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The Role of IFN γ -Producing Th1 Cells in a Type I IFN-Independent Murine Model of Autoinflammation Resulting from DNase II-Deficiency

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Abstract

Objective: Patients with hypomorphic mutations in DNase II develop a severe and debilitating autoinflammatory disease. The objectives of this study were to compare disease parameters of these patients to a murine model of DNase II deficiency, evaluate the role of specific nucleic acid sensors and identify cell types responsible for driving the autoinflammatory response.

Methods: To rescue *Dnase2*^{-/-} mice from embryonic lethality, they were intercrossed with mice that lacked the type I IFN receptor. Hematological and immune status of these mice was evaluated by CBC, flow cytometry, serum cytokine ELISAs and liver histology. Effector cell activity was determined by transferring cells from *Dnase2*^{-/-} *Ifnar*^{-/-} mice into *Rag1*^{-/-} recipients and assessing induced changes four weeks post-transfer.

Results: *Dnase2*^{-/-} *Ifnar*^{-/-} mice were shown to recapitulate many features of the DNase II-deficient patients including cytopenia, extramedullary hematopoiesis and liver fibrosis. An unusual IFN γ -producing T cell subset present in *Dnase2*^{-/-} *Ifnar*^{-/-} spleens could transfer hematological disorders to *Dnase2*^{+/+} *Rag1*^{-/-} mice (n>22). Autoinflammation did not depend on the STING pathway but was highly dependent on Unc93B1.

Conclusion: *Dnase2*^{-/-} *Ifnar*^{-/-} mice are a valid model for exploring the innate and adaptive immune mechanisms responsible for the autoinflammation that develops in *DNASE2*-hypomorphic patients. In the murine model, IFN γ is required for T cell activation and the development of clinical manifestations. The role of IFN γ in *DNASE2*-deficient patient

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populations remains to be determined, but the ability of *Dnase2*^{-/-} T cells to transfer disease to *Rag1*^{-/-} recipients points to T cells as a relevant therapeutic target in patient populations.

Introduction

The excessive accumulation of nucleic acid debris can lead to the activation of endosomal and/or cytosolic nucleic acid (NA) sensors and the subsequent development of autoimmune and autoinflammatory diseases (1, 2). Debris can accumulate as a result of inadequate scavenger cell activity and numerous scavenger receptor defects have been associated with SLE (3). Importantly, appropriate removal of DNA also depends on DNases located extracellularly or within specific cellular compartments. For example, loss-of-function (**LOF**) mutations in the extracellular DNase, DNaseIL3, have been associated with SLE in both patient populations and murine models (4, 5), while LOF mutations in the cytosolic enzyme Trex1 lead to neuroinflammation in patients with Aicardi-Goutieres syndrome (6), and to myositis and other organ damage in a murine model of autoinflammatory disease (7).

The loss of the lysosomal DNase, DNase II, has also been linked to autoinflammation. In mice, DNase II-deficiency results in an embryonically lethal anemia caused by the massive accumulation of undegraded DNA in phagocytic compartments (8). This anemia is linked to the excessive production of IFN β as mice deficient for both DNase II and the type I IFN receptor (IFN α R) survive as relatively healthy pups. This IFN production is downstream of the cytosolic DNA-detecting cGAS pathway since both STING- and cGAS-deficient mice are protected from embryonic lethality (9–11). DNA may access the cytosol as a result of lysosome instability, defects in autophagosome formation, or self-DNA that accrues in the cytosol following DNA damage or replication (12–14). However, excessive cell debris, cleared via scavenger receptors, must also accumulate in phagolysosomes and therefore access NA-sensing TLRs.

Despite surviving to adulthood, *Dnase2*^{-/-} x *Ifnar*^{-/-} double knockout (**DKO**) mice nevertheless develop immune abnormalities. These include a well-described inflammatory arthritis that results in severely swollen joints by 6–10 months of age (15, 16). This late onset arthritis depends on cGAS/STING as well as another cytosolic DNA sensor, AIM2 (10, 17, 18). DKO mice also develop an early onset of splenomegaly and extramedullary hematopoiesis, as well as the production of anti-nuclear antibodies (**ANA**), disease manifestations that are commonly found in murine models of SLE, especially those driven by TLR7. In contrast to embryonic lethality and arthritis, these disorders are not STING dependent, but depend on Unc93B1 (19, 20), a chaperone protein required for TLR trafficking from the ER to endosomal/lysosomal compartments (21), and therefore likely to involve endosomal TLRs.

