Polyubiquitin binding to ABIN1 is required to prevent autoimmunity

Sambit K. Nanda,1 Ram K.C. Venigalla,1 Alban Ordureau,1 Janet C. Patterson-Kane,2 David W. Powell,3 Rachel Toth,1 J. Simon C. Arthur,1 and Philip Cohen1

The protein ABIN1 possesses a polyubiquitin-binding domain homologous to that present in nuclear factor-κB (NF-κB) essential modulator (NEMO), a component of the inhibitor of NF-κB (IκB) kinase (IKK) complex. To address the physiological significance of polyubiquitin binding, we generated knockin mice expressing the ABIN1[D485N] mutant instead of the wild-type (WT) protein. These mice developed all the hallmarks of autoimmunity, including spontaneous formation of germinal centers, isotype switching, and production of autoreactive antibodies. Autoimmunity was suppressed by crossing to MyD88−/− mice, demonstrating that toll-like receptor (TLR)–MyD88 signaling pathways are needed for the phenotype to develop. The B cells and myeloid cells of the ABIN1[D485N] mice showed enhanced activation of the protein kinases TAK, IKK-α/β, c-Jun N-terminal kinases, and p38 mitogen-activated protein kinase and produced more IL-6 and IL-12 than WT. The mutant B cells also proliferated more rapidly in response to TLR ligands. Our results indicate that the interaction of ABIN1 with polyubiquitin is required to limit the activation of TLR–MyD88 pathways and prevent autoimmunity.

The activation of the innate immune system involves a complex interplay between protein phosphorylation and protein ubiquitylation events. For example, the activation of Toll-like receptors (TLRs) that signal through the adaptor protein MyD88 switches on protein kinases, such as IL-1 receptor–associated kinases (IRAKs), and E3 ubiquitin ligases, such as TNF receptor–associated factor (TRAF) 6 (Walsh et al., 2008) and Pellino (Ordureau et al., 2008). These E3 ligases are then thought to induce the formation of Lys63-linked polyubiquitin (K63-pUb) chains, which may be linked covalently to other proteins, such as IKKα/β and c-Jun N-terminal kinases. The NF-κB–dependent IKK complex also binds to K63-pUb chains, and mutations that abrogate its binding to polyubiquitin (e.g., NEMO[D311N]) prevent activation of the IKKs (Ea et al., 2009), enabling TAK1 to initiate activation of the canonical inhibitor of NF-κB (IκB) kinase (IKK) complex. The NF-κB essential modulator (NEMO) component of the canonical IKK complex also binds to K63-pUb chains, and mutations that abrogate its binding to polyubiquitin (e.g., NEMO[D311N]) prevent activation of the IKKs (Ea et al., 2006; Wu et al., 2006) and NF-κB–dependent gene transcription (Windheim et al., 2008) in response to inflammatory stimuli and cause immunodeficiency in man (Döflinger et al., 2001). These findings imply that the binding of polyubiquitin to NEMO is required for activation of the canonical IKKs, as well as the activation of TAK1. The K63-pUb chains may act as scaffolds to colocalize the IKK complex with TAK1, and/or their interaction with NEMO

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may induce a conformational change that facilitates phosphorylation of the activation loop of the canonical IKKs by TAK1 and/or autophosphorylation.

The canonical IKKs activate NF-κB by phosphorylating the inhibitory IκB component of this transcription factor, which marks IκBα for K48-linked polyubiquitylation by the SCF\textsuperscript{TRCP} E3 ligase and proteasomal destruction. The canonical IKKs also switch on the protein kinase Tpl2 by phosphorylating its inhibitory p105/NF-κB1 component. Similarly, TAK1 not only initiates activation of the canonical IKKs but is also required to activate c-Jun N-terminal kinase (JNK) and p38α mitogen-activated protein kinase (MAPK) via the TLR–MyD88 signaling pathway. The canonical IKKs and MAPKs then catalyze many further phosphorylation events that control the transcription, translation, processing, and secretion of inflammatory mediators (Sato et al., 2005; Shin et al., 2005).

Interestingly, the polyubiquitin-binding domain in NEMO, originally termed A20-binding inhibitor of NF-κB (ABIN) homology domain 2 (AHHD2; Heyninck et al., 2003), but later renamed the ubiquitin-binding domain in ABIN and NEMO (UBAN; Wagner et al., 2008), is found in four other human proteins, termed NRP (UBAN-related protein, also called optineurin), ABIN1, ABIN2, and ABIN3. The ABINs are so named because they were originally identified in a yeast two-hybrid screen using the protein deubiquitylase A20 as bait and because they were found to inhibit NF-κB–dependent gene transcription when overexpressed in cells (Heyninck et al., 1999).

Recently, ABIN1 knockout mice were generated and characterized (Oshima et al., 2009). These mice were found at normal Mendelian ratios up to embryonic day (E) 18.5, but the embryos were smaller and more anemic than WT embryos and died during late embryogenesis from fetal liver apoptosis and hypoplasia. Embryonic fibroblasts isolated from the ABIN1–/– mice were hypersensitive to TNF-induced programmed cell death and the lethality could be rescued by crossing to MyD88-deficient mice. They were born at normal Mendelian ratios and were of normal size and weight. However, they then developed a lupus-like autoimmune disease, which could be prevented by crossing to MyD88-deficient mice. These and other results presented in this paper demonstrate that the interaction of ABIN1 with polyubiquitin chains limits the strength of signaling downstream of TLR–MyD88 and that this is critical to prevent autoimmunity.

RESULTS

Interaction of ABIN1 with polyubiquitin chains in vitro and generation of ABIN1[D485N] knockin mice

The mutation of Asp311 of NEMO to Asn prevents it not only from binding to K63-pUb chains (Ea et al., 2006; Wu et al., 2006) but also from binding to linear polyubiquitin chains (Rahighi et al., 2009). Linear polyubiquitin chains are thought to be generated by the LUBAC E3-ligase (Kirisako et al., 2006) and to
ABIN1[D485N] mutant was unable to bind to polyubiquitin chains in vivo, we immunoprecipitated the endogenous ABIN1 from BMDM extracts, followed by immunoblotting with anti-IRAK1 (to detect polyubiquitylated-IRAK1) and anti-ubiquitin. These experiments showed that stimulation with LPS induced the binding of ABIN1 to polyubiquitylated IRAK1 (Windheim et al., 2008) but not to unmodified IRAK1. In contrast, the ABIN1[D485N] mutant did not capture polyubiquitylated IRAK1 (Fig. 1 C).

