Acquired Deficiency of A20 Results in Rapid Apoptosis, Systemic Inflammation, and Abnormal Hematopoietic Stem Cell Function

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Abstract

A20 is a negative regulator of NF-κB, and mutational loss of A20 expression is involved in the pathogenesis of autoimmune diseases and B-cell lymphomas. To clarify the role of A20 in adult hematopoiesis, we generated conditional A20 knockout mice (A20flox/flox) and crossed them with Mx–1Cre (MxCre+) and ERT2Cre (ERT2Cre+) transgenic mice in which Cre is inducibly activated by endogenous interferon and exogenous tamoxifen, respectively. A20flox/flox MxCre+ (A20Mx) mice spontaneously exhibited myeloid proliferation, B cell apoptosis, and anemia with overproduction of pro-inflammatory cytokines. Bone marrow transplantation demonstrated that these changes were caused by hematopoietic cells. NF-κB was constitutively activated in A20Mx hematopoietic stem cells (HSCs), which caused enhanced cell cycle entry and impaired repopulating ability. Tamoxifen stimulation of A20flox/flox ERT2Cre (A20ERT2) mice induced fulminant apoptosis and subsequent myeloproliferation, lymphocytopenia, and progressive anemia with excessive production of pro-inflammatory cytokines, as observed in A20Mx mice. These results demonstrate that A20 plays essential roles in the homeostasis of adult hematopoiesis by preventing apoptosis and inflammation. Our findings provide insights into the mechanism underlying A20 dysfunction and human diseases in which A20 expression is impaired.

Introduction

NF-κB plays fundamental roles in various physiological and pathological processes, such as immunity, apoptosis, inflammation, and cancer [1,2,3]. In an unstimulated state, NF-κB is sequestered in the cytoplasm by binding to IκB proteins. Upon activation by external stimuli, IκB proteins are phosphorylated by the IκB kinase (IKK) complex and then degraded by ubiquitination. NF-κB is released and translocates to the nucleus where it drives the expression of target genes [1,2,3].

A20, also known as tumor necrosis factor alpha-induced protein 3 (TNFAIP3), now emerges as a major negative regulator of NF-κB signaling [4,5]. A20 comprises an ovarian tumor (OTU) domain at its N-terminus and seven Zn-finger motifs. The OTU domain is predicted to have deubiquitinating protease activity, and the Zn finger motifs possess E3 ubiquitin ligase and ubiquitin-binding activities [4,5]. Thus, A20, acting as a ubiquitin-modifying protein, may participate in a negative feedback loop controlling NF-κB signaling [4,5]. The most compelling evidence that A20 plays an essential role in inhibiting inflammation are results of a gene knockout experiment in which A20 deficient mice prematurely died because of severe systemic inflammation and cachexia [6].

A20 is involved in various human diseases, including hematopoietic malignancies. Frequent loss of A20 expression in B-cell lymphomas caused by biallelic deletions and/or point mutations [7,8] indicates that A20 functions as a tumor suppressor in the hematopoietic system. Moreover, single nucleotide polymorphisms in A20 are associated with autoimmune and inflammatory diseases, such as systemic lupus erythematosus (SLE) [9,10,11], rheumatoid arthritis (RA) [12,13], and Crohn’s disease [14].

An approach to determine whether there is a causative association between A20 mutations and pathogenesis employs mice to target A20 in a tissue-specific manner. A number of A20 conditional knockout (cKO) mice have been generated for this purpose. For example, B cell-specific deletion of A20 using a CD19–Cre transgene results in hyper-responsiveness of B cells and
causes autoimmune disease similar to SLE [15,16,17]. Deletions of A20 from dendritic or myeloid cells using CD11c–Cre or Ly5.1M–Cre transgenes, respectively, also induced autoimmune disease. The former exhibited an SLE-like phenotype [18], and the latter developed an RA-like disease [19]. Moreover, Vinha–Cre transgenic mice harboring a deletion of A20 from their epithelial intestinal cells showed susceptibility to dextran sulfate-induced colitis [20].