Crow and colleagues have recently identified hypomorphic mutations of DNase II in patients with an uncharacterized interferonopathy (22). These patients develop a spectrum of clinical features that included severe non-regenerative anemia and thrombocytopenia at birth, splenomegaly, ANA production, glomerulonephritis, liver fibrosis, and deforming arthropathies. Based on similarities to other patients with a strong IFN signature, it has been assumed that these disease manifestations are predominantly driven by the STING-

dependent production of type I IFNs. Intriguingly, we now show that many of these disorders develop in DKO mice in the absence of conventional type I IFN signaling, and depend on Unc93B1, and endosomal TLR chaperone. It follows that a better understanding of the mechanisms responsible for the non-arthritis disease parameters of DKO mice may be highly relevant to human disease. We have now identified a major role for a novel IFN γ -producing T cell subset in the propagation of the erythrocyte and lymphocyte developmental defects characteristic of DKO mice, pointing to Th1-like cells as a therapeutic target in systemic autoimmune diseases associated with IFN signatures.

Materials and Methods

Mice

DNase II-gene targeted mice were kindly provided by Dr. S. Nagata (Osaka University) and obtained through the Riken Institute. *Dnase2*^{+/-} *x Ifnar*^{-/-} (Het), *Dnase2*^{-/-} *x Ifnar*^{-/-} (DKO), *Dnase2*^{-/-} *x Ifnar*^{-/-} *x Unc93B1*^{3d/3d} (Unc93B1 TKO), and *Dnase2*^{-/-} *x Ifnar*^{-/-} *x Tmem173*^{-/-} (STING TKO), mice have been described previously (17, 19, 20). *Ifngr*^{-/-} mice, obtained from Jackson Lab, and *Tlr3*^{-/-} and *Tlr7*^{-/-} C57BL/6 mice, kindly provided by Dr. D. Golenbock (UMass), were intercrossed with DKO mice to generate the corresponding triple KO lines. B6 *Rag1*^{-/-} (Jackson Lab) mice were intercrossed to *Ifngr*^{-/-} mice to generate *Rag1*^{-/-} *x Ifngr*^{-/-} mice. Complete Blood Counts (CBC) analysis was carried out by the UMMS Animal Medicine Core. All animal procedures were approved by and performed in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Flow cytometry

Cell suspensions were analyzed by flow cytometry as described previously (19). Spleen and bone marrow (BM) single cell suspensions were incubated in Fc block 2.4G2 supernatant (2.4G2 hybridoma, ATCC) before staining. Fluorochrome-labeled antibodies were obtained from Tonbo BioSciences: APC Ter119, PB anti-CD19 (1D3), PE CD4 (RM4-5), APC CD8 (SK1), PB CD45R/B220 (RA3-6B2), PB CD11b (M1/70); or from BioLegend: APC Ly6C (1A8), FITC Ly6G (RB6-8C5), PEcy7 CD71 (RI7217), PB CD69 (H1.2F3), PerCPcy5.5 CD62L (MEL-14), APCcy7 CD44 (IM7), APC CD93 (AA4.1), APC CD45.1 (A20). Samples were analyzed with an LSR II (BD Biosciences) and FlowJo software 9.7.6 (TreeStar). Statistical analysis was performed in GraphPad Prism (v.7.0) using a Mann-Whitney non-parametric t-test. P value <0.0001 ****; <0.001 ***; <0.01 **; <0.05 *; >0.05 ns.

Magnetic Bead purification

BD IMagTM anti-mouse magnetic particles were used for depletion or purification of CD45R/B220 (#552311), CD4 (#551539) or CD8 (#551516) cells. CD3⁺ T cells were purified using biotin-Anti-Mouse CD3e (145-2C11) (#30-0031, Tonbo Biosciences) and BDTM IMag Streptavidin Particles Plus - DM (#557812).

Histology

Trichrome staining of formalin-fixed liver sections was carried out by Applied Pathology Systems (APS), Worcester MA.

ANAs

ANAs were determined as described (19). Mouse sera diluted 1:50 was incubated on HEp-2 Ag substrate slides (MBL International), and bound Abs were detected with DyLight 488–coupled goat anti-mouse IgG Ab (Poly4053, BioLegend).

Gene expression analysis

Gene expression was quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) using the NanoString nCounter Immune Panel (#XT-CSO-MIM1–12, NanoString) according to the manufacturer's protocol (NanoString Technologies Inc.). The data were normalized to a set of 6 internal positive and 8 internal negative controls, and then to 20 housekeeping genes. Values above the threshold (mean+2SD of the negative controls) were considered for analysis. Normalized log2 transformed values were analyzed using nSolver™ analysis software 3.0 and unbiased hierarchical clustering was used to generate a heatmap in the open-source R-based software as described previously (23). Relative gene expression levels were compared using Morpheus software, <https://software.broadinstitute.org/morpheus>.

