Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis

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Rheumatoid arthritis is a chronic autoinflammatory disease that affects 1–2% of the world’s population and is characterized by widespread joint inflammation. Interleukin-1 is an important mediator of cartilage destruction in rheumatic diseases1, but our understanding of the upstream mechanisms leading to production of interleukin-1 in rheumatoid arthritis is limited by the absence of suitable mouse models of the disease in which inflammasomes contribute to pathology. Myeloid-cell-specific deletion of the rheumatoid arthritis susceptibility gene A20 in mice triggers a spontaneous erosive polyarthritis that resembles rheumatoid arthritis in patients2. Rheumatoid arthritis in A20myel-KO mice is not rescued by deletion of tumour necrosis factor receptor 1 (ref. 2). Here we show, however, that it crucially relies on the Nlrp3 inflammasome and interleukin-1 receptor signalling. Macrophages lacking A20 have increased basal and lipopolysaccharide-induced expression levels of the inflammasome adaptor Nlrp3 and proIL-1β. As a result, A20-deficiency in macrophages significantly enhances caspase-1-mediated caspase-1 activation, pyroptosis and interleukin-1β secretion by soluble and crystalline Nlrp3 stimuli. In contrast, activation of the Nlr4 and AIM2 inflammasomes is not altered. Importantly, increased Nlrp3 inflammasome activation contributes to the pathology of rheumatoid arthritis in vivo, because deletion of Nlrp3, caspase-1 and the interleukin-1 receptor markedly protects against rheumatoid-arthrits-associated inflammation and cartilage destruction in A20myel-KO mice. These results reveal A20 as a novel negative regulator of Nlrp3 inflammasome activation, and describe A20myel-KO mice as the first experimental model to study the role of inflammasomes in the pathology of rheumatoid arthritis.

A20 was deleted in myeloid cells by crossing A20lox/lox mice into lysosome M (LysM)-Cre-recombinase-expressing mice. Unlike wild-type macrophages, A20myel-KO macrophages failed to induce A20 messenger RNA (mRNA) and protein expression in response to the Toll-like receptor-4 (TLR4) ligand lipopolysaccharide (LPS) (Fig. 1a, b), demonstrating the effectiveness of LysM-driven deletion of A20 in myeloid cells. Arthritis development in A20myel-KO mice was shown to be independent of tumour necrosis factor receptor 1 (TNF-R1), whereas deletion of MyD88 markedly protected against pathology of rheumatoid arthritis2. As this signalling adaptor operates downstream of both TLRs and interleukin-1 receptor (IL-1R), we crossed Il1r−/− mice into A20myel-KO mice to assess the contribution of IL-1 signalling to arthritis pathogenesis. As expected, wild-type mice (A20lox/loxIl1r−/−) did not develop arthritis, whereas A20myel-KO mice spontaneously developed an arthritic phenotype (Fig. 1c). The incidence of A20myel-KO mice developing arthritis was 100% (Fig. 1d). In sharp contrast, A20myel-KOIl1r−/− mice were virtually devoid of clinical signs of arthritis (Fig. 1c, d). In agreement, clinical scoring of disease severity confirmed A20myel-KOIl1r−/− mice as developing severe arthritic disease (high clinical score) whereas A20myel-KOIl1r−/− mice were markedly protected (clinical score 0). A20myel-KO littermates that were heterozygous for IL-1R1 expression showed an intermediate arthritic phenotype between those of A20myel-KOIl1r+/+ and A20myel-KOIl1r−/− mice (Fig. 1d, e). These clinical assessments were supported by a histological examination of representative ankle joints. Tissue sections of diseased A20myel-KOIl1r−/− mice stained by haematoxylin and eosin showed significant synovial and periarticular inflammation and high levels of infiltrated mononuclear cells, which was associated with extensive cartilage and bone destruction (Fig. 1f). In marked contrast, ankle joints of A20myel-KOIl1r−/− littermates were strongly protected from arthritis histopathology and contained significantly reduced numbers of infiltrating inflammatory cells (Fig. 1f). Semi-quantitative scoring of these histological parameters confirmed that the severity of arthritis was substantially lower in A20myel-KOIl1r−/− mice relative to A20myel-KOIl1r+/− littermates (Fig. 1g and Extended Data Table 1). These results demonstrate that IL-1 production is detrimental for arthritis pathogenesis in mice with a myeloid cell-restricted deletion in A20.