Although these studies provide important insights into the role of A20 as a suppressor of tumorigenesis and autoimmunity, its role(s) in the normal functioning of the hematopoietic system of adults remains to be determined. To address this issue, we created mice in which A20 expression can be inducibly and preferentially ablated in hematopoietic cells.

Materials and Methods

Mice

The detailed procedures for constructing the targeting vector and generating the A20floxflox are described in Text S1. A20 cKO mice have been deposited in RIKEN BioResource Center (http://www.brc.riken.jp/en/en/index.shtml, RBBR05494). A20floxflox mice were crossed with Ly5.2–Cre (Ly5.2×Cre) transgenic mice [21] and ERT2Cre (ERT2Cre) transgenic mice (C57BL/6-Gr(Rosa)26Sor1 Cre/+ × ERT2Cre mice, purchased from Taconic) to generate A20floxflox × ERT2Cre and A20floxflox × Cre mice, respectively. Mice backcrossed with the C57BL/6-Ly5.2 background at least seven times were used here. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Hiroshima University Animal Research Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Hiroshima University (Permit Number: A13-13). All mice were maintained according to the guidelines of the Institute of Laboratory Animal Science of Hiroshima University, all surgeries were performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

Western Blotting, Flow Cytometry, and Histopathology

Western blotting, flow cytometry, and histopathology were performed as previously described [22,23,24]. Antibodies and a staining kit used in these analyses are listed in Table S1.

Measurement of Serum Cytokine Concentration

Concentrations of pro-inflammatory cytokines (TNF-α, IFN-γ, GM-CSF, IL-1β, and IL-6) were measured using a BD Cytometric Bead Array Flex Set Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions.

 Colony Formation Assay

The colony formation assay was performed as previously described [25].

Bone Marrow Transplantation (BMT), Competitive Repopulation, and Cell Cycle Analyses

Transplantation of bone marrow (BM) cells, competitive repopulation, and short-term BrdU incorporation assays were performed as previously described [24].

Results

A20floxflox MxCre+ Mice Exhibited Severe Inflammation, B Lymphocyte Apoptosis, and Premature Death

To conditionally ablate A20 function, we generated mice in which exon 3 of A20 was flanked by two loxP sites (A20floxflox, Fig. S1A and S1B). To examine the role of A20 in hematopoietic homeostasis, we crossed A20floxflox with Ly5.2–Cre transgenic (Ly5.2Cre) mice in which Cre is placed under the control of IFN-responsive Mx–I promoter [21]. Lack of A20 expression in A20floxflox × Cre mice was confirmed by western blotting of spleen extracts prepared from A20floxflox × Cre mice and A20floxflox × ERT2Cre mice (hereafter referred to as control and A20Mx mice, respectively) using an anti-A20 antibody (left panel of Fig. S1C).

Although A20Mx mice were apparently normal at birth, they exhibited spontaneous emaciation and cachexia without stimulation by polyinosinic:polycytidylic acid (pIpC), which is a strong and transient inducer of IFN, and most mice died within six months after birth (Fig. 1A). Hematological analysis of moribund mice revealed anemia, proliferation of myeloid cells, and reduction of B lymphoid cells in the peripheral blood (PB) (Table S2). The macroscopic appearance of the mice was uniformly characterized by massive hepatomegaly and enlarged spleens (indicated by an arrowhead and an arrow, respectively, in the left panel of Fig. 1B), which were frequently associated with lymph node (LN) swelling (Table S2). Pathological analysis revealed infiltration of the lung and liver by hematopoietic cells (indicated by arrows in the right top and middle panels of Fig. 1B), formation of granulomas in the liver (indicated by an arrowhead in the right middle panel of Fig. 1B), and destruction of spleen architecture caused by the proliferation of white blood cells (indicated by a white arrowhead in the right bottom panel of Fig. 1B). Higher magnification of the spleen showed that the most of the proliferated cells were with segmented or multi-lobulated nuclei, strongly suggesting that these cells were of myeloid in origin (indicated by arrowheads in the left panel of Fig. S2).