Cytokine titers

Serum cytokine titers were determined by multiplex ELISA (Procarta-plex bead assay from ThermoFisher) measured on a MAGPIX instrument with Luminex xMAP Technology.

Spleen fragment transplants

Spleen fragments were transplanted under the kidney capsule of *Rag1*^{−/−} C57BL/6 mice using established procedures (24). Briefly, spleens isolated from 4–10-day old neonatal mice were cut into 1 mm³ fragments with a scalpel. Mice first were injected s.c. with 0.1 mL of cefazolin–gentamicin antibiotic mixture and then anesthetized by i.p. injection of a ketamine–xylazine solution, 150 and 10 mg/kg, respectively. The kidney was exposed through an abdominal incision, and a 1–2 mm cut was made in the capsule. The spleen fragment was then inserted under the capsule using a surgical trocar. The kidney was returned to the abdominal cavity, the cavity wall was sutured, and the skin incision was closed with autoclips.

T cell adoptive transfer

10⁷ RBC-depleted total spleen cells, 10⁷ T or B cell-depleted spleen cells or 3–10 × 10⁶ magnetic bead purified CD4⁺, CD8⁺ or CD3⁺ T cells were injected i.v. into *Rag1*^{−/−} mice. The mice were euthanized and tissues collected for analysis at 4 wks post injection. Th1 cells were generated using standard Th1 skewing conditions (25).

Results

Hematological abnormalities in DKO mice resemble DNase II-deficient patients and are Unc93B1-dependent.

To determine the extent of cytopenia in DKO mice relative to DNase II-sufficient controls, complete blood counts (CBC) of young *Dnase2*^{-/-} *Ifnar*^{-/-} DKO mice were compared to DNase II heterozygotes (*Dnase*^{+/-} *Ifnar*^{-/-}) (**Het**). Similar to DNase II-deficient patients, young DKO mice showed a significant reduction in the total WBC, lymphocytes, platelets and RBCs compared to the other two groups, as well as increased numbers of immature erythrocytes and an increased frequency of granulocytes and monocytes (Fig. 1A and Supplemental Table 1). They also developed dramatically enlarged spleens, even by 4 weeks of age, elevated erythropoietin serum concentrations and a reduced hematocrit (Fig. 1A). This evidence of extramedullary hematopoiesis was consistent with the increased number and percentage of splenic Ter119⁺ erythroid lineage cells (19), proerythrocytes (**ProE**) (CD71^{hi} Ter119^{med}), and erythroblasts (CD71^{hi} Ter119^{hi}) at the basophilic (**EryA**) and later stages (**EryB**) of differentiation in the spleens of DKO mice compared to Het controls (Fig. 1B) (26). We also included *Dnase2*^{-/-} *Ifnar*^{-/-} *Unc93b1*^{3d/3d} triple knockout mice (**Unc93 TKO**) in the analysis and found that most of these abnormalities were reversed by Unc93B1-deficiency. However, compared to the Het mice, the percentage of both EryA and EryB erythroblasts remained high in the Unc93 TKO mice, even though total spleen weight and cell number were significantly lower than littermate DKO mice. Splenomegaly was associated with a corresponding defect in bone marrow (**BM**) erythropoiesis, as detected by low cell number (Fig. 1C) and visually (Supplemental Figure 1A). These type I IFN-independent defects also depended on Unc93B1. We conclude that the DKO mice have ineffective erythropoiesis; anemia stimulates erythropoietin production that, in turn, drives expansion of erythropoietic precursors, but nevertheless fails to generate a normal number of circulating erythrocytes.

Lymphocyte and myeloid cell composition of the spleen and BM further distinguish DKO mice.

