The deubiquitinase activity of A20 is dispensable for NF- κ B signaling

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Abstract

A20 has been suggested to limit NF- κ B activation by removing regulatory ubiquitin chains from ubiquitinated substrates. A20 is a ubiquitin-editing enzyme that removes K63-linked ubiquitin chains from adaptor proteins, such as RIP1, and then conjugates them to K48-linked polyubiquitin chains to trigger proteasomal degradation. To determine the role of the deubiquitinase function of A20 in downregulating NF- κ B signaling, we have generated a knock-in mouse that lacks the deubiquitinase function of A20 (A20-OTU mice). These mice are normal and have no signs of inflammation, have normal proportions of B, T, dendritic, and myeloid cells, respond normally to LPS and TNF, and undergo normal NF- κ B activation. Our results thus indicate that the deubiquitinase activity of A20 is dispensable for normal NF- κ B signaling.

Keywords A20; deubiquitinase; K63-linked ubiquitination; NF- κ B; signal transduction

Subject Categories Immunology; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

NF- κ B is a ubiquitously expressed, inducible transcription factor that regulates the expression of numerous target genes, particularly in the immune system. In unstimulated cells, NF- κ B is sequestered in the cytoplasm through its binding to inhibitory I κ B proteins [1]. The well-characterized K48-linked polyubiquitination triggers proteasome-mediated degradation of substrate proteins such as I κ Bs, while polyubiquitination of signaling adapter proteins with K63-linked chains has been proposed to have a critical regulatory function in NF- κ B activation pathways. RING-finger proteins such as TNF receptor-associated factors (TRAFs) are believed to act as ubiquitinating enzymes and molecules such as receptor-interacting protein 1 (RIP1) are well-characterized substrates that undergo K63-linked ubiquitination. This process is

reversible as deubiquitinating enzymes have been proposed to limit the consequence of regulatory ubiquitination by removing the polyubiquitin chains. While it is clear that K48-linked polyubiquitination leads to proteasomal degradation, the physiological consequences of regulatory K63-linked ubiquitination remain to be established in animal models. Among the various deubiquitinases that have been suggested to act on K63-linked chains in the NF- κ B pathway, the best characterized is A20 (TNFAIP3) [2].

A20 was initially identified as a TNF-inducible zinc-finger protein that protects cells from TNF-induced cytotoxicity [3]. A20 is expressed at very low levels in most cell types but is rapidly induced in response to various PAMPs or proinflammatory cytokines [3]. Subsequently, many studies, mainly in overexpression systems, have demonstrated that A20 downregulates NF- κ B signaling in multiple pathways including the tumor necrosis factor (TNF) and Toll-like receptor (TLR) pathways [4,5]. Mice deficient in A20 die prematurely from multi-organ inflammation and cachexia as a result of increased NF- κ B signaling as predicted from *in vitro* studies. Additionally, A20-deficient mouse embryonic fibroblasts (MEFs) show persistent NF- κ B signaling as evidenced by increased I κ B α degradation [6].

Intriguingly, although A20 was initially identified as a TNF-inducible gene, the spontaneous inflammation in A20-deficient mice results from TNF-independent signaling as the inflammation continues unabated even in A20^{-/-} TNF α ^{-/-} and A20^{-/-} TNFR1^{-/-} double-mutant mice. Instead, A20^{-/-} Myd88^{-/-} mice do not show the severe inflammation characteristic of A20-deficient mice, indicating that TLR signaling drives the spontaneous inflammation in these mice [7]. Treatment of A20-deficient mice with broad-spectrum antibiotics rescues the inflammatory phenotype, thereby indicating that a dysregulated intestinal flora contributes to the constitutive TLR signaling which results in perinatal lethality in these mice [8]. Thus, it has been clearly demonstrated that A20 is essential and non-redundant in restricting persistent TLR-mediated activation of NF- κ B and subsequent lethality. Therefore, while many studies have focused on the role of A20 in response to TNF, it is imperative to understand the role of A20 in the context of TLR and LPS signaling [9,10].