Gross analysis of ABIN1[D485N] knockin mice
The ABIN1[D485N] mice were the same size and weight as WT littermates (Fig. 2 A) and were born at the expected Mendelian frequencies (not depicted). However, examination of their internal organs at 2–3 mo of age revealed enlarged spleens which, by 4 mo, were four to five times the weight of those from WT mice (Fig. 2 A). Both the white pulp and the red pulp were greatly enlarged in the spleen, although the splenic architecture was maintained (Fig. 2 B).

ABIN1[D485N] knockin mice had significantly enlarged LNs (Fig. 2 C) and 60–70% of the knockin mice had large nodule-like structures on the intestinal wall, which were identified as Peyer’s Patches (Windheim et al., 2008) but not to unmodified IRAK1. In contrast, the ABIN1[D485N] mutant did not capture polyubiquitylated IRAK1 (Fig. 1 C).

Figure 2. Spontaneous development of defects in multiple immune organs in ABIN1[D485N] mice.
(A) Mean spleen weight (left) and body weight (middle) of WT and ABIN1[D485N] knockin mice with a typically enlarged spleen in 4-mo-old mutant mice (right). Bar, 1 cm. (B) Representative photomicrograph of hematoxylin and eosin-stained spleen sections from WT and ABIN1[D485N] mice. The red (R) and white (W) pulp are indicated. Bars, 0.05 mm. (C) Axillary, inguinal, and mesenteric LNs of 5-mo-old ABIN1[D485N] mice compared with WT mice. Bars, 1 cm. (D) Gut-associated lymphoid tissue (arrows) of the small intestine wall of ABIN1[D485N] (bottom) and WT (top) mice. Bars, 1 cm. (E) Hematoxylin and eosin-stained sections of Peyer’s patches (PP) in WT and ABIN1[D485N] mice. In the low-power view (top; bars, 0.5 mm), the arrows indicate Peyer’s patches in ABIN1[D485N] mice compared with WT mice. In the high-power view (bottom; bar, 0.05 mm), there are Peyer’s patches showing plasma cells (white arrows) and neutrophils (black arrow) in the ABIN1[D485N] mice. All mice used were 16–20 wk old. Data are representative of at least 12 mice (A–C) and 6 mice (D and E) of each genotype. Error bars show the mean ± SEM. **, P < 0.005 (two-tailed Students t test).
the proportions of different B cell populations in the spleen (SPL) and LNs. Profiles were gated on: lymphocytes by FSC/SSC (C–F and H); live cells (A); TCR-β+ CD4+ (D and E), and B220+ (C and F). The numbers within figures indicate the percentages of different cells. GC B, germinal center B cell. (A) Flow cytometric dot plots of spleen (top) and LN (bottom) showing frequencies of the CD11b+ Gr-1+ (macrophages and granulocytes) population in ABIN1[D485N] and WT mice. (B) Total cell numbers in spleens of ABIN1[D485N] mice compared with WT. Error bars show the mean ± SEM. ***, P < 0.001 (two-tailed Students t test). (C) Expression of B cell activation markers from WT (shaded area) and ABIN1[D485N] (black line). (D–F) Contour plots (left) and graphical analysis (right) showing percentages of cell populations in spleen (top) and LN (bottom). Each symbol represents one mouse and the horizontal bars show the mean of the values obtained. (D) Activated (CD4+ CD44hi CD62Llo) and naive (CD4+ CD62Lhi) T cells. (E) Tfh cells (CD4+ CXCR5+ PD-1 hi). (F) Germinal center B cells (B220+ GL-7+ CD95+). (G) Immunohistochemistry of spleens from 16-wk-old mice with germinal centers (brown) stained with peanut agglutinin (PNA). Bars, 0.5 mm. (H) Expression of CD138 and B220 in spleen showing plasma cells in ABIN1[D485N] mice and WT mice. All mice analyzed were 12–16 wk old, and data are representative of at least three independent experiments with three to four mice of each genotype (A–F and H) or single experiment with six mice per genotype (G).

Defects in immune cells with spontaneous germinal center formation in the ABIN1[D485N] knockin mice

To investigate immunological changes in the mice, we performed flow cytometry analyses of various lymphoid organs. Analysis of splenic and LN cells revealed more B cells and granulocytes (CD11b+ Gr-1+) in the ABIN1[D485N] mice compared with WT mice (Fig. 3, A and B; and Fig. S2 A). The expression of MHCII and CD86 receptors on B cells was also increased in the spleen (Fig. 3 C) and LNs (not depicted) of the ABIN1[D485N] mice, indicating that the B cells were activated. However, the development of B cells in the BM and the proportions of different B cell populations in the spleen, LNs, and peritoneal cavity of the ABIN1[D485N] mice did not differ significantly from those in the WT mice (Figs. S2, B–E). We also found that the ABIN1[D485N] knockin mice had four to five times the normal level of monocytes in the blood (unpublished data).

T cell development in the thymus was normal (Fig. S2 F). In contrast, analysis of the splenic and LNT cell populations demonstrated an increased proportion of activated effector T cells (CD62Llo and CD44hi) and a reduced proportion of naive T cells (CD62Lhi) compared with age-matched controls (Fig. 3 D).
Elevated levels of immunoglobulins and autoantibodies in the serum of ABIN1[D485N] mice. The hyperplasia of Tfh cells and germinal center B cells in the spleen and LN led us to investigate the serum immunoglobulin levels. A variety of immunoglobulin isotypes were elevated in the ABIN1[D485N] knockin mice compared with age-matched WT mice, which included both T cell–dependent and T cell–independent immunoglobulins. In particular, the levels of pathogenic immunoglobulins were increased significantly after 20 wk (Fig. 4 A). The circulating levels of antinuclear antibodies and antibodies against double-stranded (ds) DNA were also increased significantly after 16 wk (Fig. 4 B) or 20 wk (Fig. 4 C) in the ABIN1[D485N] knockin mice, demonstrating that some of the antibodies in the knockin mice were being produced by extraneous plasma cells and not by the Tfh cells.

It has been reported that an increased number of follicular helper T cell (Tfh cell) is associated with autoimmunity, and we therefore studied whether Tfh cell number was altered in the ABIN1[D485N] mice. These experiments revealed that the proportion of Tfh cells (CD4+ PD1hi and CXCR5+) in the spleen and LN was greatly increased (Fig. 3 E), as were the number of germinal center B cells (B220+ CD95+ and GL7+; Fig. 3 F). Consistent with more germinal center B cells, there was increased formation of germinal centers in the spleens of ABIN1[D485N] mice (Fig. 3 G). There was also an increase in the proportion of extrafollicular plasma cells in the spleen of ABIN1[D485N] mice (Fig. 3 H).
and peripancreatic arteries (not depicted).

