Suppression of IRAK1 or IRAK4 Catalytic Activity, but Not Type 1 IFN Signaling, Prevents Lupus Nephritis in Mice Expressing a Ubiquitin Binding–Defective Mutant of ABIN1

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Polymorphisms in the TNIP1 gene encoding A20-binding inhibitor of NF-κB1 (ABIN1) predispose to lupus and other autoimmune diseases in at least eight human populations. We found previously that knock-in mice expressing a ubiquitin-binding–defective mutant of ABIN1 (ABIN1[D485N]) develop autoimmunity as they age and succumb to a disease resembling lupus nephritis in humans. In this article, we report that Flt3-derived dendritic cells from these mice overproduced type 1 IFNs upon stimulation with ligands that activate TLR7 or TLR9. However, crossing ABIN1[D485N] mice to IFNAR1-knockout mice that do not express the α-subunit of the type 1 IFNR did not prevent splenomegaly, the appearance of high serum levels of autoantibodies and other Igs, or liver inflammation and only reduced kidney inflammation modestly. In contrast, crossing ABIN1[D485N] mice to knock-in mice expressing catalytically inactive mutants of IRAK1 or IRAK4 prevented splenomegaly, autoimmunity, and liver and kidney inflammation. Our results support the notion that IRAK1 and/or IRAK4 are attractive targets for the development of drugs to prevent, and perhaps treat, lupus nephritis and other autoinflammatory diseases caused by the decreased ability of ABIN1 or other proteins to restrict the strength of MyD88 signaling.

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Abbreviations used in this article: ABIN1, A20-binding inhibitor of NF-κB1; Aβ, anti-nuclear Ab; BMDM, bone marrow–derived macrophage; DC, dendritic cell; IKK, IκB kinase; KO, knockout; PAS, periodic acid–Schiff; pDC, plasmacytoid dendritic cell; SLE, systemic lupus erythematosus; TAK1, TGF-β–activated kinase 1; WT, wild-type.

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transcription factors NF-κB and IRF5 (25, 26), whereas TAK1 also induces activation of the JNKs and p38 MAPKs, which switch on additional transcription factors and stimulate posttranscriptional events controlling the synthesis, processing, and secretion of inflammatory mediators. ABIN1 interacts with Lys63- and Met1-linked ubiquitin oligomers and is thought to compete with the TAK1 and IKK complexes for binding to the hybrid ubiquitin chains formed when this signaling pathway is activated. This explains why TAK1 and IKK are hyperactivated and the secretion of proinflammatory cytokines, such as IL-6, IL-12, and TNF-α, is enhanced in dendritic cells (DCs) expressing the ubiquitin-binding-defective ABIN1[D485N] mutant (1). These findings suggested that ABIN1[D485N] mice could be used to identify components of the MyD88 signaling pathway that might be attractive targets for the development of drugs to prevent and treat lupus. Therefore, we also crossed ABIN1[D485N] mice to mice expressing catalytically inactive mutants of IRAK1 (27) or IRAK4, as well as a functionally defective mutant of IRAK2 (28), the results of which are also reported in this article.

Materials and Methods

Generation and maintenance of mouse lines
ABIN1[D485N]-knock-in (1), IRAK2[E525A]-knock-in (28), and IRAK1[D359A]-knock-in (27) mice were described. ABIN1[D485N] mice were back-crossed to C57BL6/J mice (Jackson Laboratory) for up to 10 generations, IRAK1[D359A] mice up to 10 generations, and IRAK4[D329A]-knock-in mice were developed by Taconic-Artemis using embryonic stem cells from C57BL/6 mice and were further back-crossed to C57BL/6J mice for six generations. The C57BL/6 strain from Jackson Laboratory that was used for back-crossing these mice is free of the DOCK2 mutation that is reported to be present in C57BL mice from Harlan Laboratories and that affects the immune system (29). IFNAR1-KO mice (Crick Institute, London, U.K.) (30) were on a C57BL/6 background and were further back-crossed to C57BL/6J mice for six generations. Mice were maintained in individually ventilated cages under specific pathogen–free conditions, given free access to food and water, and housed in accordance with U.K. and European Union regulations. All procedures were carried out under a U.K. Home Office Project License and were subjected to local ethical review.