Dysregulation of A20 has now been implicated in various autoimmune diseases and cancer. Polymorphisms in the A20 locus increases disease susceptibility in multiple autoimmune diseases including type I diabetes, psoriasis, rheumatoid arthritis, systemic lupus erythematosus [11]. Recently, A20 polymorphisms (A125V

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and F127C) were discovered in the DUB domain that increases susceptibility to autoimmunity by impairing A20-mediated deubiquitination [12].

It has been shown by various biochemical assays that A20 can function both as a deubiquitinase (DUB) and as an ubiquitin ligase. A20 has a N-terminal OTU domain followed by seven C-terminal zinc-finger domains. The C103 residue in the OTU domain has been shown to be essential for the deubiquitinating function of A20 [10,13]. Previous biochemical studies using recombinant A20 has demonstrated that A20 is a unique ubiquitin-modifying enzyme regulating both the activity (by removing K63-linked chains) and stability (by adding K48-linked chains) of signaling molecules such as RIP1 and TRAF6 [10]. These studies showed that in response to TNF stimulation, A20 acts by first deubiquitinating the regulatory K63-linked polyubiquitin chains from RIP1, thereby initially attenuating NF- κ B signaling. Subsequently, A20 acts as an E3 ubiquitin ligase, adding K48-linked polyubiquitin chains to RIP1 leading to degradation of RIP1 and terminating any residual NF- κ B signaling. In the TLR and the IL-1R pathways, A20 inhibits NF- κ B signaling by disrupting the binding of the E3 ligase, TRAF6, with E2 ubiquitin-conjugating enzymes like Ubc13 or UbcH5c. The deubiquitinase activity mediated by the C103 residue has been shown to be essential in mediating this interaction as well [14]. However, A20's deubiquitinase activity is not limited to K63-linked polyubiquitin chains; it has been shown to disassemble K48-linked polyubiquitin chains as well [15,16].

Mutating C103 to alanine eliminates the deubiquitinase activity of A20. However, despite the A20-C103A mutant proteins being completely deficient in DUB activity, some studies showed that overexpression of A20-C103A can inhibit NF- κ B signaling [4,13,17]. We were intrigued by these observations because they suggest that the ability of A20 to inhibit NF- κ B activation might not be due to the ability of A20 to deubiquitinate K63-ubiquitinated substrates. Therefore, to accurately delineate the physiological ramifications of A20's deubiquitinating function, we generated a gene-targeted mouse with a C103A point mutation to eliminate the DUB activity of A20. Given the severe multi-organ inflammation in A20^{-/-} mice that led to perinatal lethality [6], we hypothesized that these A20-C103A knock-in (KI) deubiquitinase mutant mice would show a phenotype similar to the A20 knock-out mice. Furthermore, since dysregulation of A20 has been implicated in a large number of diseases [11], these knock-in mice could prove to be a useful model for studying these disorders in a physiologically relevant setting. However, as described in this report, the A20-C103A KI mice do not show any aberrant pro-inflammatory phenotype and demonstrate a normal life span. Analysis of NF- κ B signaling in cells isolated from these mice shows no discernible difference from normal, wild-type cells, including the degree of ubiquitination of signaling adapter proteins. Therefore, our studies reveal that the deubiquitinase function of A20 is not important for the well-described role of A20 in NF- κ B signaling. It is important to appreciate that the phenotype observed in the knock-in mice reflects regulation of the C103A mutant protein by endogenous mechanisms as compared to previous *in vitro* experiments, which relied on overexpression systems and exogenous gene regulatory mechanisms.