Early onset extramedullary hematopoiesis was associated with additional shifts in the myeloid and lymphocyte compartment in the spleen and BM that were clearly apparent by 10 weeks of age. The % B cells in the DKO spleens after RBC-depletion was significantly lower than in either the Het or Unc93B1 TKO spleens (19). This decrease reflected a disruption in B cell development in the BM where the DKO mice had comparable numbers of total B220⁺ cells but a much lower proportion of AA4.1^{neg} mature B cells (Fig. 2A). The percentage of splenic T cells was also reduced, and residual T cells were highly activated as shown by the increased frequency of CD69^{hi} T cells and T cells expressing the effector memory phenotype CD44^{hi} CD62^{lo} (Fig. 2B). There was a corresponding increase in the percentage and/or number of CD11b⁺ cells in both the spleen and BM, concomitant with a dramatic increase in the % neutrophils in the spleen (Fig. 2C). These hematopoietic defects will henceforth be referred to as the “DKO phenotype” and can be readily assessed by: increased spleen weight; increased frequency of Ter119⁺, CD11b⁺ and granulocytes in the spleen; the decreased percentage of total B cells in the spleen and mature B cells in the BM; increased percentage of CD11b⁺ cells in the BM; and ineffective erythropoiesis in the BM.

DKO mice produce proinflammatory cytokines and develop liver fibrosis.

DNase II-deficient patients show elevated serum titers of IFN γ and IFN-induced cytokines and chemokines, as well as IFN-independent cytokines (22). Similarly, titers of IFN γ and IFN λ /IL-28, IFN-inducible cytokines and chemokines (eg. IL-12, CXCL10/IP10), IFN-independent cytokines (eg. TNF and IL-6), chemokines associated with the recruitment of myeloid cells (CCL5/RANTES, CCL3/MIP1a, and CXCL1/KC), and immunoregulatory type 2 cytokines (IL-10 and IL-19) (27) were significantly elevated in the sera of DKO mice compared to the Het and Unc93B1 TKO groups (Fig. 3A). Therefore, even cytokine/chemokine production seems to be Unc93B1 dependent, raising the possibility that cytokine production is downstream of TLR signaling.

DNase II-deficient patients also develop liver fibrosis. The DKO mice again reflected the patient populations as shown by extensive periportal trichrome staining of the livers of 6-month-old DKO mice, but not the Het or Unc93B1 mice (Fig. 3B).

Spleen fragment transplants from neonatal DKO mice induce the DKO phenotype in *Dnase2^{+/+} x Rag1^{-/-}* host.

It has been generally assumed that the failure to clear apoptotic debris has a major impact on hematopoietic as opposed to stromal cell subsets. However, in previous radiation chimera studies we found that the DKO phenotype depended on DNase II-deficiency in both the stem cell donor and the recipient (20), suggesting that stromal elements might contribute to the splenic abnormalities. To address this possibility, we transplanted spleen fragments from CD45.1 DKO or Het mice under the kidney capsule of CD45.2 *Rag1^{-/-}* mice. We assumed that if splenic stromal cells promoted the DKO phenotype by producing proinflammatory chemokines, we would see more severe hematological abnormalities in the transplanted DKO spleen than in the host spleen.

The mice were analyzed 5 months post-transplantation. 8/9 of the Het spleens had visibly engrafted, but residual subcapsular spleen fragments could not be found in any of the 9 DKO-transplanted mice. However, closer examination of the recipient mice revealed that the host spleens were significantly enlarged in *Rag1^{-/-}* mice initially transplanted with DKO spleen fragments, but not Het spleen fragments. These enlarged spleens had a significant increase in the % erythroblasts when compared to Het fragment-transplanted or non-transplanted control *Rag1^{-/-}* mice (Fig. 4A). The BM of the DKO spleen-transplanted mice was also abnormal as shown by a decreased percentage of erythroblasts and an increased percentage of CD11b⁺ cells. Importantly, >99% of the CD11b⁺ cells were CD45.1^{neg} (data not shown) and therefore derived from recipient stem cells (Fig. 4A). These data indicated that hematopoietic cells present in the DKO but not Het spleens could induce the aberrant development of DNase II-sufficient cells, and the ensuing DKO-associated splenic and BM abnormalities, in *Rag1^{-/-}* mice.

DKO hematopoietic cells induce the DKO phenotype in *Dnase2^{+/+} x Rag1^{-/-}* mice.

To determine whether a DKO spleen cell suspension alone was sufficient to induce the DKO phenotype, 10⁷ spleen cells isolated from CD45.1 DKO or Het mice were injected i.v. into CD45.2 B6 *Rag1^{-/-}* mice and the recipient mice were analyzed 4 wks later. Erythropoiesis