TLR agonists
The TLR agonists Pam3CSK4 (TLR1/2), R848 (TLR7), CpG type A (ODN1585) (TLR9), and CpG type B (ODN1826) (TLR9) were from InvivoGen, and poly(dU) (TLR7) was from Sigma-Aldrich. Poly(dU) was added to the culture medium conjugated with Lipofectamine 2000 (Invitrogen), as described (28).

Characterization of Flt3-derived DCs
The percentage of pDCs in Flt3-derived cell populations was assessed by staining with B220, PDCA1, CD11c, and CD11b Abs (Becton-Dickinson) and analyzed by flow cytometry.

Abs
Abs recognizing p105/NFκB1 phosphorylated at Ser533, p38α MAPK phosphorylated at its Thr-Gly-Tyr motif, total p38α MAPK, total IRAK4, and GAPDH were from Cell Signaling Technology. An Ab recognizing IRAK4 phosphorylated at Thr345 and Ser346 was provided by V. Rao (Pfizer) (31). An

FIGURE 1. Enhanced IFN secretion in Flt3-derived DCs from ABIN1[D485N] mice. Flt3-derived DCs (3.5 × 10³ cells) from 6–8-wk-old WT mice or ABIN1[D485N] mice were stimulated for 12 h with the indicated concentrations of poly(dU) (A and D), CpG A (B and E), or CpG B (C and F). The concentrations of IFN-β (A–C) and IFN-α (D–F) in the cell culture medium was measured by ELISA using a mouse IFN-β kit (BioLegend) or VeriKine Mouse IFN Alpha ELISA Kit, respectively. Error bars represent the mean ± SEM for four separate experiments carried out on cells from a total of 12 mice for each genotype. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student t test.
Ab recognizing JNKs phosphorylated at their Thr-Pro-Tyr motif was from Invitrogen, and a rabbit secondary Ab conjugated to HRP was from Pierce.

Histopathological analysis

Mice were euthanized by increasing CO₂ levels. The kidneys and liver from each mouse were removed, fixed in 10% neutral buffered formalin for $48$ h, and then embedded in paraffin. For liver, one sample from the left lobe and two from the median lobe were trimmed and processed. Sections (4 μm) were prepared, and liver and kidney sections were stained with H&E. Kidney sections were also stained with periodic acid–Schiff (PAS) reagent, and liver sections were stained with Sirius Red. Kidney and liver tissue sections were assessed by a veterinary pathologist (F.M.) who was blinded to the genotype of the mice in the different cohorts. The multiparametric semiquantitative scoring system used to assess renal and liver changes was based on the principles and guidelines for histopathologic scoring in research (32).

**FIGURE 2.** Enhanced *ifn-β* and *ifn-α* mRNA in Flt3-derived DCs from ABIN1[D485N] mice. Flt3-derived DCs (3.5 × 10⁵ cells) from 6–8-wk-old WT mice or ABIN1[D485N] mice were stimulated for 12 h with the indicated concentrations of poly(dU) (A and D), CpG A (B and E), or CpG B (C and F). The total RNA was extracted from the cells, and mRNA encoding *ifn-β* (A–C) or *ifnα* (D–F) was measured by quantitative RT-PCR. Error bars represent the mean ± SEM for four separate experiments carried out on cells from a total of 12 mice for each genotype. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, Student t test.

**FIGURE 3.** Hallmarks of autoimmunity are not reduced in ABIN1[D485N] × IFNAR1-KO mice. Three WT mice, six or seven ABIN1[D485N] mice, six or seven ABIN1[D485N] × IFNAR1-KO mice, and four IFNAR1-KO mice were used for all of the experiments. (A) Spleen weights of 6-mo-old WT mice (○), ABIN1[D485N]-knock-in mice (△), ABIN1[D485N] × IFNAR1-KO mice (●), and IFNAR1-KO mice (▲). Each symbol represents data from one mouse, and the horizontal lines show the average ± SEM. Total ANAs (B), anti-dsDNA Abs (C), and Ig isotypes (D) in the serum of 6-mo-old mice of the indicated genotypes were measured by ELISA. Each symbol represents one mouse, and the horizontal lines show the average ± SEM. *p < 0.05, Mann–Whitney U test. ns, not significant.
Other methods

Generation and culture of bone marrow–derived macrophages (BMDMs) and Flt3-derived DCs, cell lysis, immunoblotting and cytokine measurements, cDNA synthesis, and RNA extraction were carried out as described (28). Quantitative PCR was performed as described (28), with the exception that SsoFast EvaGreen Supermix (Bio-Rad) was used. The primers used to measure \( \text{ifnb} \), \( \text{ifna}^4 \), and \( \text{ifna}^6 \) mRNA were described previously (33). Autoantibodies were measured by ELISA (1), and Ig isotypes were measured using a kit from Millipore.