The normal architecture of the walls of affected splenic arterioles was largely replaced by fibrinous material with scattered nuclear debris and mononuclear inflammatory cells (fibrinoid necrosis), and by extravascular leakage (Fig. 4 F). Similar changes were noted in heart-base arteries but with more severe inflammatory cell infiltrates, including neutrophils, macrophages, and reactive fibroblasts, which not only segmentally obliterated arterial wall architecture but also extended into the adjacent atrial myocardium (Fig. 4 G). Similarly, in lung tissue of most mice, the peri-bronchial lymphoid tissue was variably expanded by increased numbers of plasma cells (Fig. 4 H). Other histological lesions included expansion of hepatic portal tracts by variably (but often markedly) increased numbers of hematopoietic cells, lymphocytes, plasma cells, macrophages, and neutrophils with bridging fibrosis and biliary hyperplasia (Fig. 4 I).

The combination of lesions may explain why all the ABIN1[D485N] mice developed severe symptoms of autoimmune disease, requiring them to be culled within 6 mo, if they had not died already. In contrast, no WT control mice had died at this age.

Enhanced proliferation, cytokine production, and activation of signaling pathways in B cells and myeloid cells in ABIN1[D485N] mice

To understand why abnormal levels of immunoglobulins were present in the serum, we studied B cell function in ABIN1[D485N] mice produced against self-cellular components, a feature of autoimmune disease. The heterozygous ABIN1[D485N]+/− mice, which did not develop any pathological abnormalities, only had marginally elevated levels of anti-dsDNA (Fig. 4 B).

High levels of pathogenic immunoglobulins in the serum are known to be deposited in the kidney and the blood vessels, initiating an inflammatory reaction in these organs. We found that there was immune complex deposition in the kidney of the ABIN1[D485N] mice, leading to activation of the complement pathway (Fig. 4 D) and the development of severe renal disease at 20–24 wk of age. Histologically, this was revealed by severe generalized global membranoproliferative glomerulonephritis of the kidney with infiltration of neutrophils and plasma cells (Fig. 4 E, top). There was also thickening of glomerular capillary loops and Bowman’s capsule basement membrane regions by periodic acid–Schiff (PAS)–positive material (Fig. 4 E, bottom).

Vascular lesions were noted at the same age in multiple sites, including splenic arterioles (Fig. 4 F) and heart-base arteries (Fig. 4 G), with occasional involvement of Peyer’s Patch arterioles and peripancreatic arteries (not depicted). The normal architecture of the walls of affected splenic arterioles was largely replaced by fibrinous material with scattered nuclear debris and mononuclear inflammatory cells (fibrinoid necrosis), and by extravascular leakage (Fig. 4 F). Similar changes were noted in heart-base arteries but with more severe inflammatory cell infiltrates, including neutrophils, macrophages, and reactive fibroblasts, which not only segmentally obliterated arterial wall architecture but also extended into the adjacent atrial myocardium (Fig. 4 G). Similarly, in lung tissue of most mice, the peri-bronchial lymphoid tissue was variably expanded by increased numbers of plasma cells (Fig. 4 H). Other histological lesions included expansion of hepatic portal tracts by variably (but often markedly) increased numbers of hematopoietic cells, lymphocytes, plasma cells, macrophages, and neutrophils with bridging fibrosis and biliary hyperplasia (Fig. 4 I).

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Enhanced proliferation, cytokine production, and activation of signaling pathways in B cells and myeloid cells, but not T cells, from ABIN1[D485N] knockin mice

To understand why abnormal levels of immunoglobulins were present in the serum, we studied B cell function in
more marked in the ABIN1[D485N] knockin cells (Fig. 5 B), and this might contribute to the increased activation of T cells via interaction with the CD28 receptor on T cells.

In contrast, stimulation of purified CD4+ T cells with anti-CD3, alone or in combination with either CD28 or IL-2, resulted in similar proliferation rates in WT and ABIN1[D485N] knockin cells (Fig. 5 B), and this might contribute to the increased activation of T cells via interaction with the CD28 receptor on T cells.

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The stimulation of B cells with LPS alone, or the combination of LPS with anti-CD40, also induced higher levels of IL-6 and IL-12p40 secretion in the ABIN1[D485N] mice compared with WT mice. Similar results were found with LTA and R848 (Fig. 5 C). Moreover, TLR stimulation of other immune cells of myeloid origin, such as BM-derived DCS (BMDCs), also increased production of IL-6 and other proinflammatory cytokines, such as TNF, more markedly in the cells from the ABIN1[D485N] mice (Fig. 5 D).

The results described in the previous paragraph suggested that signaling events required for the production of cytokines by B cells and myeloid cells were being switched on more strongly in the ABIN1[D485N] mice compared with WT, and they led us to examine the activation of these pathways. Stimulation of naive splenic B cells with the TLR7 ligand R848 (Fig. 6 A), the TLR4 ligand LPS (Fig. S4 A), the B cell receptor (BCR) ligand (anti-IgM; Fig. 6 B), or the CD40 agonist (anti-IgM; Fig. 6 B), or the CD40 agonist (anti-CD40; Fig. 6 C) caused enhanced phosphorylation (activation) of the canonical IKKs (IKK-α/β) and the phosphorylation of their substrates p105/NF-κB1 and IκBα in the ABIN1[D485N] mice compared with WT mice, and, consistent with enhanced phosphorylation of IκBα, there was a more rapid proteasomal degradation of this protein. There was also modestly enhanced phosphorylation (activation) of the MAPKs JNK1/2 and p38 MAPK. Enhanced activation of the canonical IKKs and MAPKs was also observed in BMDC and BMDM from ABIN1[D485N] mice compared with WT mice after stimulation with LTA or the TLR7 agonist R848 (Fig. 6, D and E; and Fig. S4, B and C).

The finding that activation of MAPKs, as well as the canonical IKK complex, was enhanced in immune cells from the ABIN1[D485N] mice after TLR stimulation indicated that ABIN1 must be exerting its effect by suppressing the activation of a more upstream component of the pathway. This led us examine the activation of TAK1 and to show that it is also enhanced in BMDC from the knockin mice compared with WT mice (Fig. 6 F).