was disrupted in mice injected with DKO and STING TKO spleen cells, when compared to uninjected mice or mice injected with Het or Unc93B1 TKO spleen cells, as shown by an increased percentage of Ter119⁺ erythroblasts in the spleen and the corresponding splenomegaly (Fig 4B). The DKO- and STING TKO spleen cell-injected mice also had fewer erythroblasts and more CD11b⁺ cells in the BM; almost all of CD11b⁺ cells were CD45.1^{neg}/CD45.2⁺ and therefore derived from Rag1^{-/-} progenitors (Fig 4B and Supplemental Fig. 1B). Early B cell development in the BM was also abnormal. In Rag1^{-/-} mice, B cells normally proceed to the pro-B cell stage of B cell development (IgM^{-/-} CD19⁺ AA4.1⁺) (28). This early step in B cell differentiation was impaired in the DKO splenocyte-injected mice and B lineage cells failed to move into the pro-B cell compartment (IgM^{neg} CD19^{neg} B220^{neg} AA4.1⁺) (Fig. 4B and Supplemental Fig. 1B). Consistent with the phenotype of the parental strains, Unc93B1 TKO cells did not transfer these defects while STING TKO cells elicited the same outcome as DKO T cells.

DKO CD4⁺ T cells induce the DKO phenotype in *Dnase2^{+/+} x Rag1^{-/-}* mice.

Previous studies documented the presence of activated thymocytes in neonatal DKO mice (8), consistent with our own data (Fig. 2B) on splenic T cells. To determine whether T cells or B cells could be responsible for inducing the DKO phenotype, the DKO spleen cell suspension was depleted of B cells or both CD4 and CD8 T cells and then injected into B6 Rag1^{-/-} mice. CD4/CD8 cell depletion, but not B cell depletion, abrogated the ability of the DKO spleen cells to transfer the DKO phenotype (Fig. 4C). To confirm that the relevant cells were T cells and not CD4⁺ or CD8⁺ dendritic cells, we also used magnetic beads to isolate CD3⁺ T cells and injected 3×10⁶ cells/mouse into Rag1^{-/-} hosts. These CD3⁺ cells could transfer the DKO phenotype as effectively as total DKO spleen cells. Magnetic bead enriched CD4⁺ T cells, but not enriched CD8⁺ cells, also transferred autoinflammation (Fig. 4C).

To better define the autoinflammation elicited by DKO CD4 T cells, we further compared Rag1^{-/-} mice injected with DKO CD4-magnetic bead enriched cells to mice injected with comparably isolated cells from littermate Het controls. By 4 weeks post T cell transfer, only the DKO T cell injected mice, and not the Het T cell-injected mice, exhibited extramedullary hematopoiesis as demonstrated by splenomegaly, and the increased frequency of pro-erythrocytes as well as both EryA and EryB erythroblasts. They also showed an increased percentage of CD11b⁺ cells in the spleen, consistent with myeloid cell activation. This outcome was not due to a failure of the Het T cells to engraft since the Het CD4-injected mice had a much higher percentage of T cells (Fig. 5A,B). The DKO CD4 T cell recipients further exhibited BM hyperplasia, not seen in the Het CD4 T cell recipients, clearly evident by overall reduced cellularity and fewer RBCs (Fig. 5A and Supplemental Fig. 1C). The BM of the DKO-injected mice also contained fewer Ter119⁺ cells, a reduced percentage of EryA and EryB erythroblasts within the Ter119 subset and an increased percentage of CD11b⁺ cells (Figs. 5C). Pro-B cell differentiation was also suppressed as shown by the accumulation of B lineage cells in the AA4.1⁺ B220^{neg} compartment. Together these data show that DKO CD4⁺ T cells can induce developmental abnormalities in both the BM and spleen of DNase II-sufficient mice, and that low numbers of CD4 DKO T cells can disrupt the BM niche responsible for erythroid and B cell development, independently of cytokines

produced by erythroid island macrophages (or any other cells) present in the BM of DNase II-deficient mice.

Role of Unc93B1-dependent TLR.

Exactly how Unc93B1 contributes to the inflammatory response in DKO mice is not clear. TLR9 has been proposed to play a T cell intrinsic role in the generation of Tregs (29) and it is very possible that the DKO phenotype reflects an absence of Tregs in DKO mice that would normally limit the inflammatory response. However, two separate reports have found that TLR9 in DKO mice cannot detect natural DNA ligands, presumably due to the inability of DNase II-deficient mice to degrade dsDNA into fragments that can be detected by TLR9 (19, 30). TLR7 has been shown to contribute to autoantibody production in numerous models of SLE. To assess the role of TLR7 in DKO mice, we generated *Dnase2*^{-/-} x *Ifnar*^{-/-} x *Tlr7*^{-/-} triple KO mice. These TLR7 TKO mice lost the ability to make the autoantibodies normally detected in DKO sera, but they still developed splenomegaly and autoinflammation (Fig. 6A). To further explore the role of RNA-sensing TLRs, we also generated TLR3 TKO mice, but TLR3 deficiency had no effect on autoantibody production or, the development of splenomegaly (Fig. 6A).