Macrophages are a prime source of proIL-1β, and generally depend on caspase-1 for maturation and secretion of the biologically active cytokine. Caspase-1 is produced as a cytosolic zymogen, the activation of which is controlled by different inflammasomes3. To study the role of A20 in inflammasome signalling, we assessed caspase-1 processing in bone-marrow-derived macrophages (BMDMs) of wild-type and A20myel-KO mice. Notably, caspase-1 activation was substantially increased in LPS-primed A20myel-KO macrophages that were treated with soluble (ATP and nigericin) or crystalline (silica) stimuli of the Nlrp3 inflammasome compared with wild-type BMDMs (Fig. 2a). Concurrently, the levels of secreted IL-1β in the culture medium of ATP- and nigericin-treated A20myel-KO macrophages were about twice those of wild-type cells, and silica triggered nearly four times higher levels of secreted IL-1β (Fig. 2b). Enhanced caspase-1 autoprocessing (Fig. 2c, d) and IL-1β secretion (Fig. 2e, f) by the Nlrp3 inflammasome was evident within 10 min after ATP or nigericin addition, and continued to increase in a time-dependent fashion. Similarly, the induction of caspase-1-dependent pyroptosis was enhanced in A20myel-KO macrophages (Fig. 2g, h). Despite their hypersensitivity for Nlrp3 inflammasome activation, A20myel-KO macrophages failed to process caspase-1 and secrete IL-1β and IL-18 when treated with LPS, ATP or nigericin alone (Extended Data Fig. 1). The increased responsiveness of A20myel-KO macrophages towards inflammasome activation was restricted to the Nlrp3 inflammasome because caspase-1 processing and pyroptotic cell death by the Nlrc4 inflammasome were similarly induced in wild-type and A20myel-KO macrophages that had been infected with Salmonella enterica serovar Typhimurium (Fig. 2i, j). Similarly, stimulation of the AIM2 inflammasome with cytosolic double-stranded DNA (dsDNA) did not result in differential levels of caspase-1 processing and pyroptosis induction in wild-type and A20myel-KO macrophages (Fig. 2k, l). It is worth noting, however, that despite normal caspase-1 activation and pyroptosis levels in response

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to S. enterica serovar Typhimurium infection and dsDNA transfection, secretion of IL-1β in response to these treatments was consistently higher in A20\(^{myel-KO}\) macrophages compared with wild-type controls (Fig. 2m, n), which could be explained by increased induction of proIL-1β mRNA in A20\(^{myel-KO}\) macrophages (data not shown). Together, these results demonstrate that A20 negatively regulates activation of caspase-1 by the Nlrp3 inflammasome, but not by the Nlr4 and AIM2 inflammasomes.

Activation of the Nlrp3 inflammasome in wild-type macrophages is tightly regulated at different levels. A priming signal (referred to as step 1 and usually provided by TLRs) upregulates Nlrp3 expression levels along with the inflammasome substrate proIL-1β via the pro-inflammatory transcription factor NF-kB. A20 negatively regulates LPS-induced NF-kB activation (refs 5–8 and Extended Data Fig. 2a), which was also reflected in increased secretion of the NF-kB-dependent cytokines IL-6 and TNF in A20\(^{myel-KO}\) macrophages (Extended Data Fig. 2b, c). We further showed A20-deficiency to markedly enhance LPS-induced mRNA expression levels of Nlrp3 (Fig. 3a) and proIL-1β (Fig. 3b). In contrast, LPS-induced transcript levels of caspase-1 and the inflammasome adaptor ASC were respectively mildly upregulated and normal in A20\(^{myel-KO}\) macrophages (Extended Data Fig. 3a, b). Analysis of protein expression levels confirmed Nlrp3 and proIL-1β to be significantly higher in LPS-primed A20\(^{myel-KO}\) macrophages than wild-type cells, whereas caspase-1 and ASC were not differentially modulated in the two genotypes (Fig. 3c).