Results

Generation of A20-OTU knock-in mice

To evaluate the physiological contribution of A20's deubiquitinating function, we used a BAC-based approach to generate a knock-in mouse harboring a point mutation (cysteine to alanine) at position 103 (Supplementary Fig S1). The sequencing chromatogram of the genomic DNA isolated from heterozygous mice shows both cysteine and alanine, while the homozygous mice contain only alanine at position 103, confirming the presence of the correct C103A point mutation (Fig 1A). In this manuscript, we refer to the wild-type littermates as C/C meaning they have a cysteine at position 103 in both alleles; the heterozygous mice as C/A as they have a cysteine and alanine in the two alleles; and the homozygous knock-in mice as A/A as they have only the mutated alanines in both alleles. The genotyping strategy and the littermates are shown in Fig 1B.

We next isolated BMDMs from both WT and A/A littermates and found roughly equal levels of A20 mRNA (Supplementary Fig S2) and protein (Fig 1C) following LPS and TNF stimulation. A20 was initially found at relatively low levels but is rapidly induced upon LPS stimulation in equal amounts in both the C/C and A/A littermates, as would be expected in a gene-targeted knock-in system. This also shows that the stability of the protein is not affected by the C103A mutation.

Characterization of A20-OTU knock-in mice

Although A20^{-/-} mice were runted by 1 week of age and died perinatally of multi-organ inflammation and cachexia, the homozygous A/A knock-in mice were normal (Fig 1B) and did not display any external signs of inflammation (for the observed period of 10 months after birth). They were born in Mendelian ratios, and both the C/C and A/A littermate adults weighed the same (Supplementary Figs S3 and S4). Histological examination of 6-week-old A20^{-/-} mice had revealed severe tissue damage in multiple organs [6]. However, histology of major organs (kidney, liver, spleen, thymus, lung, and heart) of WT (C/C), heterozygous knock-in (C/A), and homozygous knock-in (A/A) littermates did not reveal any differences (Fig 1D). Hence, the deubiquitinating function of A20 is not important for maintaining basal tissue homeostasis. This was surprising as the C103 residue of A20 has been proposed to be crucial for downregulating NF- κ B signaling *in vitro* [10,14]. Thus, it was expected that the A20-C103A mice would at least partially resemble the A20^{-/-} mice [6].

Characterizing the cells of the immune system of C103A knock-in mice in the steady state

Unlike A20^{-/-} mice that have increased numbers of myeloid lineage cells in the bone marrow and spleen [6], the C/C, C/A, and A/A littermates had comparable frequencies of myeloid lineage cells (CD11b⁺). Within the myeloid compartment, the frequencies of monocytes (CD11b⁺Ly6c⁺Ly6G⁻) and granulocytes (CD11b⁺Ly6c⁺Ly6G⁺) were similar across all indicated genotypes (Fig 2A, top-left panels). Similarly, analysis of B-cell development indicated that the differentiation pattern of CD19⁺B220⁺ and CD19⁻B220⁺ cells

Figure 1. Generation of the A20-OTU/OTU mice.

- A Sequencing of genomic DNA from homozygous (A/A) and heterozygous littermates (C/A).
 B Genotyping strategy and photograph of wild-type, heterozygous, and homozygous littermates showing normal development and growth.
 C Similar induction of A20 protein upon stimulation of macrophages from C/C or A/A littermates, with TNF or LPS.
 D Histology of kidney, liver, spleen, thymus, lung, and heart of littermates.

in heterozygous and homozygous knock-in mice was comparable to the WT littermates. In addition, analysis of surface IgM and IgD expression in CD19⁺ B cells of the bone marrow indicated similar expression patterns among wild-type, heterozygous, and homozygous knock-in mice (Fig 2A, top-right panels). Consistently, analysis of erythroid (Ter119⁺) and megakaryocyte (CD41⁺) lineage cells suggested comparable frequencies in the bone marrow (Supplementary Fig S5).