We then analyzed the time-dependent changes in hematopoietic parameters in control and A20Mx mice. The white blood cell count (WBC), hemoglobin concentration (Hgb), and platelet (Plt) number in the PB, and the absolute numbers of B lymphoid (B220+), T lymphoid (Thy1.2+), and myeloid (Mac1+Gr1+) cells in the PB and spleen were analyzed at three and six months after birth. Although no significant difference was observed in the WBC count and Plt number between the two groups, A20Mx mice exhibited progressive anemia (top panels of Fig. 1C). In addition, lineage analysis of WBCs in the PB and spleen revealed that the numbers of myeloid cells were significantly increased, whereas those of B lymphoid cells were significantly decreased in A20Mx mice compared with control mice (middle and bottom panels of Fig. 1C). To clarify the mechanism of B-cell reduction in A20Mx mice, spleens of A20Mx mice and control littermates were subjected to and anti-B-cell and TUNEL double staining. As shown in Fig. S3, a significant portion of B lymphocytes in the A20Mx spleen were positive for TUNEL, indicating that the B cell reduction in A20Mx mice was due to apoptosis.

Because A20 inhibits NF-κB signaling and suppresses inflammatory pathway activity [4,5], we reasoned that the aforementioned findings were caused by sustained inflammatory responses. Therefore, serum concentrations of pro-inflammatory cytokines, including TNF-α, IFN-γ, GM-CSF, IL-1β, and IL-6, were measured and compared between the two groups. The concentrations of these cytokines were higher in A20Mx mice than in control mice, and those of TNF-α, IFN-γ, and GM-CSF were significantly increased (Fig. 1D). These results indicate that...
cytokine-mediated inflammation induced the proliferation of myeloid cells and other hematopoietic abnormalities, which subsequently damaged internal organs and eventually caused premature death of A20Mx mice.

Transfer of the Aberrant A20Mx Hematopoietic Phenotype to Naïve Mice

To determine the cellular origin of the severe inflammation observed in A20Mx mice, we performed BMT assays. Mononuclear BM cells derived from control and A20Mx mice were transplanted into lethally irradiated syngeneic recipient mice (hereafter, mice transplanted with control and A20Mx BM cells are referred to as control and A20Mx BMT mice, respectively). One month after BMT, all A20Mx BMT mice became moribund and were sacrificed to assess hematopoietic and pathological changes. Macroscopically, A20Mx BMT mice exhibited thymic atrophy and splenic enlargement (indicated by an arrowhead and an arrow, respectively, in the right panel of Fig. 1B). Pathological analysis revealed marked infiltration of hematopoietic cells in the lung and liver (indicated by arrows and arrowheads, respectively) and massive proliferation of the same cell types in the spleen (Fig. 1D). Hematopoietic analysis showed elevated WBC count, reduced Hgb concentration, and decreased Plt number in A20Mx BMT mice (top panel of Fig. 2B). Lineage analysis of the PB and spleen revealed a significant increase in the numbers of myeloid cells and a significant decrease in the numbers of B and T lymphoid cells in both tissues (middle and bottom panels of Fig. 2B). The abnormal phenotypes of A20Mx BMT mice were similar to those of A20Mx mice, indicating that the inflammatory changes detected in A20Mx mice were caused by hematopoietic cells.

Impaired Repopulation Ability, Enhanced Cell Cycle Entry, and Constitutive NF-κB Activation in Hematopoietic Stem Cells of A20Mx Mice

We then investigated whether the abnormal phenotypes of A20Mx and A20Mx BMT mice were induced by hematopoietic stem cells (HSCs). HSCs are usually defined as “lineage marker (Lin)-, Sca-1+ and c-Kit+ (LSK)” cells. However, in A20Mx mice,
this method is not appropriate, since serum concentration of IFN-\(\gamma\) is elevated and the expression level of Sca-1 was reported to be up-regulated by IFNs [26]. Thus, to isolate HSCs, we used other markers, “Lin\(^-\), CD48\(^-\), CD150\(^+\)”, called as SLAM-code, which distinguishes HSCs from progenitor cells [27].