TLR stimulation during brief time intervals (10 min or less) was recently shown to license activation of the Nlrp3 inflammasome independently of new protein synthesis\(^ {6,12}\). Rapid Nlrp3 inflammasome activation resulted in procaspase-1 processing and secretion of pre-synthesized proIL-18 in the absence of the NF-kB-dependent cytokines IL-1β, TNF and IL-6 (refs 9–11). To address whether A20 modulated rapid Nlrp3 inflammasome activation, cells were exposed to ATP or nigericin after being primed with LPS for 10 min. As reported\(^ {6,12}\), wild-type BMDMs activated caspase-1 (Fig. 3d) and secreted significant amounts of IL-18, but not IL-1β, TNF or IL-6 (Fig. 3e). Moreover, we noted Nlrp3 protein levels were lowered after stimulation both in wild-type and A20\(^{myel-KO}\) macrophages (Fig. 3d), supporting the notion that acute Nlrp3 inflammasome activation occurred independently of LPS-induced new protein synthesis. Both caspase-1 processing and IL-18 secretion were markedly increased in A20\(^{myel-KO}\) macrophages in the absence of substantial IL-1β, TNF and IL-6 secretion (Fig. 3d, e). This was probably due to increased basal expression of Nlrp3 and proIL-18 in these cells (Fig. 3c, d). In agreement, basal mRNA levels of Nlrp3, proIL-1β and proIL-18 were increased in untreated A20\(^{myel-KO}\) macrophages (Fig. 3a, b and Extended Data Fig. 3c). Together, these results suggest that A20 negatively regulates Nlrp3 inflammasome signalling by suppressing NF-kB-dependent production of Nlrp3 and the inflammasome substrates proIL-1β and proIL-18. In agreement, the pharmacological inhibitor of kappa B kinase (IKK) inhibitor BMS-345541 significantly reduced Nlrp3 levels in LPS-primed A20\(^{myel-KO}\) macrophages (Fig. 3f). Moreover, both BMS-345541 and the selective IKK2 inhibitor TCPA-1 significantly reduced ATP- and nigericin-induced caspase-1 autoprocessing, IL-1β secretion and pyroptosis induction in LPS-primed A20\(^{myel-KO}\) macrophages (Fig. 3g–i).

Having established A20 as a negative regulator of Nlrp3 inflammasome activation, we hypothesized that excessive A20\(^{myel-KO}\) activation might drive pathology of rheumatoid arthritis in A20-deficient mice upstream of IL-1R1. To test this hypothesis, Nlrp3\(^{−/−}\) mice were crossed into A20\(^{myel-KO}\) mice and the levels of four cytokines (IL-1α, IL-1β, IL-6 and TNF) relevant to rheumatoid arthritis were monitored. Although IL-1α levels were not significantly different in A20-sufficient and A20\(^{myel-KO}\) mice (Extended Data Fig. 4a), the latter group of mice had significantly higher levels of IL-1β in circulation (Fig. 4a). In addition, the levels of IL-6 and TNF were also significantly higher in A20\(^{myel-KO}\) mice compared with A20\(^{lox/lox}\) littermates (Extended Data Fig. 4b, c). Notably,
deletion of Nlrp3 in A20\textsuperscript{myel-KO} mice markedly reduced IL-1β secretion to baseline levels of A20\textsuperscript{floX/floX} mice, thereby demonstrating that the Nlrp3 inflammasome contributes critically to excessive IL-1β production in A20\textsuperscript{myel-KO} mice in vivo (Fig. 4a). Intriguingly, A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} mice also were protected from excessive IL-6 production, suggesting that high IL-6 levels are consequent to excessive inflammasome-mediated IL-1β production (Extended Data Fig. 4b). In contrast, TNF production was not significantly affected in A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} mice (Extended Data Fig. 4c). In agreement, TNF-R1 signalling was previously shown to be dispensable for pathology of rheumatoid arthritis in A20\textsuperscript{myel-KO} mice, whereas IL-6 neutralization provided protection\cite{2}.