In mice, T-cell development occurs in the thymus and undergoes distinct stages of differentiation including the earliest, the double-negative (DN) stage (CD4⁻CD8⁻), the double-positive (DP) stage (CD4⁺CD8⁺), and subsequently the CD4⁺CD8⁻ and CD4⁻CD8⁺ single-positive stages [18]. Analysis of the thymus of the three genotypes indicated a roughly equal differentiation of CD4 and CD8 cells

between the genotypes as shown in Fig 2B. Consistent with the normal differentiation of myeloid, lymphoid and erythroid lineages in the BM and thymus, the spleen also showed normal proportions of B cells, T cells, dendritic cells, and myeloid cells (Fig 2C). Therefore, these results suggest that the deubiquitinase function of A20 does not play a role in differentiation and maintenance of immune cell types.

As mice age to 6 months, the A/A deubiquitinase mutant mice develop splenomegaly and had larger spleens as compared to their C/C littermates. Both the wild-type and mutant mice weigh around the same (Supplementary Fig S6). Analysis of the bone marrow and spleen of older mice showed that the homozygous mutant mice showed an increase in the numbers of myeloid cells (Supplementary Fig S7).

Figure 2. Characterization of the cells of the immune system of C103A knock-in 6-week-old mice in the basal state.

- A Characterizing the monocytes, granulocytes, and B cells of the bone marrow.
 B Characterizing the CD4 and CD8 cell populations of the thymus.
 C Characterizing the myeloid cells, dendritic cells, T cells, and B cells of the spleen.

Activation of wild-type and mutant BMDM and BMDCs in response to LPS and TNF *in vitro*

We differentiated bone marrow cells of wild-type (C/C), heterozygous (C/A), and homozygous (A/A) mice into dendritic cells and macrophages in the presence of either GM-CSF or M-CSF, respectively. After 7 days of *in vitro* culture, we divided the cells into two plates and stimulated one plate with TNF, while the other plate was stimulated with LPS. ELISA analysis revealed that LPS stimulation produced roughly equal amounts of TNF, IL-6, and

IL-12 by BMDMs (Fig 3A) and BMDCs (Supplementary Fig S8A) of both C/C and A/A mice. Similar results were obtained following TNF stimulation on the same (IL-6 and IL-12 were roughly similar for both C/C and A/A littermates) as shown in Fig 3B and Supplementary Fig S8B.

In addition, we also analyzed the DCs and macrophages by flow cytometry for their activation status (as determined by upregulation of CD40, CD80, CD86, and MHC-Class II) after 48 h of stimulation with LPS and TNF. Both DCs and macrophages showed upregulation of CD40, CD80, CD86, and MHC-Class II

Figure 3. Activation of wild-type and mutant BMDM and BMDC in response to LPS and TNF *in vitro*; response of littermates to LPS shock *in vivo*.

A TNF- α , IL-6, and IL-12 produced by BMDM in response to 1 μ g/ml LPS were measured by ELISA. Error bars represent standard deviation representative of three independent experiments.

B IL-6 and IL-12 produced by BMDM in response to 10 ng/ml TNF- α were measured by ELISA. Error bars represent standard deviation representative of three independent experiments.

C Analysis of activation status of BMDMs by flow cytometry (black: wild-type; red: heterozygous; green: homozygous).

upon stimulation, and the levels of expression were comparable among C/C, C/A, and A/A littermates (Fig 3C and Supplementary Fig S8C).

Taken together, these data indicated that the C103A deubiquitinase function is not involved in limiting inducible NF- κ B activation following stimulation with LPS and TNF.

Response of A20-OTU knock-in mice to LPS shock

Both the C/C/ and the A/A littermates succumbed to LPS shock with similar kinetics (Fig 4A). Analysis of the serum levels of key acute-phase cytokines (TNF- α , IL-12, and IL-6) after LPS injection showed that while TNF- α peaked after 1 h, IL-6 and IL-12 peaked later, in agreement with previous studies [19]. However, there was no significant difference in the levels of the cytokines between WT and the A/A mice after LPS shock (Fig 4B). Thus, while A20 is important for restricting Myd88-mediated signaling *in vivo* and A20^{-/-} mice succumb to sublethal doses of LPS, the deubiquitinating function of A20 is not important in limiting inflammatory responses following LPS administration.