Gene expression analysis of DKO T cells identifies a strong IFN γ signature.

CD4⁺ cells obtained from Het, DKO and Unc93B1 spleens were evaluated for gene expression using the Nanostring® immune code set. The isolated cells were mainly CD4⁺ T cells but included 5% CD4⁺ CD11c⁺ DCs (Supplemental Fig. 2A). Genes associated with the Th1 subset were uniquely upregulated in the DKO sample. Examples include the transcription factor Tbet (Tbx21), the canonical cytokine IFN γ , Th1 chemokine receptors CXCR3 and CCR5, Th1 chemokine receptor ligands CXCL9 and CXCL10, IL-12 cytokine and receptor subunits, and other genes known to be induced by IFN γ (Fig. 6B). The increased production of IFN γ by the DKO CD4 T cells was confirmed by cytoplasmic staining (Supplemental Fig. 2B). CXCL9 and CXCL10 are produced by dendritic cells and not T cells, and most likely made by CD11c⁺ CD4⁺ cells included in the CD4-magnetic bead purification. A number of inflammatory chemokines (CCL3, CCL4, CCL5, CCL2, CCL8, CCL19), cytotoxic molecules (Ctsg, Gzmb, Camp) and complement components were upregulated 10–100- fold in DKO as compared to Het. Additional highly upregulated genes included the RAGE ligands S100A and S100B and several caspases, further pointing to a general state of inflammation. The majority of these genes were not upregulated in the Unc93B1 TKO samples. The complete data set is provided in Supplemental Fig. 2C. These data further support an important role of Unc93B1 and therefore endosomal TLRs in the DKO inflammatory response.

IFN γ signaling is required but not sufficient for the DKO phenotype.

To determine whether IFN γ is required for the development of the DKO phenotype, we intercrossed DKO mice with mice lacking an IFN γ receptor. The IFN γ R TKO mice developed less splenomegaly and had a reduced frequency of Ter119⁺ cells in the spleen, compared to the DKO mice (Fig. 6A). Also, the percentage and activation status of splenic T cells returned to the normal range, as reflected by the MFI of CD69 (Fig. 6C). By contrast, the BM of the IFN γ R TKO mice was still disrupted. Thus, IFN γ is required for the

generation of the DKO effector Th1 cell subset, but not for DKO-associated BM abnormalities and BM abnormalities can occur in DNase II^{-/-} mice in the absence of activated T cells. The absence of IFN γ signaling did not impact the TLR-independent development of inflammatory arthritis; clinical disease scores of IFN γ R TKO mice were comparable to age-matched DKO mice (Supplemental Figure 3), consistent with the development of inflammatory arthritis in Rag2^{-/-} TKO mice (16).

The next question was whether IFN γ , or some other factor made by the DKO T cells, was responsible for the ability of adoptively transferred activated DKO CD4 T cells to induce the DKO phenotype in Rag1^{-/-} mice. IFN γ R is expressed on most hematopoietic progenitors including HSCs, MPPs, common myeloid progenitors (CMPs), GMPs, MEPs and CLPs, and IFN γ can have direct stimulatory or inhibitory impact on hematopoiesis (31). To assess the role of IFN γ R expression in the Rag1^{-/-} recipients, we generated Rag1^{-/-} Ifnar^{-/-} mice and injected these mice with DKO spleen cells. The loss of IFN γ R in recipient mice had only a modest impact on the ability of these mice to acquire the DKO phenotype (Fig. 6D). Altogether, the data demonstrate that T cell production of IFN γ is required for the development/expansion of activated DKO T cells and splenomegaly but cannot completely account for the capacity of DKO T cells to elicit BM-associated inflammation. We also attempted to transfer the DKO phenotype with Th1 cells that were generated in vitro. These cells did not transfer the DKO phenotype despite the successful engraftment of T cells producing copious amounts of IFN γ (Fig. 6E). This suggests the DKO CD4 T cells have a function apart from IFN- γ production that mediates the transfer of autoinflammation.