Enhanced activation of the nucleotide oligomerization domain (NOD) 1–NOD2 signaling pathway

The cytoplasmic proteins NOD1 and NOD2 detect peptidoglycan components from intracellular bacteria that have evaded the TLR defense system (Inohara et al., 2005). The activation of these receptors induces their interaction with the protein kinase RIP2, leading to the activation of NF-κB1 and 1κBα in the ABIN1[D485N] mice compared with WT mice, and, consistent with enhanced phosphorylation of 1κBα, there was a more rapid proteasomal degradation of this protein. There was also modestly enhanced phosphorylation (activation) of the MAPKs JNK1/2 and p38α MAPK. Enhanced activation of the canonical IKKs and MAPKs was also observed in BMDC and BMDM from ABIN1[D485N] mice compared with WT mice after stimulation with LTA or the TLR7 agonist R848 (Fig. 6, D and E; and Fig. S4, B and C).

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Figure 7. Comparison of the phenotypes of WT, ABIN1[D485N], ABIN1[D485N]/MyD88−/−, and MyD88−/− mice. (A) Spleen sizes of WT, ABIN1[D485N]/MyD88−/−, ABIN1[D485N]/MyD88−/−, and MyD88−/− mice. Bar, 1 cm. A typical result from many mice analyzed is shown. (B) Flow cytometric analysis was performed as in Fig 2, and a representative set of contour plots is presented (top) showing the proportion of germinal center (GC) B cells [B220+ GL-7+ CD95+] in WT, ABIN1[D485N]/MyD88−/−, ABIN1[D485N]/MyD88−/−, and MyD88−/− mice. Similar results were obtained in three independent experiments. On the bottom, the percentage of GC B cells is shown for each WT type (filled squares), ABIN1[D485N]/MyD88−/− (filled triangles), ABIN1[D485N]/MyD88−/− (open triangles), and MyD88−/− (open squares) mouse examined. Each symbol represents an individual mouse and the horizontal bar shows the mean of the values obtained. (C) Anti-dsDNA antibodies and ANA antibodies in ABIN1[D485N]/MyD88−/− mice (filled squares) and ABIN1[D485N]/MyD88−/− mice (filled triangles). Data are shown for a single experiment with six to eight mice of each genotype. ***, P < 0.005; **, P > 0.005 (Mann-Whitney U test). Each symbol represents an individual mouse and the horizontal bar shows the mean of the values obtained. (D) Hematoxylin and eosin (H&E) staining (first two panels) and PAS staining (third and fourth panels) of kidney sections from ABIN1[D485N]/MyD88−/− and ABIN1[D485N]/MyD88−/−. Bars, 0.05 mm.
The absence of MyD88, but not RIP2, suppresses the autoimmune phenotype of ABIN1[D485N] mice

The results presented in the previous section suggested that enhanced activation of one or more cell signaling pathways might underlie or contribute to the autoimmune phenotype of the ABIN1[D485N] mice. To investigate the importance of the TLR–MyD88–dependent and NOD1/2–RIP2 signaling pathways, we crossed the ABIN1[D485N] mice with mice that do not express the adaptor MyD88 (required for TLR–MyD88 signaling) signaling or with mice that do not express RIP2. The ABIN1[D485N]xRIP2−/− mice still displayed all the gross phenotypes of the ABIN1[D485N] mice (spleenomegaly, lymphadenopathy, and intestinal nodules) and flow cytometry analysis did not show rescue of any immune phenotype (unpublished data). The ABIN1[D485N]xMyD88−/−/− mice, however, did not develop splenomegaly (Fig. 7 A), enlarged LNs, and intestinal nodules (not depicted). They had greatly reduced numbers of germinal center B cells in the spleen (Fig. 7 B), and serum levels of anti-dsDNA, antinuclear antibodies (Fig. 7 C), and different immunoglobulins (not depicted) were also greatly reduced in the ABIN1[D485N]xMyD88−/−/− mice. Moreover, no glomerulonephritis was observed in the kidneys of the ABIN1[D485N]xMyD88−/−/− after 20–24 wk (Fig. 7 D). Interestingly, the increased number of activated T cells and Tfh cells in the ABIN1[D485N] mice also returned to the level found in WT mice in the ABIN1[D485N]xMyD88−/−/− mice (Figs. S5, A and B).

CD40 is not thought to signal via MyD88 (He et al., 2010a), but using B cells from age-matched ABIN1[D485N] mice, MyD88−/−/− mice, ABIN1[D485N]/MyD88−/−/− mice, and WT mice, we found that the enhanced activation of the canonical IKKs and JNK observed in CD40-stimulated B cells from the ABIN1[D485N] mice was reduced in B cells from the ABIN1[D485N]/MyD88−/−/− mice to the same level found in either WT mice or MyD88−/−/− mice (unpublished data). This suggests that the over-reactivity of the ABIN1[D485N]-expressing B cells to CD40 depends on MyD88 signaling. This suggests that there may be cross talk between the CD40 and MyD88 signaling pathways, which would be interesting to investigate in future studies.

Generation of ABIN1[D478-606] mice

The way in which the targeting vector for the ABIN1[D485N] mice was designed allowed the targeted region to be removed by crossing to CRE deleter mice, which generated a form of ABIN1 in which residues 478–606 were deleted. This deletion did not cause the truncated protein to misfold and be degraded, and the ABIN1[D478-606] mutant was actually expressed at higher levels than WT ABIN1 in BMDM (Fig. S6 A) and embryonic fibroblasts (not depicted). The ABIN1[D478-606] mice were also born at near Mendelian frequencies and had a similar phenotype to the ABIN1[D485N] mice (Fig. S6 B), although it was slightly more severe, in as much as the mice became moribund about a month earlier than the ABIN1[D485N] mice. As found in the ABIN1[D485N] mice, there was enhanced activation of TLR signaling pathways in BMDM from the ABIN1[D478-606] mice (Fig. S6 A).

TNF-induced apoptosis in embryonic fibroblasts from ABIN1[D485N] mice

ABIN1−/− mice were reported to be found at normal Mendelian ratios up to E18.5, but the embryos then died during late embryogenesis from fetal liver apoptosis and hypoplasia. Embryonic fibroblasts isolated from the ABIN1−/− mice were hypersensitive to TNF-induced apoptosis, and the lethality could be rescued by crossing to mice that did not express the TNFR1 receptor (Oshima et al., 2009). The TNF-stimulated apoptosis could be restored to normal levels by reintroducing WT ABIN1 but not by reintroduction of the ABIN1[D485N] mutant. Consistent with these findings, we found that embryonic fibroblasts from the ABIN1[D485N] knockin mice also showed enhanced TNF-induced apoptosis (Fig. S7, C and D). The reason why the ABIN1[D485N] and the ABIN1[D478-606] mice that we generated did not display embryonic lethality is unclear but could be the result of a difference in the background of the mice.