Based on these findings, we assessed the contribution of Nlrp3 to pathogenesis of rheumatoid arthritis in A20\textsuperscript{myel-KO} mice. Swelling and redness of the hind paws of A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} mice became evident around 11 weeks of age (Fig. 4b), and had afflicted all animals of this genotype when they were 20 weeks old (Fig. 4c). Disease severity continued to progress, and became increasingly pronounced in A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} mice aged 21–40 weeks (Fig. 4d). In contrast, A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} mice of similar age were markedly protected from rheumatoid arthritis, and their hind paws had a normal appearance and lacked clinical signs of pathology of rheumatoid arthritis (Fig. 4b–d). Histological analysis of the ankle joints of these mice showed significantly reduced synovial and periarticular inflammation, and substantially less infiltrated mononuclear cells compared with tissue sections of A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{+/+} mice of comparable age (Fig. 4e, f and Extended Data Table 2). In agreement, three-dimensional micro-computed tomography imaging showed that the extent of bone erosion in hind paws of representative A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} and A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{+/−} mice was markedly different. Unlike in Nlrp3-deficient A20\textsuperscript{myel-KO} mice, hind paws of Nlrp3-sufficient mice exhibited severe loss of bone density in the metatarsal region (Fig. 4g), demonstrating a key role for Nlrp3 in the pathology of rheumatoid arthritis.

We also analysed the impact of caspase-1/11 deficiency on IL-1β secretion and pathology of rheumatoid arthritis in A20\textsuperscript{myel-KO} mice. IL-1β levels in circulation were significantly reduced in A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{−/−} mice compared with A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{+/+} mice (Fig. 4h). As in A20\textsuperscript{myel-KO} Nlrp3\textsuperscript{−/−} mice, serum levels of IL-6 were markedly reduced in A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{−/−} mice, whereas TNF production was not significantly different compared with A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{+/+} mice (Extended Data Fig. 4). Moreover, hind paws of all analysed A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{−/−} mice were clearly inflamed and swollen (Fig. 4i–k). In contrast, 50% of A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{−/−} mice were devoid of clinical signs of arthritis, and disease symptoms in the remaining A20\textsuperscript{myel-KO} Casp1/11\textsuperscript{−/−} mice were very mild (Fig. 4i–k). In agreement, analysis of
A20myel-KO mice critically relies on the Nlrp3 inflammasome/IL-1 signalling axis. Because both A20/Tnfrp3−/− and Nlrp3−/− are rheumatoid arthritis susceptibility genes, this suggests that A20myel-KO mice might be a suitable pre-clinical model for validating the effectiveness of experimental therapies for rheumatoid arthritis targeting inflammasomes and/or IL-1 signalling.

METHODS SUMMARY

Mice. Nlrp3−/− (ref. 22), Casp1/11−/− (ref. 23), Il1r1−/− (ref. 24) and A20myel-KO mice with a lysozyme M-Cre–targeted deletion of A20 in myeloid cells were described. BMDM studies. BMDMs were isolated and the Nlrp3 inflammasome was activated by LPS in combination with ATP (Roche), nigericin (Sigma-Aldrich) or Silica (US Silica). The AIM2 or Nlr4 inflammasomes were activated by infection with Salmonella enterica serovar Typhimurium or transfection with plasmid DNA, respectively.

Antibodies. The following antibodies were used: A20 (Santa Cruz Biotechnology), caspase-1, Nlrp3, ASC (Adipogen), IL-1β (Genetex), IL-18 (Biovision), IκBα, Phospho-IκBα (Ser32) (Cell Signaling) and β-actin (Novus Biologicals).

Cytokine analysis and lactate dehydrogenase measurement. Cytokine levels in culture medium of BMDMs and in serum were determined by LumineX assays (Bio-Rad) and IL-1β–enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Lactate dehydrogenase (LDH) activity (Promega) was used to quantify pyroptosis.

Reverse transcription PCR. RNA was isolated using RNeasy kit (Qiagen) and mRNA levels were determined by quantitative reverse transcription PCR (qRT-PCR).

Clinical scoring. Mice were randomly scored in a blinded fashion for development of peripheral arthritis. The severity of arthritis was assessed using a visual scoring system.