HSCs isolated from control and A20Mx mice (Ly5.2\(^+\)) were transplanted into lethally irradiated Ly5.1\(^+\) recipients together with Ly5.1\(^+\) BM MNCs as competitors (hereafter the recipient mice transplanted with control and A20Mx HSCs are referred to as control and A20Mx HSC BMT mice, respectively). Interestingly, in contrast that the percentage of Ly5.2\(^+\) cells in the PB of control HSC BMT mice increased after transplantation and accounted for approximately 40% of WBCs at two months, that of A20Mx HSCs BMT mice was significantly less and did not reach 10% of WBCs during the observation period (Fig. 3A). These results indicate severely impaired repopulating ability of HSCs in A20Mx mice and suggest abnormal proliferation/differentiation status and cell cycle kinetics of A20Mx HSCs.

To address this issue, HSCs isolated from control and A20Mx mice were subjected to cell counting and short-term 5-bromo-2\(^\text{9}\)-deoxyuridine (BrdU) uptake assay. As shown in Fig. 3B, although the absolute number of HSCs was considerably decreased in A20Mx mice as compared to control mice, BrdU uptake by A20Mx HSCs was significantly higher than that of control HSCs, indicating that A20Mx HSCs entered the cell cycle at a higher rate.

A20 is a negative regulator of NF-\(\kappa\)B [4,5] and NF-\(\kappa\)B activation is detected by its translocation from the cytoplasm to the nucleus [1,28]. To investigate whether NF-\(\kappa\)B activation underlies the abnormal behaviors of HSCs, the intracellular localization of NF-\(\kappa\)B in HSCs of control and A20Mx mice were examined by immunofluorescently staining with an anti-NF-\(\kappa\)B antibody. In contrast that NF-\(\kappa\)B signals in control HSCs were mainly observed in the cytoplasm, those in A20Mx HSCs were significantly detected in the nucleus (Fig. 3C). These results indicate that NF-\(\kappa\)B was constitutively activated in A20Mx HSCs, which caused increased cell cycle entry, perturbed their stemness and eventually impaired the repopulating activity.

**Rapid Apoptosis and Subsequent Anemia, Myeloid Proliferation, and Reduced Numbers of Lymphocytes Induced by Acquired A20 Deficiency in Hematopoietic Cells**

Because the expression of Cre in Mx\(\text{Cre}\) mice was controlled by the IFN-responsive element [21], the activity of Cre was very likely influenced by endogenous IFN. Therefore, we used an \(\text{ERT2Cre}\) transgenic (\(\text{ERT2Cre}\)) system in which Cre is fused to a mutated estrogen receptor and can only be activated by exogenously administered tamoxifen. For this purpose, we crossed \(\text{A20flox/flox}\) mice with \(\text{ERT2Cre}\) mice and generated \(\text{A20flox/flox ERT2Cre}\) mice (hereafter referred to as \(\text{A20ERT2}\) mice). \(\text{A20flox/flox ERT2Cre}\) littermates were used as controls. \(\text{A20ERT2}\) mice did
Figure 3. Analysis of HSCs in control and A20Mx mice. (A) The percentages of Ly5.2+ cells in the PB of control and A20Mx BMT mice at 1 and 2 months after BMT are shown. **p < 0.01 (Student’s t-test). (B) Cell number and BrdU incorporation in control and A20Mx HSCs. Representative results of flow cytometry is shown in the left panels and the HSC number and percentage of BrdU-positive cells are shown in the right column. **p < 0.01 (Student’s t-test). (C) Intracellular localization of NF-κB in control and A20Mx HSCs. Immunofluorescence staining of NF-κB and percentages of the NF-κB-positive area.
not spontaneously develop abnormal phenotypes in contrast to A20Mx mice. However, after tamoxifen administration, A20ERT2 mice became rapidly moribund, exhibited a marked decrease in the number of hematopoietic cells and died within several days (not shown). Macroscopical examinations revealed that the thymus was atrophic, the spleen was pale, and liver had white spots (not shown). Pathological analysis revealed that massive apoptosis occurred in major organs, including hematopoietic tissues such as the thymus, spleen, liver, and BM (Fig. S4A). Microemboli and necrotic areas were also observed in the liver (indicated by an arrow and arrowheads in Fig. S4A), suggesting that the white spots in the liver were caused by ischemic necrosis. The measurement of pro-inflammatory cytokines revealed that the concentrations of these cytokines were higher in A20ERT2 mice than in control mice, and those of TNF-α, IFN-γ, and IL-6 were significantly increased (Fig. S4B). These results indicate that loss of A20 in adult mice induced fulminant apoptosis, possibly via rapid elevation of pro-inflammatory cytokines.