Statistical significance

Statistical significance was calculated using the two-tailed Student \( t \) test or Mann–Whitney \( U \) test with GraphPad Prism software. Additional details are given in the figure legends.

Results

Type 1 IFNs are overproduced in Flt3-derived DCs from ABIN1\[D485N\] mice

pDCs are a major source of the type 1 IFNs produced by the MyD88 signaling network. Therefore, we compared IFN production in Flt3-derived DCs from ABIN1\[D485N\] and WT mice, which are widely used as a model for pDC function (34). We found that Flt3-derived DCs from ABIN1\[D485N\] mice secreted much higher levels of IFN-\( \beta \) (Fig. 1A–C) and IFN-\( \alpha \) (Fig. 1D–F) and produced much higher levels of \( \text{ifnb} \) (Fig. 2A–C), \( \text{ifna}^4 \) (Fig. 2D–F) and \( \text{ifna}^6 \) mRNA (Supplemental Fig. 2) than pDCs from WT mice (note that the ordinates in Fig. 2 and Supplemental Fig. 2 are plotted on a log scale). Enhanced type 1 IFN production was not explained by an increased proportion of B220\(^+\) and PDCA\(^+\) cells in the preparations from ABIN1\[D485N\] mice. Indeed, the proportion of these cells relative to B220\(^+\) and PDCA\(^+\) cells was lower in preparations from these mice (27.76 \pm 2.02\% versus 37.18 \pm 2.77\% in WT mice). In contrast, the proportion of B220\(^+\) and PDCA\(^+\) cells increased from 4.8 \pm 0.73\% in WT mice to 6.1 \pm 1.0\% in ABIN1\[D485N\] mice. Taken together, our results demonstrate that ABIN1 restricts the production of type 1 IFNs in Flt3-derived DCs.

Lupus nephritis in ABIN1\[D485N\] \( \times \) IFNAR1-KO mice

These experiments raised the question of whether the elevated levels of type 1 IFNs in ABIN1\[D485N\] mice were responsible for the development of lupus nephritis in these animals. Therefore, we crossed them to IFNAR1-KO mice, a gene essential for every known signaling event triggered by type 1 IFNs. At 6 mo of age, ABIN1\[D485N\] \( \times \) IFNAR1-KO mice showed enlarged spleens that were similar in size to those observed in ABIN1\[D485N\] mice (Fig. 3A), and the high serum levels of anti-nuclear Abs (ANAs), anti-self dsDNA, or other Ig isotypes in ABIN1\[D485N\] mice were not reduced in ABIN1\[D485N\] \( \times \) IFNAR1-KO mice (Fig. 3B–D). In contrast, there was a partial reduction in the extent of glomerulonephritis in ABIN1\[D485N\] \( \times \) IFNAR1-KO mice compared with ABIN1\[D485N\] mice (Fig. 4A, 4C). However, the liver inflammation found in ABIN1\[D485N\] mice at 6 mo of age was not reduced in ABIN1\[D485N\] \( \times \) IFNAR1-KO mice (Fig. 4B, 4D).

Lupus nephritis and liver inflammation in ABIN1\[D485N\] mice are prevented by crossing to mice expressing catalytically inactive mutants of IRAK1 or IRAK4

We showed previously that autoimmunity in ABIN1\[D485N\] mice is prevented by crossing to MyD88-KO mice (1). Because the other
components of the Myddosome are IRAK1 and IRAK4 and the inactive pseudokinase IRAK2, we studied whether the loss of IRAK1 or IRAK4 catalytic activity, or IRAK2 function, prevented lupus in ABIN1[D485N] mice.

