Hbb-b2 expression in BM, spleen and liver tissues collected from 7 to 13-week-old WT and D18N mice ($n = 5$). (D) Circulating erythroblast levels were determined through CD71-Ter119 staining of whole blood samples ($n = 8$, gating strategy provided in supplemental figure 1(A)). (E) Modified Perls' Prussian Blue Iron staining of liver samples was used to identify iron containing phagocytes (***) ($p < .001$).

Figure 2.

Normocytic normochromic anaemia and enlarged spleen in TREX1 D18N mice. Blood samples were analyzed for (A) Hematocrit ($n = 11$), (B) hemoglobin ($n = 4$) and (C) MCH, MCHC, and MCV ($n = 5$) levels in 7 to 13-week-old WT and D18N mice. (D) Percentage of reticulocytes compared to total RBCs in WT and D18N blood. (E) Epo transcript in whole kidney samples and (F) hepcidin transcript in whole liver samples collected from 7 to 13-week-old WT and D18N mice ($n = 6$). (G) Gross pictures, immunohistochemistry (IHC) and weights of spleen tissues from 7 to 13-week-old WT and D18N mice. (H) Percentages of ProE, EryA, EryB, and EryC populations in the BM and spleen of 7 to 13-week-old WT and D18N mice ($n = 5$). (** $p < .01$, *** $p < .001$).

Figure 3.

Presence of pyrenocyte nuclei and expression of ISGs in cultured erythroblastic islands from WT and D18N mice. (A) Fluorescence microscopic analysis of DNA (DAPI and TUNEL), macrophage (F4/80) and erythroblast (CD71) markers in isolated EBI clusters. (B) Quantification of pyrenocytes per cluster in isolated EBIs over time (60× objective). Images were acquired using a Nikon Eclipse TE300 fluorescent microscope with a QImaging Retiga EX camera and analyzed using ImageJ. A Nikon Plan Apo 4× objective was used with a numerical aperture (NA) of 0.2. (C) Flow cytometry analysis of DNA content (Hoechst) in isolated EBIs (gating strategy provided in supplemental figure 1(B)). (D) ISG expression, including IFI44, IFIT1, ISG15 and Usp18, over time in cultured EBIs purified from WT (dashed lines) and D18N (solid lines) spleens 5 days after anaemic challenge (* $p < .05$, ** $p < .01$, *** $p < .001$).

Figure 4.

Survival and gross response to anaemic challenge of WT and TREX1 D18N mice. (A) Survival ($n = 18$) and (B) spleen weights ($n = 4$) of 7 to 13-week-old PHZ treated WT (solid line) and D18N (dashed line) mice. (C) Percentage of reticulocytes compared to total RBCs in control untreated and PHZ treated WT (white bars) and D18N (black bars) mice. (D) H&E staining of liver (4 $\times$ objective). EBIs (arrows) were observed within the liver parenchyma (spherical or ellipsoidal shape) and quantified (bar graph) before and after PHZ injections. Images were acquired using a Nikon Eclipse TE300 fluorescent microscope as above. (E) Percentages of ProE, EryA, EryB, and EryC populations in the BM and spleen of 7 to 12-week-old WT and D18N mice 3 days after 0.3 mL facial vein bleeds ($n = 3$, * $p < .05$, ** $p < .01$, *** $p < .001$).

Figure 5.

Expression of hematotoxicity genes in the liver of PHZ treated WT and TREX1 D18N mice. mRNA expression levels of hematotoxicity genes, Ahsp, Hmox1 and Hbb-b1, in the livers of (A) control untreated, (B) day 1, (C) day 5 and (D) day 10 after PHZ injections in WT (white bars) and D18N (black bars) mice. Data are normalized to housekeeping gene (RPLPO) for each sample and displayed as $2^{\Delta\Delta Ct}$. (* $p < .05$, *** $p < .001$).

Figure 6.

Histological analysis and ISG expression in spleen of PHZ treated WT and TREX1 D18N mice. (A) H&E and (B) Ter119 and DAPI IHC staining of spleen tissue obtained from control WT (top panels) and D18N (bottom panels) mice. Lymphoid follicles indicating appropriate splenic architecture are denoted by arrows and dashed white lines (4× objective). Images were acquired using a Nikon Eclipse TE300 fluorescent microscope as described above. (C) ISG expression, including IFI44, IFIT1, ISG15 and Usp18, in whole spleen samples collected from WT (dashed lines) and D18N (solid lines) mice 0, 1, 5 and 10 days after PHZ injections (* $p < .05$, ** $p < .01$, *** $p < .001$).

Table 1.

Taqman assays.

Gene	Full name	Assay number
Hbb-b1	Hemoglobin, beta adult major chain	Mm01611268_g1
Hbb-b2	Hemoglobin, beta adult minor chain	Mm00731743_mH
Abcb10	ATP binding cassette subfamily B member 10	Mm00497926_m1
Epo	Erythropoietin	Mm01202755_m1
Alhsp	Alpha hemoglobin stabilizing protein	Mm04214740_u1
Hmox1	Heme oxygenase-1	Mm00516005_m1
Alas2	5'-aminolevulinate synthase 2	Mm00802083_m1
IFI44	Interferon induced protein 44	Mm01320796_g1
IFIT1	Interferon induced protein with tetratricopeptide repeats 1	Mm00515153_m1
ISG15	Interferon-stimulated gene 15	Mm01705338_sl
Usp18	Ubiquitin specific peptidase 18	Mm01188805_m1