Discussion

Autoinflammation due to DNase II deficiency falls in the category of type I interferonopathy as defined by increased expression of IFN-inducible genes, elevated serum IFN α titers, and increased phosphorylation of STAT1 and STAT3 (22). We now show that DKO mice recapitulate many of the features of DNase II-deficient patients, including splenomegaly, lymphopenia, thrombocytopenia, anemia, elevated serum titers of non-IFN induced inflammatory cytokines and liver inflammation/fibrosis. Moreover, DNase II-deficient mice develop deforming arthropathies remarkably similar to that seen in patients with type I interferonopathies (15). Autoinflammation in these mice is independent of type I IFN and at least partially dependent on Unc93B1 expression. Therefore, autoinflammation in the DNase II patient population may be more dependent on type II IFN (or type III IFN) than type I IFN. Recent studies have shown that gene-targeted mice expressing gain-of-function mutations in STING also develop an inflammatory disease through type I IFN-independent mechanisms (32, 33). These STING mutations were originally identified in children who developed an autoinflammatory syndrome referred to as STING-associated vasculitis of infancy or SAVI. Thus, a better understanding of the interplay between aberrant activation of NA-sensing receptors and activation of the adaptive immune system is needed.

The adoptive transfer studies in the current study now reveal an unanticipated capacity of DKO T cells to induce autoinflammation upon adoptive transfer into Rag1^{-/-} mice. Although the percentage of splenic T cells was reduced in DKO mice, the persisting T cells were highly activated as indicated by elevated CD44 and CD69 levels. Gene expression

analysis of a spleen cell fraction, highly enriched for T cells, revealed a strong Th1/IFN γ signature, consistent with the high levels of IFN γ detected in the serum of both the murine model and patient populations. According to the Nanostring data, the DKO CD4⁺ cells also produced IL-21 and IFN γ /IL-21 double producing CD4 T cells have been shown to promote IL-12 production in the context of inflammation and infections (34, 35). It is also possible that a distinct subset of IL-21-producing CD4⁺ cells contributes to the activation of autoreactive B cells.

DKO T cell activation depended on IFN γ R as IFN γ R TKO T cells did not upregulate either CD69 or CD44 expression and splenomegaly and extramedullary hematopoiesis was dramatically reduced in the IFN γ R TKO mice when compared to DKO mice. T cell transfer of DKO, and not Het, CD4⁺ T cells into naïve *Dnase2*^{+/+} $\times$ *Rag1*^{-/-} recipients resulted in disrupted erythropoiesis and B cell development in the BM, even though the recipient cells all expressed DNase II. Both recipient and T cell donors were on a C57BL/6 background and the DKO and Het T cell donors were littermates, so the outcome was not likely to be due to a GVH response. Nevertheless, IFN γ R TKO mice still developed BM abnormalities despite the lack of activated T cells, suggesting that loss of DNase II in non-T cells contributed to the development of autoinflammation.

Limitations of this study include a minimal understanding of the conditions that drive the activation of CD4⁺ T cells in DKO mice and the mechanism(s) by which T cells induce autoinflammatory defects in B6 *Rag1*^{-/-} mice. The absence of autoinflammation in Unc93B1 TKO mice, and the minimal impact of STING-deficiency, implicates NA-sensing TLRs in these events. Since prior studies had shown that TLR9-deficient cells could not respond to dsDNA (19, 30), we reasoned that the excessive accumulation of cell debris in DKO mice, as the result of an inability to degrade DNA, could provide a source of RNA that in turn engaged one of the RNA-sensing TLRs. This premise was consistent with our previous data showing that DKO mice make autoantibodies that frequently stain HEP2 cells with a nucleolar staining pattern (19). We found that TLR7-deficiency prevented autoantibody production but not other features of autoinflammation. Moreover, TLR3-deficiency appeared to have no impact on either autoantibody production or autoinflammation. Therefore, we assume that either the RNA sensing TLRs are redundant, and that the combined deletion of TLR3, TLR7 and TLR8 will be needed to ameliorate disease; so far we have been unable to generate these mice. Alternatively, it will be necessary to re-explore the role of TLR9 in the context of the DKO model.

Importantly, endosomal TLRs have been shown to act synergistically in the induction of the type III IFN expression, particularly in dendritic cell subsets and epithelial cells (36–38). Therefore IL-28 activity may account for DKO DC production of IFN-induced chemokines in Figure 6B. Type III IFNs have been further linked to chemokine production and renal injury in SLE patient populations (39, 40). In renal sections obtained from patients with severe lupus nephritis, the presence of IFN λ was associated with crescent formation, thereby connecting type III IFN production with fibrosis (40). Type III IFNs are also produced by liver cells and in vivo administration of IFN λ specifically reduced the viral load in a murine model of HSV-2 infection (41). Whether type III IFN contributes to the liver fibrosis

exhibited by DKO mice is a question that warrants further study and could be addressed in DKO mice that fail to express the type III IFN receptor.