To investigate the importance of IRAK4 catalytic activity, we generated a knock-in mouse in which IRAK4 was replaced by the kinase-inactive IRAK4[D329A] mutant (Supplemental Fig. 1). This mutation prevents interaction of Asp329 with the magnesium ion of Mg-ATP without significantly affecting kinase conformation (35). Similar to mice expressing different kinase-inactive IRAK4 mutants (36, 37), IFN-β or IFN-α secretion induced by TLR ligation was undetectable in Flt3-derived DCs from IRAK4[D329A] mice (Supplemental Fig. 3A, 3B), and TNF-α, IL-6, and IL-12p40 secretion (Supplemental Fig. 3C–E) and MyD88 signaling (Fig. 3F, 3G) in BMDMs were also greatly reduced. IRAK4[D329A] mice, as well as the kinase-inactive IRAK1[D359A] mice (27), were then crossed to ABIN1[D485N] mice.

Neither ABIN1[D485N] nor ABIN1[D485N] × IRAK4[D329A] mice developed splenomegaly (Fig. 5A) and, consistent with these observations, the levels of ANAs (Fig. 5B), anti-dsDNA Abs (Fig. 5C), and Ig isotypes (Fig. 5D) in the serum of 24-wk-old mice of the indicated genotypes were measured by ELISA. (B and C) Each symbol represents one mouse, and the horizontal lines show the average ± SEM. **p < 0.005, ***p < 0.005, Mann–Whitney U test. ns, nonsignificant.

However, the high level of IgA found in the serum of ABIN1[D485N] mice was suppressed by crossing to IRAK4[D329A] mice but not by crossing to IRAK1[D359A] mice (Fig. 5D). Moreover, no kidney inflammation was detectable in ABIN1[D485N] × IRAK4[D329A] mice or ABIN1[D485N] × IRAK1[D359A] mice at 6 mo of age (Fig. 6) (see Materials and Methods for scoring system), and the liver inflammation present in ABIN1[D485N]–knock-in mice was also largely reduced after crossing to IRAK4[D329A] or IRAK1[D359A] mice (Fig. 7B). Liver fibrosis along with inflammatory changes observed in ABIN1[D485N] mice were drastically reduced after crossing to IRAK4[D329A] or IRAK1[D359A] mice but were not reduced by crossing to IFNAR1-KO mice (Supplemental Fig. 4).

We also crossed ABIN1[D485N] mice to knock-in mice expressing the IRAK2[E525A] mutant, which is unable to interact with TRAF6. Proinflammatory cytokine production induced by several TLR agonists is greatly reduced in BMDMs from IRAK2[E525A]–knock-in mice (28). ABIN1[D485N] × IRAK2[E525A] mice still displayed splenomegaly and increased levels of autoantibodies and other IgGs at 6 mo of age, similar to ABIN1[D485N] mice (Fig. 5). Moreover, the extent of kidney inflammation in ABIN1[D485N] × IRAK2[E525A] mice was similar to that in...
ABIN1[D485N] mice (Fig. 6). Interestingly, liver inflammation and attending fibrosis were partially reduced in ABIN1[D485N] × IRAK2[E525A] mice (Fig. 7, Supplemental Fig. 4).

Discussion

Type 1 IFNs, which have important roles in regulating innate and adaptive immunity, also were implicated in the pathogenesis of lupus. However, we report that, although crossing ABIN1[D485N] mice to IFNAR1-KO mice modestly reduced kidney inflammation, it did not prevent the splenomegaly, elevated levels of Igs (including autoantibodies), and the liver pathology that occur spontaneously in ABIN1[D485N] mice (Figs. 3, 4, Supplemental Fig. 4). These findings indicate that elevated levels of IFN-regulated genes are not the primary cause of autoimmunity in ABIN1[D485N] mice and that enhanced IFN signaling induces the severity of glomerulonephritis by a mechanism that is independent of autoantibody production and the liver pathology that occur spontaneously in ABIN1[D485N] mice (Figs. 3, 4, Supplemental Fig. 4). These findings indicate that elevated levels of IFN-regulated genes are not the primary cause of autoimmunity in ABIN1[D485N] mice and that enhanced IFN signaling induces the severity of glomerulonephritis by a mechanism that is independent of autoantibody production. Our findings suggest that SLE in patients expressing ABIN1 variants known to predispose to this disease may derive relatively little benefit from anti-type 1 IFN therapy. However, it cannot be excluded that the overproduction of type 2 and/or type 3 IFNs is also required to drive autoimmunity.