Histology and histological scoring. Paraffin sections of murine paws were stained with haematoxylin and eosin for evaluation of inflammation and bone erosion. Histological scores were based on evaluation of two parameters, calcaneal erosion and inflammation at the synovio-entheseal complex, each ranging from 0 (normal) to 3.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.L. (mohamed.lamkanfi@vib-ugent.be).
METHODS

Mice. Nlrp3<sup>−/−</sup> (ref. 22) and Casp1<sup>−/−</sup> (ref. 23) mice have been described, and were provided by V. Dixit and R. Flavell. Ilr1<sup>−/−</sup> (ref. 24) mice were purchased from Jackson Laboratories and bred in house. A20<sup>myel-KO</sup> mice with a lysozyme M-Cre-targeted deletion of A20 in myeloid cells were as described<sup>2</sup>. In vivo experiments were controlled with age- and sex-matched littermates. The sample size was chosen to validate statistical analyses. Mice were housed in individually ventilated cages and kept under pathogen-free conditions at the animal facilities of Ghent University. All animal experiments were conducted with permission of the ethical committees on laboratory animal welfare of Ghent University.

Macrophase differentiation and stimulation. BMDMs were generated by culturing bone marrow cells in IMDM medium containing 10% heat-inactivated FBS, 30% L cell-conditioned medium, 100 U ml<sup>−1</sup> penicillin, and 100 mg ml<sup>−1</sup> streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. Six days later, cells were collected and seeded in 12-well plates in IMDM containing 10% heat-inactivated FBS and 1% non-essential amino acids in the presence of antibiotics. The next day, BMDMs were either left untreated or treated with 5 μg ml<sup>−1</sup> ultrapure LPS from Salmonella minnesota (InvivoGen) for 3 h followed by 5 mM ATP (Roche) or 20 μM Nigericin (Sigma-Aldrich) for 30 min or 0.5 mg ml<sup>−1</sup> silica (Min-U-Sil 5, Bio-Rad) for 3 h. For inhibition of NF-κB, A20<sup>myel-KO</sup> cells were pre-incubated for 30 min with 1.25 or 2.5 μM of the selective IKK inhibitors BMS-345541 (ref. 25) or TP-CA-1 (ref. 26) (Sigma-Aldrich) before stimulation with LPS.

Western blotting. Cell lysates and culture supernatants were incubated with cell lysis buffer (20 mM Tris HCl pH 7.4, 200 mM NaCl, 1% NP-40) and denatured in Laemmli buffer. Subsequently the protein samples were boiled at 95 °C for 10 min and separated by SDS–polyacrylamide gel electrophoresis. Separated proteins were transferred to PVDF membranes. Blocking, incubation with antibody and washing of the membrane were done in PBS supplemented with 0.05% Tween-20 (v/v) and 3% (w/v) non-fat dry milk. Immunoblots were incubated overnight with primary antibodies against caspase-1 (AG-20B-0042-C100, Adipogen), Nlrp3 (AG-20B-0014-C100, Adipogen), ASC (AG-25B-0006, Adipogen), IL-1β (GTX74034, Genetex), IL-18 (GTX74035, Genetex), IkBα (9242S, Cell Signaling), Phospho-IκBα (2859S, Ser22) (Cell Signaling), β-actin (NB600-501H, Novus Biologicals) and A20 (sc-166692, Santa Cruz Biotechnology). Horseradish-peroxidase-conjugated goat anti-mouse (115-035-146, Jackson Immunoresearch Laboratories) or anti-rabbit secondary antibody (115-035-144, Jackson Immunoresearch Laboratories) was used to detect proteins by enhanced chemiluminescence (Thermo Scientific).

Cytokine analysis and LDH measurement. Cytokine levels in culture medium and serum were determined by magnetic bead-based multiplex assay using Luminex technology (Bio-Rad) and IL-1β ELISA (R&D Systems), according to the manufacturers’ instructions. Cell death was determined by measuring LDH activity in culture medium according to the manufacturer’s instructions (Promega).