Overall, we believe that the current data are consistent with a biphasic disease process in which DNase2 deficiency initially leads to excessive accumulation of cell debris and the subsequent Unc93B1-dependent activation of antigen presenting cells that in turn induce the IFN -dependent development of an unusual Th1 subset. Once these T cells develop, they have the capacity to promote autoantibody production, and mediate tissue damage. They can also independently maintain an “autoinflammatory state”, and induce inflammation in DNase2-sufficient mice I (Supplemental Fig. 4).. If true, this framework has important implications for the treatment of patients with autoinflammatory conditions where it will be important to block both the initiating innate stimuli that lead to T cell activation as well as the T cells that can then independently promote hematopoietic and immune cell abnormalities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Unc93B1-dependent type I IFN-independent hematological defects in *Dnase2*^{-/-} mice. (A) Total WBC, lymphocytes, platelets and hematocrit of Het, DKO and Unc93B1 TKO mice in blood collected from 5–7 wk old mice (n=6). EPO serum levels of 10-week-old mice (n=4–6); spleen weight of 4-week-old mice (n=12–19). (B) FACS analysis of total spleen from 10-week-old mice to identify: Erythroid progenitors (ProE, CD71⁺ Ter119^{lo}, n=12–14); Erythroid lineage cells (Ter119^{hi}, n=13–16); and EryA erythroblasts (Ter119⁺ CD71⁺ FSC-A^{hi}, n=12–14). (C) Number of cells recovered from 4 leg bones/mouse and EryB

erythroblasts (Ter119⁺ CD71⁺ FSC-A^{lo}) in total BM of 10 wk old mice (n=7–16 mice/group).

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Figure 2. Lymphocyte and myeloid cell abnormalities in DKO mice.

(A) FACS analysis of Ter119^{neg} spleen cells stained for B cell marker CD19 (top) and FACS analysis of total BM cells stained for B220 and AA4.1 to identify immature (AA4.1^{hi}) and mature (AA4.1^{lo}) B cells (bottom). (B) Analysis of Ter119^{neg} spleen cells stained for CD3, CD4, CD8, CD69 (activated T cell), and effector memory subsets (CD44⁺, CD62L⁻). (C) Analysis of Ter119^{neg} spleen cells to identify myeloid cells (CD11b⁺) and neutrophils (CD11b⁺ Ly6G⁺) in spleen (top) and BM (bottom). Bar graphs for A-C summarize 5 independent experiments using 10 wk old mice (n=5–22 mice/group).

Figure 3. Proinflammatory cytokines and liver fibrosis in DKO mice.

(A) Cytokine levels in sera from 8-week-old Het (white bars), DKO (black bars) and Unc93B1 TKO (grey bars) mice (n=5). (B) Trichrome stain of liver sections from 6 mon old mice (representative of n=3). Magnification is 40x and enlarged insert is 100x.

Figure 5. Induction of Autoinflammation by DKO CD4⁺ T cells transfer into Rag1^{-/-} mice. (A) Rag1^{-/-} mice were injected with 10^7 magnetic bead-purified CD4⁺ T cells isolated from either Het or DKO mice and the spleen and bones were obtained from euthanized mice 4 weeks later and compared to tissues from uninjected Rag1^{-/-} mice. (B, C) Bone marrow and spleen cell suspensions isolated from the same mice were analyzed by flow cytometry. Plots are representative of 3–5 mice/group and results are summarized in the bottom row.

Figure 6. Role of TLRs and IFN γ in autoinflammation.

(A) Immunofluorescent stain of HEp-2 cells by sera from 4–5-month-old mice of the indicated strains (left). Spleen weights of 4–10-week-old mice of the indicated strains (n=8–31 mice/group) (right). (B) RNA was extracted from Het, DKO and Unc93B1 TKO CD4-bead purified T cells and expression levels were quantified using the Nanostring Immune Code Set. Morpheus software used to generate heat map of Th1-related genes and additional genes upregulated 10–100 fold in the DKO vs Het CD4 $^{+}$ cells (n=3). (C) Comparison of 5 wk old mice from the indicated strains (n=3–10 mice/group). (D) Comparison of uninjected

Rag1^{-/-} mice, either Rag1^{-/-} or Rag1^{-/-} IFN γ R^{-/-} mice injected with DKO spleen cells (n=3–19). (E) Comparison of uninjected Rag1^{-/-} mice, Rag1^{-/-} mice injected with DKO T cells or Rag1^{-/-} IFN γ R^{-/-} mice injected with in vitro generated Th1 cells (n=3–17).

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