In contrast to our findings, studies reported that crossing two other lines of lupus-prone mice to IFNAR1-KO mice reduced the levels of autoantibodies and glomerulonephritis and, therefore, increased life span (38, 39). However, the mutations causing autoimmunity in these mice are unknown. Interestingly, in one of these lines, blockade of IL-6 also suppressed the development of autoimmunity (40, 41), indicating that elevated levels of more than one cytokine are required to trigger SLE. The lupus nephritis that develops in TANK-KO mice, and which is driven by hyperactivation of the MyD88 signaling network (42, 43), was similarly prevented by crossing to IL-6–KO mice (43). A recent study divided human SLE into a number of categories: some display elevated levels of IFN-regulated genes, whereas others have elevated levels of different inflammatory cytokines and chemokines (44). Taken together, these observations suggest that the effective treatment of SLE patients will require the development of a number of therapies, depending on the mutations that underlie the disease.

The most striking finding in the current study was that the autoimmune phenotype of ABIN1[D485N] mice was prevented by crossing to knock-in mice in which IRAK4 or IRAK1 was replaced by catalytically inactive mutants. These results are consistent with autoimmunity in ABIN1[D485N] mice being driven by hyperactivation of the MyD88 signaling network and raise the possibility that small molecule inhibitors of IRAK4 and/or IRAK1 prevent (and perhaps reverse) autoimmune diseases in which alterations in the TNIP1 gene encoding ABIN1 (Introduction) or other genes causing hyperactivation of MyD88 signaling are a contributory.
factor. IRAK1 and IRAK4 do not participate in the cytoplasmic pathways by which viral RNA (RIGI, MDA5 pathways) or DNA (STING pathway) induce the production of type 1 IFNs (45, 46). Therefore, SLE patients treated with IRAK1 or IRAK4 inhibitors may have a lower risk for developing severe viral infection compared with patients given anti-IFN therapy. IRAK4-deficient children are susceptible to infection by pyogenic bacteria until they reach puberty, but they are not more susceptible to viral infection (47).

Although genome-wide association studies identified IRAK1 polymorphisms as a risk factor for SLE (48–50), to our knowledge the current study is the first to provide evidence that specific inhibition of IRAK1 kinase may have therapeutic potential for the prevention of SLE. Such inhibitors might have significant advantages compared with IRAK4-specific inhibitors or dual inhibitors of IRAK1 and IRAK4, which are likely to impair the MyD88-dependent production of proinflammatory cytokines more drastically than the loss of IRAK1 catalytic activity alone. For example, the production of proinflammatory cytokines by several TLR agonists was found to be unimpaired in BMDMs from IRAK1(D359A) mice (28). In contrast, the production of type 1 IFNs and proinflammatory cytokines by ligands that activate the MyD88 signaling network specifically was virtually abolished in BMDMs from IRAK4(D329A)–knock-in mice (Supplemental Fig. 3), as well as in knock-in mice expressing different catalytically inactive mutants of IRAK4 (36, 37). Therefore, patients treated with an IRAK1-specific inhibitor might have a reduced risk for infection by microbial pathogens than patients treated with an IRAK4 inhibitor.

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**Disclosures**

The authors have no financial conflicts of interest.

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**FIGURE 7.** Liver inflammation in ABIN1[D485N] mice is reduced by crossing to mice expressing functionally inactive mutants of IRAK1, IRAK2, or IRAK4. (A) Representative H&E staining of liver sections from 6-mo-old WT mice, ABIN1[D485N]–knock-in mice, ABIN1[D485N] × IRAK4[D329A] mice, ABIN1[D485N] × IRAK1[D359A] mice, ABIN1[D485N] × IRAK2[E525A] mice, IRAK4[D329A] mice, IRAK1[D359A] mice, and IRAK2[E525A] mice. Scale bar, 0.05 mm. (B) Assessment of liver pathology scores (see Materials and Methods). All of the results shown were carried out using four mice of each genotype. *p > 0.05, Mann–Whitney U test.
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