Similar activation of NF- κ B in BMDMs from both wild-type and homozygous mice

Before biochemically testing the activation of NF- κ B in C/C and A/A littermates, we first did an *in vitro* deubiquitinase assay incubating recombinant K48- or K63-linked polyubiquitin chains with immunoprecipitated A20 analogs (wild-type and C103A-A20) from LPS-stimulated BMDMs of littermates and showed that the C103A mutation eliminates deubiquitinase activity in A20. Our results clearly demonstrate that the C103A mutation eliminates DUB activity of A20, as C103A-A20 is unable to deubiquitinate either K48-linked or K63-linked ubiquitin chains (Supplementary Fig S9). However, wt-A20 can deubiquitinate both K48-linked and K63-linked ubiquitin chains in the *in vitro* DUB assay in agreement with previous results [15,16,20].

Next, we wanted to test the role of the A20 deubiquitinase activity in NF- κ B activation in BMDMs stimulated with TNF- α . We prepared nuclear fractions of BMDMs from C/C and A/A littermates to test NF- κ B binding to DNA by EMSA (Fig 5A). Both the WT and the A/A BMDMs showed elevated (but similar) binding of p65-p50

Figure 4. LPS shock of wild-type and homozygous littermates.

A Age- and sex-matched mice ($n = 5$; representative of 3 independent experiments) were given intraperitoneal injections of 50 $\mu\text{g/ml}$ LPS, and survival was scored every 6 h for 72 h.

B Serum cytokine levels of TNF- α , IL-6, and IL-12 in mice injected with 50 $\mu\text{g/ml}$ LPS were measured by ELISA for indicated time points. Error bars represent standard deviation representative of three independent experiments.

heterodimers to the DNA following stimulation. The nuclear extract isolated after stimulating the WT BMDMs for 30 min was used for the super-shift assay and analysis with the unlabeled probe. Western blotting also showed similar activation of NF- κB as tracked by degradation of I $\kappa\text{B}\alpha$. pJNK, p38, and pERK signaling were also found to be similar between wild-type and A/A littermates. As RIP1 in stimulated cells has been suggested to be deubiquitinated by A20, we immunoprecipitated RIP1 from stimulated cells and tested it for ubiquitination. Both the WT and the C103A-A20 were recruited to RIP1 in roughly equal amounts and with similar kinetics following TNF- α stimulation (Fig 5B), showing that protein structure and stability are unaffected by the mutation. Intriguingly, we also found roughly equal amounts of ubiquitinated RIP1 in the BMDMs of both genotypes following TNF- α stimulation. These results suggest that the deubiquitinase activity of A20 does not substantially affect RIP1 ubiquitination in cells.

Since A20 has also been reported to inhibit TNF-induced cell death [3,21], we treated BMDMs derived from C/C and A/A littermates with TNF- α . Staining with Annexin V and propidium iodide showed that cell death is similar in both the genotypes (Supplementary Fig S10). Hence, we conclude that the deubiquitinase activity of A20 is not responsible for inhibiting TNF- α -induced cell death.

Similar results were also obtained upon stimulation of BMDMs with LPS. Both the wild-type and the homozygous mutant showed similar activation of NF- κB as analyzed by EMSA (Fig 5C). The result is consistent with the kinetics of disappearance of I $\kappa\text{B}\alpha$ as analyzed by Western blotting (Fig 5D). Since TRAF6 has been shown to be deubiquitinated by A20 in response to stimulation with LPS, we immunoprecipitated TRAF6 from stimulated cells and found that the levels of ubiquitination (and K63-linked ubiquitination) of TRAF6 are more in BMDMs derived from A/A mice as compared to their wild-type littermates after stimulation with LPS.