We next investigated the effect of deleting A20 from hematopoietic cells by transplanting control and A20ERT2 BM cells into lethally irradiated syngeneic mice and then administering tamoxifen (mice transplanted with A20ERT2 BM cells are hereafter referred to as A20ERT2 BMT mice). Before tamoxifen stimulation, the number of transplanted Ly5.2+ cells was comparably increased in control BMT and A20ERT2 BMT mice, and there was no significant difference in the values of hematopoietic parameters between them (not shown). Tamoxifen was administered eight weeks after transplantation [29] (Fig. 4A), and two days later, all A20ERT2 BMT mice became moribund with marked decrease in WBC count and Plt number in the PB ("2 days" at top panels of Fig. 4A).

Tamoxifen-treated A20ERT2 BMT mice survived this stage, in contrast to A20ERT2 mice that died after several days after tamoxifen administration, probably because there were no severe apoptotic changes in the non-hematopoietic tissues. Examination of PB parameters showed that A20ERT2 BMT mice exhibited progressive anemia, proliferation of myeloid cells, and reduction in lymphocyte number, particularly that of B cells, as observed in A20Mx and A20Ms BMT mice (bottom panels of Fig. 4A). The analysis of the spleen four months after tamoxifen stimulation showed a marked reduction of B and T lymphocytes and a significant increase of myeloid cells (Fig. 4B).

To further investigate whether the changes in the hematopoietic cell population in tamoxifen-treated A20ERT2 BMT mice were caused by HSCs, competitive repopulation assays were performed. Unlike to the MxCre system, the ERT2Cre system does not respond to endogenous IFNs and no hematological abnormalities were found in the unstimulated state, we used LKS markers to isolate HSCs. LSK cells isolated from control and A20ERT2 mice were transplanted to the recipient mice and tamoxifen was administered 20 days after transplantation [30]. The recipient mice transplanted with A20ERT2 LSK cells (hereafter referred to as A20ERT2 LSK BMT mice, Fig. 5A). While control LSK BMT mice were healthy during the observation period, A20ERT2 LSK BMT mice became gradually emaciated and were sacrificed at 3.5 months after transplantation. The mice showed thymic atrophy and splenomegaly (not shown), and hematopoietic analysis demonstrated anemia and decreased numbers of Ly5.2+ lymphocytes in the PB, elevation of myeloid cell number, and reduction of lymphoid cell numbers in the total and Ly5.2+ fractions of the spleen (Fig. 5A and 5B). These abnormalities were similar to those observed in A20ERT2 BMT as well as A20Mx and A20Ms BMT mice. Therefore, we conclude that the abnormalities observed in A20ERT2 LSK BMT mice can be primarily attributed to the HSCs. Moreover, these abnormalities were found not only in the donor (Ly5.2+) cells but also in the competitor cells (Ly5.1+), indicating that the changes were propagated by cell-cell contact and/or humoral factors, such as pro-inflammatory cytokines.

### Discussion

A20 plays an essential role in inflammation, immune system, apoptosis, and tumor suppression [4,5]. The biological roles of A20 in development and in individual tissues have been analyzed using A20 KO mice [6] and A20 cKO mice crossed with various Cre transgenic mice [15,16,17,18,19,20,31], respectively. However, to the best of our knowledge, the effect of acquired A20 deficiency on the adult hematopoietic system has not been reported. To address this issue, we generated A20 cKO mice and crossed them with two different inducible Cre transgenic mice; Mx1-Cre and ERT2Cre transgenic mice in which Cre is activated in response to endogenous IFN or exogenous tamoxifen, respectively.