In contrast to the effect of TNF on apoptosis, the TNF-stimulated activation of NF-κB was reported to be similar in embryonic fibroblasts from ABIN1−/− mice and WT mice (Oshima et al., 2009). Consistent with this finding, we did not see enhanced activation of MAPKs or the canonical IKK complex in embryonic fibroblasts from the ABIN1[D485N] mice, although these signaling pathways were activated a little more strongly in IL-1–stimulated fibroblasts from the ABIN1[D485N] mice (Fig. S7).

DISCUSSION

In this paper, we demonstrate that replacing a single aspartate in the UBAN of ABIN1 (Asp485) by an asparaginyl residue, which suppresses the binding of ABIN1 to K63-pUb chains or linear pUb chains, causes autoimmunity in mice. Immune cells from the ABIN1[D485N] knockin mice showed enhanced activation of signaling pathways in response to TLR agonists that signal via MyD88, leading to increased B cell proliferation and enhanced production of IL-6 and IL-12p40 production by B cells and elevated IL-6 and TNF by myeloid cells. The critical role of TLR signaling pathways in driving autoimmunity was indicated by the finding that the phenotype was suppressed completely when the ABIN1[D485N] knockin mice were crossed to MyD88−/− mice. These observations raised the question of how increased signaling via TLR–MyD88 pathways can lead to autoimmunity.

Autoimmunity probably results from the formation of many germinal centers that were present in the spleens of the ABIN1[D485N] mice and are responsible for antibody isotype switching and the production of pathogenic antibodies. As suggested by others (Vinuesa et al., 2005, 2009), the formation...
of germinal centers may be triggered by the presence of abnormally high numbers of T<sub>fh</sub> cells, which was a feature of the spleen and LNs of the ABIN1[D485N] knockin mice. An increase in the number of T<sub>fh</sub> cells can result from the aberrant expression of ICOS molecules on T cells (Vinuesa et al., 2005) and be stimulated by IL-6 and IL-12 (Nurieva et al., 2008; Ma et al., 2009; Schmitt et al., 2009). We did not observe any changes in T<sub>fh</sub> cell receptor activation or increased production of ICOS by T cells in the ABIN1[D485N] mice compared with WT mice after TCR stimulation. It therefore seems more likely that increased IL-6 and IL-12 secretion in B cells and myeloid cells via the TLR–MyD88 pathway, coupled with increased expression of co-stimulatory molecules (CD80 and CD86) by antigen-presenting cells, underlies the increased number of T<sub>fh</sub> cells in the mutant animals. Enhanced TLR signaling could, however, lead to germinal center formation in other ways. For example, the migration of B cells to form new germinal centers can be driven by stimulation of TLR4 (Hwang et al., 2009) or TLR7 (Bessa et al., 2010) in vivo, even in IL–21<sup>−/−</sup> mice where T<sub>fh</sub> cell function is defective. Another potentially important mechanism by which TLR–MyD88 signaling pathways contribute to autoimmunity is via the hyperactivation of T reg cells, leading to loss of their ability to suppress effector T cell responses (Pasare and Medzhitov, 2004; Peng et al., 2005; Sutmuller et al., 2007). In summary, our studies with the ABIN1[D485N] mice provide striking support for the concept that abnormally high TLR–MyD88 signaling can trigger autoimmunity (Marshak-Rothstein, 2006).

The increased production of interferon α in plasmacytoid DCs by TLR7 and/or TLR9 agonists is reported to be involved in the development of lupus-like autoimmunity (Banchereau and Pascual, 2006), raising the question of whether ABIN1 prevents autoimmunity by limiting TLR7/TLR9-stimulated interferon α production by pDC. However, we were unable to detect any increase in interferon α production by pDCs from ABIN1[D485N] mice compared with pDCs from WT mice in response to the TLR7 agonist poly(dU) or the TLR9 agonist ODN1826 (unpublished data).

An important unresolved question is the identity of the endogenous ligands that initiate activation of the TLR–MyD88 pathways in these animals in vivo. One possibility is that endogenous host RNA and/or DNA is able to activate TLR7 and TLR9 in the ABIN1[D485N] mice but not in WT mice. Alternatively, components of commensal bacteria may activate the TLR–MyD88 pathways resulting in autoimmunity, as observed in mice that do not express TANK (TRAF-associated NF-κB activator), which also display enhanced activation of NF-κB in response to ligands that signal via the TLR–MyD88 dependent pathway (Kawagoe et al., 2009). To investigate whether commensal bacteria were involved in the development of the phenotype, we fed the mice from birth with broad spectrum antibiotics, but this had no effect on the phenotype. The level of anti–dsDNA in the serum after 16 wk was 20.47 ± 3.69 kU/ml (n = 8) and 19.42 ± 8.19 kU/ml (n = 9), respectively, in control and antibiotic-fed WT mice, and rose to 61.54 ± 33.26 kU/ml (n = 8) and 78.55 ± 31.57 kU/ml (n = 5), respectively, in control and antibiotic-fed ABIN1[D485N] mice. Moreover, the antibiotic-fed ABIN1[D485N] mice had similar enlargement of the spleen and LNs. In contrast, the control IL–10<sup>−/−</sup> mice, which were fed the same combination of antibiotics in parallel, were protected against the development of ulcerative colitis and anal prolapse, as expected.

How polyubiquitin binding to WT ABIN1 limits the strength of TLR–MyD88 signaling is a complex issue and a full molecular understanding will require further analysis. However, the studies we have performed in this paper indicate that ABIN1 exerts its inhibitory effects downstream of MyD88 but upstream of TAK1, explaining why the activation of JNK and p38α MAPK, as well as the canonical IKKs, is enhanced in immune cells from the ABIN1[D485N] mice. Enhanced activation of TAK1 can also explain why the NOD1/2–RIP2 signaling pathway was activated more strongly in peptidoglycan-stimulated BMDM from ABIN1[D485N] mice and why B cells from the mutant mice proliferated more rapidly than B cells from the WT mice in response to BCR and CD40 agonists because TAK1 is known to be required for NOD1/2–RIP2 (Windheim et al., 2007), BCR, and CD40 signaling (Sato et al., 2005).