This shows that while the Cys103 might play a role in deubiquitinating TRAF6, it does not play a role in inhibiting NF- κB . The kinetics and amounts of WT and the C103A-A20 recruited to TRAF6 were similar after stimulation with LPS (Fig 5D), showing the structural integrity of C103-A20 protein.

Conclusions

Our results are in agreement with a recent study that also found that mice lacking the deubiquitinating activity of A20 are grossly normal for at least 4 months and contain a normal number of lymphocytes [9]; however, older mice (6 months) develop splenomegaly and show increased number of myeloid cells. It is therefore possible that Cys103 plays an indirect role in older mice. However as our data show, it does not play a role in directly inhibiting NF- κB . In this regard, defects in the NF- κB pathway would manifest itself in much younger mice. For example, it is worth pointing out that complete knockouts of A20 are perinatally lethal from persistent inflammation resulting from NF- κB activation. These investigators also concluded, similar to our observations, that the deubiquitinase activity of A20 was not required for prevention of spontaneous cachexia and premature death. Additionally, while their study focused exclusively on the role of C103 in limiting TNF-induced NF- κB activation [9], our study examined the role of C103 in limiting LPS-mediated NF- κB activation. We feel this is an important distinction as it has been reported that A20 functions *in vivo* in limiting TLR/MyD88-dependent pathways [7,8]. Hence, our study focusing on the role of A20-C103 in LPS/TLR-mediated responses is vital for understanding the physiological role of the deubiquitinating property of A20 in restricting persistent TLR-mediated activation of NF- κB . The study, however, showed that RIP1 was slightly more ubiquitinated in

Figure 5. Biochemical analysis of NF-κB activation in wild-type and homozygous BMDMs.

- A** NF-κB binding to DNA in response to TNF-α stimulation was analyzed by electrophoretic mobility shift assay. EMSA was performed with nuclear extracts after stimulating BMDMs isolated from wild-type and homozygous littermates with 10 ng/ml of TNF-α for the indicated time points. The nuclear extract isolated at 30 min from wild-type BMDMs was used for the super-shift assay (with p65 and p50 antibodies) and analysis with the unlabeled κB probe.
- B** BMDMs isolated from wild-type and homozygous littermates were stimulated with 10 ng/ml TNF-α for the indicated time points. The lysates were immunoprecipitated with RIP1 and immunoblotted with indicated antibodies.
- C** EMSA was performed as in (A); cells stimulated with 1 μg/ml of LPS.
- D** BMDMs isolated from wild-type and homozygous littermates were stimulated with 1 μg/ml of LPS for the indicated time points. The lysates were immunoprecipitated with TRAF6 and immunoblotted with indicated antibodies.

TNF-stimulated cells in MEFs derived from mutant mice. We used BMDMs for our stimulation, and the minor difference might have resulted from cell-specific variations.

As our studies clearly demonstrate, the deubiquitinase activity of A20 is dispensable for its regulation of NF-κB signaling. This is not necessarily surprising, as if A20 were to play a profound role in limiting a tightly regulated process such as NF-κB activation, it would be expected that the deubiquitinating function of A20 would be specific for K63-linked ubiquitin chains. However, the deubiquitinase activity mediated by C103 has been shown to promiscuously cleave unanchored K11-, K48-, and K63-linked polyubiquitin chains in addition to disassembling K63-linked chains from *in vitro* substrates [7,10,15,16,20].

It is possible that the E3 ligase function of the ZnF4 domain could partially compensate for the lack of deubiquitinating function *in vivo*, even though A20 is thought to ubiquitinate substrates such as

RIP1 after first deubiquitinating them. A recent study using mice with A20 mutations demonstrated that neither the C103 residue nor the ZnF4 motif was exclusively responsible for all of A20's functions in restricting TNF signaling [9]. Such a model casts doubt on the singular importance of the deubiquitinase function of A20. As we know that the A20 protein is important in inflammatory signaling, we would also like to explore the possibility that A20 might play a role that does not involve ubiquitination/deubiquitination in exerting its regulatory function in inflammatory signaling. Alternative mechanisms might be important for the biological function of A20. For example, the seventh zinc-finger motif (ZnF7) of A20 was recently proposed to be involved in direct inhibition of IKK by a non-catalytic mechanism [22], perhaps by suppressing LUBAC-mediated NF-κB activation by binding to linear ubiquitin chains [23,24]. This hypothesis will need to be explored in greater depth in the future.