A20flox/flox MxCre<sup>+</sup> (A20Mx) mice gradually debilitated and died several months after birth (Fig. 1A) and hematopoietic, pathological, and serological analyses revealed massive inflammatory changes in these mice (Fig. 1B-D). These images of the lung and liver (Fig. 1B), strongly suggest that the mice died of respiratory failure and/or liver dysfunction due to excessive infiltration of hematopoietic cells. Because the Mx-1 promoter is an IFN-responsive element, it is likely that cells that responded to endogenous IFNs, such as macrophages and lymphocytes, lost the A20 gene, did not attenuate IFN-mediated signaling, and continuously produced pro-inflammatory cytokines, which eventually caused severe systemic inflammation and premature death.

To determine whether the phenotypes of A20Ms mice could be primarily attributed to hematopoietic cells, BMT assays were performed. Mice transplanted with A20Mx BM cells exhibited abnormal phenotypes similar to those of A20Ms mice, indicating that these changes were induced by hematopoietic cells (Fig. 2). We also determined the ability of hematopoietic cells of control and A20Ms mice to form colonies (Fig. S5). When cultured in vitro with SCF+IL3+Epo or SCF+IL7, hematopoietic cells from control and A20Ms mice formed similar numbers of colonies with similar morphology. Thus, the hematopoietic abnormalities observed in A20Ms mice were mainly attributed to excessive production of pro-inflammatory cytokines and systemic inflammation.

To further investigate the effect of A20 deficiency on HSC activity, we performed competitive repopulation assays in which Lin<sup>−</sup>, CD48<sup>−</sup>, CD150<sup>−</sup> HSCs cells in control or A20Mx mice (Ly5.2) were transplanted into recipients with Ly5.1 competitors. Interestingly, the repopulation ability of A20Mx HSCs was significantly impaired as compared to that of control HSCs (Fig. 3A). In addition, BrdU incorporation assays showed an enhanced proliferative ability of A20Ms HSCs (Fig. 3B). Furthermore, NF-κB was activated in a substantial portion of A20Ms LT-HSCs as revealed by its presence in the nucleus (Fig. 3C). These results indicated that the loss of A20 in the hematopoietic system activated HSCs, entered them into the cell cycle, and eventually impaired the repopulation ability. Quiescence is an important...
Figure 4. Analysis of control and A20ERT2 BMT mice. (A) Time schedule of BMT, tamoxifen ip, blood sampling, and time-dependent changes in hematopoietic parameters in the PB. **p<0.01 (Student's t-test). (B) Percentages of Thy1.2+, B220+, and Mac1+/Gr1+ cells in the spleen 4 M after transplantation. **p<0.01 (Student's t-test). doi:10.1371/journal.pone.0087425.g004
Figure 5. Analysis of control and A20ERT2 LSK BMT mice. (A) Time schedule of BMT, tamoxifen ip, blood sampling, and time-dependent changes of hematopoietic parameters and percentages of Ly5.2+ cells in the PB. *p<0.05 and **p<0.01 (Student’s t-test). (B) Percentages of different hematopoietic compartments and the population of Ly5.2+ cells in the spleen 3.5 M after transplantation. **p<0.01 (Student’s t-test). doi:10.1371/journal.pone.0087425.g005
characteristic for HSCs to maintain their stemness and engraftment activity. Our findings in this regard are consistent with those of previous studies demonstrating that cycling HSCs lose its repopulating ability [32,33,34]. Whether HSCs sense and directly respond to inflammation is controversial [35,36]. Our results suggest that HSCs could be a direct target of systemic inflammation and support the idea that TNF-α and IFN-γ are major regulators of inflammatory responses of HSCs, as documented in previous studies [34,37].