ABIN1 was originally identified as a protein that interacts with the deubiquitylase A20. Like ABIN1, A20 is known to function as a negative regulator of the innate immune system and mice with a conditional knockout of A20 in B cells develop autoimmunity (Boone et al., 2004; Tavares et al., 2010; Chu et al., 2011). Moreover, human polymorphisms in A20 predispose to autoimmune diseases (Plenge et al., 2007; Thomson et al., 2007; Nair et al., 2009). A priori, one might therefore have imagined that by binding to K63–pUb chains/proteins generated in response to TLR activation (see Introduction), ABIN1 may recruit A20 to its substrates, facilitating the A20-catalysed hydrolysis of K63–pUb chains/proteins and the inhibition of TLR–MyD88–dependent signaling. Cells expressing the polyubiquitin binding–defective ABIN1[D485N] mutant would be unable to perform this function, leading to increased levels of K63–pUb chains/proteins and enhanced recruitment and activation of the TAK1 complex. However, whether this is the way in which ABIN1 limits the strength of signaling is unclear for several reasons. First, the bacterially expressed A20 catalytic domain cleaves K48–pUb chains and not K63–pUb chains or linear polyubiquitin chains in vitro (Komander et al., 2009), although A20 may be able to hydrolyze K63–pUb chains when overexpressed in mammalian cells (Wertz et al., 2004), in which case there may be a mechanism for altering the specificity of A20 in vivo. Second, a truncated form of ABIN1, ABIN1[444–601], which does not interact with A20, inhibits TNF-stimulated NF-κB–dependent gene transcription as effectively as WT ABIN1 in overexpression experiments (Heyninck et al., 2003). Third, the level of A20 is low in unstimulated cells and increases greatly after stimulation for 1 h with TLR agonists (Fig. 6, C and D), but the enhanced activation of signaling pathways in immune cells from the ABIN1[D485N] mice can be observed.
after only 10–15 min (Fig. 6), demonstrating that this effect is independent of the induction of A20. Fourth, although the B cell–specific knockout of A20 leads to autoimmunity, the onset of the pathology seems to be delayed by several months compared with the ABIN1[D485N] mice (Tavares et al., 2010). Therefore, if the phenotype of the ABIN1[D485N] mice is driven only by alterations in B cell function, it would be difficult to explain this solely by the loss of ABIN1-dependent recruitment of A20 to its substrates.

ABIN1 binds linear polyubiquitin chains, as well as K63-polyubiquitin chains (Fig. 1), and LUBAC, an E3 ligase which produces linear polyubiquitin specifically in vitro, appears to participate in at least one MyD88-dependent signaling pathway because the IL-1–stimulated activation of the canonical IKK complex was reported to be impaired in MEFs deficient in HOIL-IL, a component of LUBAC (Tokuenga et al., 2009). Therefore, the possibility that ABIN1 restricts signaling to NF-κB by binding to linear polyubiquitin chains as well as, or instead of, K63-pUb chains cannot be excluded at this stage. However, the activation of JNK by TNF was not inhibited in HOIL-IL−/− MEFs, implying that LUBAC is not required for the activation of TAK1. Moreover, the TAB2 component of the TAK1 complex binds to K63-pUb tetramers >100-fold more strongly than to linear ubiquitin tetramers (Kanayama et al., 2004; Kulathu et al., 2009; Sato et al., 2009). WT ABIN1 might therefore compete with the polyubiquitin-binding components of the TAK1 complex (TAB2–TAB3) for binding to the same K63-pUb chains/proteins, limiting the extent of activation of TAK1. We also observed in the present study that the endogenous polyubiquitylated IRAK1 could be communoprecipitated with WT ABIN1, but not with ABIN1[D485N], from the extracts of LPS-stimulated BMDM. Therefore, another possibility is that the interaction of WT ABIN1 with polyubiquitylated IRAK1 may hinder the ability of IRAK1 to interact with and activate TRAF6, reducing the TRAF6-mediated formation of K63-pUb chains and limiting the activation of TAK1. IRAK1 undergoes Lys63-linked polyubiquitylation in response to IL-1 (Windheim et al., 2010), but the possibility that it also undergoes linear polyubiquitylation has not been excluded.

Interestingly, while this study was in progress, human polymorphisms were identified in the gene encoding ABIN1 that predispose to lupus–like autoimmune diseases, psoriasis (Han et al., 2009; Nair et al., 2009), and vasculitis (He et al., 2010b). This suggests that ABIN1 also plays a key role in preventing autoimmunity in man. The ABIN1[D485N] mice may therefore be a good model for human autoimmune disease and could be used to assess the efficacy of drugs that target components of TLR–MyD88 signaling pathways. Drugs that increase the expression of ABIN1 could also have therapeutic value because the adenovirus–mediated delivery of ABIN1 to the lung epithelium has been reported to reduce allergen–induced eosinophil infiltration of the lungs in a mouse model of allergen–induced asthma (El Bakkouri et al., 2005).

MATERIALS AND METHODS

Expression and purification of ABIN1 and NEMO and binding to polyubiquitin. Human ABIN1 and ABIN1[D472N] were cloned and expressed as fusion proteins with glutathione S-transferase (GST) at the N terminus and a His tag at the C terminus. The expressed proteins were purified on nickel–nitrilotriacetate agarose (QIAGEN) followed by chromatography on glutathione–Sepharose (GE Healthcare). Human NEMO and NEMO[D311N] were expressed as GST fusions and purified on glutathione–Sepharose. 8 μg of these proteins were immobilized individually on a 10-μl packed volume of glutathione–Sepharose and incubated for 1 h at 21°C with 2 μg of K48-linked, K63-linked (Boston Biochem), or linear ubiquitin oligomers (Enzo Life Sciences) in 300 μl 25 mM HEPES, pH 7.5, 1 mM EGTA, 0.5% (vol/vol) Triton X-100, and 2 mM MgCl2 (Buffer A) plus 150 mM NaCl. The beads were washed five times with Buffer A plus 250 mM NaCl and once with Buffer A without Triton X-100.

Generation of ABIN1[D485N] knockin mice. The Asp485 to Asn knockin mutation was generated in exon 14 of the ABIN1 locus and, at the same time, LoxP sites were inserted in the introns adjacent to exons 14 and 16 by standard gene targeting methods (Fig. 5A). The following primers were used for making the targeting vector (underlined regions indicate the presence of the LoxP restriction site): sense (5′-GCCCTACTCAAATCTCCAATGC-3′), and antisense (5′-GCCCTACTCAAATCTCCAATGC-3′), Exon14–16 sense (5′-AGGATCCCGGTGGCAGTCCTG-3′) and antisense (5′-AGGATCCCGGTGGCAGTCCTG-3′), splicing donor sense (5′-GCCCTACTCAAATCTCCAATGC-3′), and acceptor antisense (5′-AGGATCCCGGTGGCAGTCCTG-3′). All animals were expressed as GST fusions and purified on glutathione–Sepharose. 8 μg of these proteins were immobilized individually on a 10-μl packed volume of glutathione–Sepharose and incubated for 1 h at 21°C with linear ubiquitin oligomers (Enzo Life Sciences) in 300 μl 25 mM HEPES, pH 7.5, 1 mM EGTA, 0.5% (vol/vol) Triton X-100, and 2 mM MgCl2 (Buffer A) plus 150 mM NaCl. The beads were washed five times with Buffer A plus 250 mM NaCl and once with Buffer A without Triton X-100.