Materials and Methods

Generation of A20C103A knock-in mice

The A20C103A mice were generated using conventional gene-targeting approaches. Site-directed mutagenesis (Stratagene) was used to mutate the cysteine residue at position 103 to alanine, and the relevant part of the mutated A20 construct was transferred to the BAC using homologous recombination. The BAC construct harboring the Lox-Neo-Lox cassette along with the C103A mutation was retrieved into a pMCS_DTA plasmid (containing a diphtheria toxin selection gene), linearized with NotI, and electroporated into CSL2J2, albino C57BL/6J ES cells. Correctly screened ES cell clones were injected into blastocysts derived from C57BL/6 mice to give rise to chimaeras (in the Columbia University Transgenic Core facility). Genetic transmission of the allele was confirmed by PCR analysis and subsequent sequencing to confirm the presence of the C103A mutation. The LoxP-flanked neomycin sequences were deleted by crossing the transgenic mice with the EIIA-Cre deleter mice. The C/A heterozygous mice were bred to generate age-matched C/C wild-type and A/A homozygous mice for various experiments.

Cells

Bone marrow cells from 6- to 8-week age-matched wild-type (C/C), heterozygous (C/A), and homozygous (A/A) mice were differentiated into dendritic cells and macrophages in the presence of either GM-CSF or MCSF, respectively. In the *in vitro* experiments, BMDMs were stimulated with 10 ng/ml TNF or 1 µg/ml LPS.

Biochemical experiments

Immunoprecipitations and Western blotting were performed as has been described previously [10]. For the *in vitro* DUB assay, immunoprecipitated A20 was incubated 37°C with recombinant K48- or K63-linked polyubiquitin chains in 20 µl of DUB buffer (25 mM Hepes pH 7.4, 1 mM DTT, and 5 mM MgCl₂) for 1 h. Gel shift assay was done using the Li-cor EMSA kit as per the user manual. The κB probe was purchased from Li-cor, while the Oct1 probe was custom-made from Integrated DNA Technologies (IDT). Antibodies used in this study included an anti-murine RIP monoclonal (BD Bioscience, clone 610458), anti-ubiquitin (Santa Cruz, sc-8017), anti-IκBα (Santa Cruz, sc-371), anti-A20 (Imgenex, 161A), anti-β-tubulin (Abcam).

LPS shock

LPS-induced shock: LPS was injected intraperitoneally at a concentration of 50 mg/kg of body weight. The mice were monitored for survival every 8 h. In a separate experiment, the mice were bled at 1, 2, and 6 h after LPS treatment, and the serum cytokine levels were measured by ELISA [19].

Flow cytometry, ELISA, and qRT-PCR

Cell preparations and flow cytometric and ELISA analyses were performed as previously described [19,25]. ELISA was performed with kits from BD Biosciences. Cells were analyzed by flow cytometry using LSRII and FlowJo software (Tree Star). For qRT-PCR,

BMDMs were stimulated and RNA was isolated using the RNA Easy kit (Qiagen). RNA was reverse-transcribed (SuperScriptIII reverse transcriptase; Invitrogen-Life Technologies), and SYBR Green master mix (QuantiTect SYBR green; Invitrogen) was used to quantify relative gene expression of the corresponding mRNA with normalization to β-actin [19].

Supplementary information for this article is available online:

<http://embor.embopress.org>

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Author contributions

AD and TD generated the knock-in mice, AD and CVR conducted the experiments. AD, CVR and SG analyzed the data, AD and SG wrote the manuscript. SG conceived the research and experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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