Next, we used ERT2Cre mice that respond to exogenously administered tamoxifen but not to endogenous estrogens. As mimics B-cell lymphomas harboring A20 defects, A20 function must be abrogated in early progenitor cells to induce apoptosis in adult tissues. There is evidence that the interaction of cytokines and inflammatory cytokines with HSCs, as major regulators of inflammatory responses of HSCs, act as a direct target of systemic inflammation, such as TNF-α, and induce apoptosis. Our findings in this regard are consistent with those of previous studies demonstrating that cycling HSCs lose its stemness and engraftment activity. In summary, we generated mice in which A20 can be inducibly and preferentially deleted from hematopoietic cells and found that acquired loss of A20 induced fulminant apoptosis and subsequent systemic inflammation. Our results demonstrate the essential role of A20 in the maintenance of adult hematopoietic homeostasis and provide insights into the mechanism responsible for A20 dysfunction and human diseases with A20 mutations.

Supporting Information

Figure S1 Generation of A20 conditional knockout mice. (A) Targeting strategy. Exon 3 of mouse A20 was floxed and the FRT-flanked Neo-resistance gene was removed using Fsp recombinase. The positions of a 5′ probe for Southern blotting and P1 and P2 primers for 5′ genomic PCR analysis are shown. Restriction sites: Bsa, BamHI; Sac, SacI; Stu, StuI; Sal, SalI; Bg, BglII. (B) Southern blotting and genomic PCR using a 5′ probe and 3′ primers, respectively, to detect homologous recombination in two independent ES clones (#1 and #2). Germline (GL) and targeted allele-derived bands are indicated by arrows (left panel), and the recombination-specific PCR product is indicated by an arrowhead (right panel). (C) Western blotting of A20 expression in A20lox/lox MxCre− and A20lox/lox MxCre+ mice. Proteins extracted from the spleens of LPS-stimulated A20lox/lox MxCre− and A20lox/lox MxCre+ mice were blotted and probed with anti-A20- (upper panel) or anti-β-actin antibodies (lower panel). The position of A20 is indicated by an arrow. (PDF)

Figure S2 Proliferation of mature myeloid cells in the spleen of A20lox/lox and A20lox/lox BMT mice. Higher magnification of HE-stained sections of the spleen of A20lox/lox and A20lox/lox BMT mice. Mature myeloid cells with segmented or multi-lobulated nuclei are indicated by arrowheads. (PDF)

Figure S3 Apoptosis of B cells. Representative results of double staining with an anti-B cell antibody and TUNEL in control and A20lox/lox spleens (three weeks old). Blue and brown staining patterns show B and apoptotic cells, respectively. The boxed areas in the upper panels are magnified in the lower panels. (PDF)

Figure S4 Analysis of control and A20ERT2 mice. (A) HE-stained A20ERT2 tissues that show severe apoptosis. Microemboli and necrotic areas in the liver are indicated by arrowheads and an arrow, respectively. $ below standard range and out of invertable range. (B) Serum concentrations of pro-inflammatory cytokines. $p<0.05 and **p<0.01 (Student’s t-test). $ below standard range and out of invertable range. (PDF)

Figure S5 Colony formation assay. (A) The colony numbers generated in the presence of SCF+IL3+Epo, and those generated with SCF+IL7 are shown. No significant difference was observed between control and A20lox/lox mice. (B) Representative images of colonies. Colonies derived from both types of mice are similar in size and shape. (PDF)
Table S1 Antibodies. All the antibodies used in this study are listed.

(PDF)

Table S2 Characteristics of A20Mx mice. Hematopoietic parameters of moribund A20Mx mice are described.

(PDF)

Text S1 Construction of a targeting vector and generation of A20 knockout mice. Detailed procedure of Construction of a targeting vector and generation of A20 knockout mice is described.

(PDF)

References

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Author Contributions
Conceived and designed the experiments: AN ZH TS TI HH. Performed the experiments: AN TU NY YE K. Tsuji K. Takubo HO HH. Analyzed the data: AN YN TU NY YE K. Tsuji HO HH. Wrote the paper: TU YE K. Takubo HH.