Flow cytometry. Single-cell suspensions were made from thymus and spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipetting, whereas BM samples were prepared from spleen by gentle sieving and pipette...
Histopathology and immunohistochemistry. Formalin-fixed tissues were stained with hematoxylin & eosin or with the PAS reagent. For immunohistochemical labeling of germinal centers, sections were dewaxed in xylene and rehydrated through graded alcohols. Proteinase K (Dako) was applied for 20 min at 20°C for antigen retrieval, and slides were washed in buffer and a peroxidase blocker applied (Dako) for 5 min, followed by further washes. The slides were incubated for 1 h with biotinylated peanut agglutinin (Vector Laboratories) diluted 1:100. After washing, Avidin-Biotin Complex was applied for 30 min, washed again, and visualized by incubation for two 5-min periods in diaminobenzidine (Dako). After further washing, counterstaining was performed for 30 s using Gill’s hematoxylin. Stained sections were washed, dehydrated, and mounted under glass coverslips with DPX.

For immunofluorescence studies, kidney sections were washed three times in PBS and blocked for 30 min at 20°C with 2.5% BSA, 0.05% Tween 20, and 0.05% Triton X-100. Sections were washed three times in PBS and mounted with Vectashield. FITC-conjugated anti-mouse C3 (C7503F; Cedarlane Laboratories Limited), C1q (JL-1; Hyclut Biotech), and IgG (F2266; Sigma-Aldrich) were used for immunostaining. Images were taken on a microscope (BX51; Olympus) with a camera (DP71; Olympus) fitted using acquisition software (Olympus). Images were processed with Photoshop software (CS3; Adobe).

Measurement of autoantibodies and immunoglobulin isotypes by ELISA. Blood was collected from mice by cardiac puncture, allowed to clot, and the serum was separated by centrifugation. Immunoglobulins (Immunology Consultants Laboratory) and autoantibodies to dsDNA and antinuclear antibodies (total Ig; Alpha Diagnostics International) were measured by ELISA.

Multiplex cytokine assays. Cytokines in cell culture supernatants and mouse serum were measured using either Luminex-based Bio-Plex Mouse Grp 1 Cytokine 8-Plex, 6-Plex Panel, or 13 plex (Bio-Rad Laboratories) or mouse serum were measured using either Luminex-based Bio-Plex Mouse Cytokines (Bio-Rad Laboratories) diluted 1:100. After washing, Avidin-Biotin Complex was applied for 30 min, washed again, and visualized by incubation for two 5-min periods in diaminobenzidine (Dako). After further washing, counterstaining was performed for 30 s using Gill’s hematoxylin. Stained sections were washed, dehydrated, and mounted under glass coverslips with DPX.

For immunofluorescence studies, kidney sections were washed three times in PBS and blocked for 30 min at 20°C with 2.5% horse serum, 1% BSA, 0.05% Tween 20, and 0.05% Triton X-100. Sections were washed three times in PBS and mounted with Vectashield. FITC-conjugated anti-mouse C3 (C7503F; Cedarlane Laboratories Limited), C1q (JL-1; Hyclut Biotech), and IgG (F2266; Sigma-Aldrich) were used for immunostaining. Images were taken on a microscope (BX51; Olympus) with a camera (DP71; Olympus) fitted using acquisition software (Olympus). Images were processed with Photoshop software (CS3; Adobe).

Measurement of anti-TNF activity. This was performed as described previously (Cheung et al., 2003), except that α-TNF was used to immunoprecipitate TNF1 instead of α-TAB1. In brief, TNF1 complexes were immunoprecipitated from 0.15 mg BMDC extract protein, using 0.45 µg anti-TNF antibody. The TNF1 activity in the immunoprecipitates was measured by the activation of MAPK kinase 6 and coupled to the activation of p38 MAPK. The active p38 MAPK generated in this step was quantified in a secondary assay by measuring its ability to incorporate [32P] phosphate from γ-[32P]ATP into myelin basic protein.

Statistical analysis. Statistical significance was calculated either using the two-tailed Student’s t test or the Mann-Whitney U test using Prism software (GraphPad Software). Further details are provided in the relevant figure legends.

Online supplemental material. Fig. S1 shows design of the targeting vector and the expression pattern of ABIN1 protein in various cells from ABIN1[D485N] mice. Fig. S2 shows immune phenotyping of ABIN1[D485N] mice. Fig. S3 shows no alteration in T cell receptor signaling in ABIN1 knockin mice. Fig. S4 shows enhanced activation of LPS signaling in B cells and also enhanced activation of TLR-MYD88 and NOD1-RIP2 signaling pathways in BMDM from ABIN1[D485N] mice. Fig. S5 shows rescue of the T cell phenotype observed in ABIN1[D485N]/MyD88−/− mice. Fig. S6 shows enhanced activated TLR signaling in BMDM from ABIN1[D485N]/MyD88−/− mice. Fig. S6 suggests that the T cell phenotype observed in ABIN1[D485N]/MyD88−/− mice and enhanced TNF-induced apoptosis in embryonic fibroblasts from ABIN1[D485N] mice. Fig. S7 shows TNF and IL-1 signaling in embryonic fibroblasts from ABIN1[D485N] mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101777/DCl.

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Figure S1. Generation of ABIN1[D485N] knockin mice. (A) Strategy for generating the D485N mutation in the ABIN1 gene. A vector was constructed containing a 5’ arm of homology followed by exons 14–16 containing the D485N mutation in exon 14 (asterisk) flanked by LoxP sites, an Frt-flanked neomycin resistance cassette without a polyadenylation sequence (neo), and a 3’ arm of homology and thymidine cassette (TK). This vector was transfected into E14 embryonic stem cells and selection performed by standard techniques. Colonies were screened for the presence of the 5’ LoxP site by genomic PCR and mRNA corresponding to the region from the end of the neo cassette to exon 18 of the ABIN1 gene. The primer positions are shown by light gray arrows (for sequences see Materials and methods). Heterozygous embryonic stem cells were used to generate chimeric mice, and chimeras giving germline transmission were crossed to flp transgenic mice to excise the neo cassette. Routine genotyping was performed using primers across the 3’ LoxP site (P5-P6). (B) The primers generate a 400-bp band for a WT allele and 450 bp for a knockin allele (or 600 bp for a knockin allele before excision of the neo cassette). The figure shows an example of genotyping for a WT (wt), heterozygous (het), and homozygous knockin (ki) allele. (C–E) Immunoblotting of WT ABIN1 and ABIN1[D485N] in BMDM (C), B cells (D), and T cells (E). GAPDH was the loading control.
Figure S2. Characterization of immune cells in ABIN1[D485N] mice. Flow cytometric profiles from spleen (SPL) and LN of WT and ABIN1[D485N] mice. All mice used were 9–12 wk old except in B and F (4–6 wk old). Profiles were gated on lymphocytes by FSC/SSC. The lymphocytes were further gated on Thy1+ (F) and TCR-β+ (G). The numbers contained adjacent to or within the boxed areas in each figure indicate the percentages of different subset of cells. Other abbreviations are: PC, peritoneal cavity; ImatB, immature B; MatB, mature B; MzB, marginal zone B; FoB, follicular B; DN, double negative; DP, double positive. (A) Dot plot of B (B220) and T (TCR-β) cells in spleen and LN. (B) Contour plot showing B cell development in the BM. (C and D) B cell maturation into different peripheral subsets in spleen and LN of WT and ABIN1[D485N] mice. (E) Proportion of different B cell subsets in PC of WT and ABIN1[D485N] mice. (F) Dot plots of thymocytes gated on Thy1 and analyzed for CD4 and CD8 expression to show the percentage of CD4−CD8−, DN, CD4+, CD8+, and CD4+CD8+ DP cells. (G) Dot plot showing CD4 and CD8 cells in spleen and LN. The results shown are representative of at least three independent experiments.
Figure S3. The T cell phenotype of WT and ABIN1[D485N] mice. In A and B, the data are representative of two independent experiments each with three mice. (A) Splenic plus LN CD4+ T cells from WT or ABIN1[D485N] mice were stimulated for 72 h with 2 or 5 µg/ml of plate-bound anti-CD3 alone or with 1 µg/ml anti-CD3 plus 1 µg/ml anti-CD28. The cells were pulsed with 0.5 µCi [3H]-thymidine for the last 16 h. [3H]-thymidine incorporation was measured using a β-scintillation counter. (B) Splenic plus LN CD4+ T cells were stimulated with 5 µg/ml anti-CD3 alone or in combination with 1 µg/ml anti-CD3 plus 1 µg/ml anti-CD28 or 20 ng/ml PMA plus 1 µM ionomycin for 48 h, and secretion of the indicated cytokines was measured by multiplex luminex assay. (C) Contour plot (top) and graphical analysis (bottom) of Treg cells (CD4+ CD25+ FoxP3+) of WT and ABIN1[D485N] mice. Two independent experiments are shown using a total of five mice of each genotype. Error bars represent mean ± SD. Each symbol represents an individual mouse and horizontal bar shows the mean of the values obtained.
Figure S4. Signaling via TLR and NOD1 receptors in B cells and macrophages from WT and ABIN1[D485N] mice. (A) LPS signaling in B cells from WT and ABIN1[D485N] mice. Purified B cells were stimulated with 20 µg/ml LPS for the times indicated and cell lysates were probed with the antibodies shown. Data are representative of two independent experiments. *p<0.05, nonspecific band. (B–D) Immunoblotting of BMDM extracts from WT and ABIN1[D485N] mice after stimulation with 2 µg/ml of the TLR2/6 agonist LTA (B), 2 µM of the TLR9 agonist ODN1826 (C), or 10 ng/ml of the NOD1 agonist meso-diaminopimelic acid (meso-DAP; D), for the times indicated. The cell lysates were probed with the antibodies indicated. Data are representative of two to four independent experiments in all panels.
Figure S5. Comparison of the T cell phenotypes of WT, ABIN1[D485N], ABIN1[D485N]/MyD88−/−, and MyD88−/− mice. (A and B) Flow cytometric analysis presented as contour plots (top) and graphically (bottom) showing expression and percentages of activated (CD4+ CD44hi CD62Llo) and naive (CD4+ CD62Lhi) T cell populations (A), and T<sub>fh</sub> cells (CD4+ CXCR5+ PD-1hi; B) in spleen of WT (filled squares), ABIN1[D485N]xMyD88−/− (filled triangles), ABIN1[D485N]xMyD88−/− (open squares), and MyD88−/− (open triangles) mice. Data presented are from three independent experiments. Each symbol represents an individual mouse and horizontal bars show the mean of the values obtained.
Figure S6. Phenotype of the ABIN1Δ478–606 mice and TNF-stimulated apoptosis in MEFs from WT and ABIN1[D485N] mice. (A) Immunoblotting of BMDM lysates from WT and ABIN1[Δ478–606] mice after stimulation with 2 µg/ml of the TLR agonist LTA for the times indicated. The cell lysates were probed with the antibodies indicated. Data are representative of two independent experiments. (B) Hematoxylin and eosin–stained sections of spleen (bars, 0.05 mm), kidney (bars, 0.05 mm), lungs (bars, 0.2 mm), and liver (bars, 0.1 mm) from 20-wk-old mice from WT (top) and ABIN1[Δ478–606] (bottom) mice. The pictures are from a single experiment with three mice being analyzed per genotype. (C) MEFs from WT, ABIN1[D485N], or heterozygous (HET) mice were either treated with 10 µg/ml cycloheximide (CHX) plus TNF for the times indicated or left untreated. Cell lysates were immunoblotted with the antibodies indicated and the positions of full-length caspase 3 (37 kD) and truncated caspase 3 (19 kD) are indicated. The data are representative of two independent experiments. * , nonspecific band. (D) The graph shows the percentage of MEFs with activated caspase 3 (caspase3* cells) after treatment with TNF plus CHX (filled bars) compared with MEFs treated with CHX alone (open bars) for 6 h. The analysis was performed by flow cytometry. The results shown are for MEFs isolated from two to three individual embryos with the experiment being performed in triplicate. ***, P < 0.005 (two-tailed Student’s t test). Error bars represent mean ± SD.
Figure S7. IL-1 and TNF-signaling in mouse embryonic fibroblasts from WT and ABIN1[D485N] mice. Mouse embryonic fibroblasts (MEFs) were stimulated with either 10 ng/ml TNF (A) or 5 ng/ml IL-1α (B) for the times indicated. Cell lysates (20 µg protein) were immunoblotted with the antibodies indicated. Data are representative of at least two independent experiments for all panels.