Reverse transcription PCR. Total RNA was isolated from BMDMs using RNeasy kit (Qiagen). Complementary DNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). A20 mRNA expression in wild-type and A20<sup>myel-KO</sup> BMDMs, treated with 5 μg ml<sup>−1</sup> LPS for 6 h, was determined by RT–PCR using the following primers: 5′-CTCGGACCTTTAATCCGCC-3′ and 5′-GGTAAAGTTAGCTTCATCC-3′. To analyse mRNA levels of Nlrp3, proIL-1β, ASC and caspase-1, wild-type and A20<sup>myel-KO</sup> BMDMs were left untreated or treated with 5 μg ml<sup>−1</sup> LPS for 3 h and qRT–PCR was performed using a LightCycler 480 SYBR Green I Master Mix Kit (Roche Applied Science) in a LightCycler 480 real-time PCR machine (Roche Applied Science). The cycling conditions were 95 °C for 5 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. Gene expression levels were normalized to GAPDH. qRT–PCR sense/antisense primers used were as follows: mouse GAPDH, 5′-GGTGAAAGTCTCGGTGAACG-3′ and 5′-CTCCTGTCTCGGAAGATGGTG-3′; mouse Nlrp3, 5′-ATTACCAGCCCGGAGAAGG-3′ and 5′-TGGACACAGAAGATCCACAGACG-3′; mouse proIL-1β, 5′-TGCGGGCTCAGGGAAAGA-3′ and 5′-GGTGTGCTGTGACATTCA-3′; mouse ASC, 5′-CTTGACGAGGATGACCTAAA-3′ and 5′-GCCATAGAGACCTGGATGAGCG-3′; mouse Casp1, 5′-AAAGGACCGGGGACATGCTG-3′ and 5′-TCCAGGTCGTAAGATGCTG-3′. Quantitative analysis of mouse proIL-18 mRNA was performed by Taqman gene expression assay (Mm00434225_m1, Applied Biosystems).

Clinical scoring. Mice were randomly scored in a blinded fashion for development of peripheral arthritis once a week and scores were subsequently linked to genotypes. A score ranging from 0 to 3 was assigned to each paw, with 0 being normal, 0.5 being swelling of one or more toes, 1 being mild swelling of the wrist and/or ankle or carpus and/or tarsus, 2 being moderate swelling of the wrist and/or ankle or carpus and/or tarsus or mild swelling of both, and 3 being severe swelling of the entire paw.

Micro-computed tomography imaging. Micro-computed tomography micrographs of paws fixed in formalin were made using an ex vivo micro-computed tomography scanner (LocusSP Specimen CT, GE Healthcare) at 28-μm isotropic voxel size, with 720 projections, an integration time of 1,700 ms, photon energy of 80 keV and a current of 70 μA.

Histology and histological scoring. Mice paws were dissected, fixed in 4% formaldehyde and de-calcified in 5% formic acid until bones were visible. Paraffin sections were stained with haematoxylin and eosin for evaluation of inflammation and bone erosions. Histological sections were evaluated by two blinded assessors, and scores were determined by combining assessment of two parameters, calcaneal erosion and inflammation, at the synovio-entheseal complex, each ranging from 0 (normal) to 3 (erosion; 0, none; 1, minimal; 2, intermediate; 3, into bone marrow; inflammation; 0, none; 1, minimal one or two cell layers; 2, two to five cell layers; 3, more than five cell layers).

Statistics. GraphPad Prism 5.0 software was used for data analysis. Data are shown as mean ± s.d. Data were compared by an unpaired two-tailed Student’s t-test when values followed a Gaussian distribution, or with the non-parametric Mann–Whitney U test. P < 0.05 was considered to indicate statistical significance.
Extended Data Figure 1 | A20 deficiency does not cause spontaneous Nlrp3 inflammasome activation. a–c, Wild-type and A20myel-KO BMDMs were stimulated with 5 μg ml⁻¹ LPS for 3 h, treated with 5 mM ATP or 20 μM nigericin for 60 min, or stimulated with 5 μg ml⁻¹ LPS for 3 h and then treated with 5 mM ATP or 20 μM nigericin for 60 min. Cell extracts were immunoblotted for caspase-1 (a) and culture supernatants were analysed for secretion of IL-1β (b) and IL-18 (c). Black arrows on western blots denote procaspase-1 (p45); white arrows denote the processed p20 subunit (p20). ELISA data are shown as mean ± s.d. of one out of three biological replicates, with three technical replicates each (***P < 0.001; Student’s t-test).
Extended Data Figure 2 | A20 negatively regulates NF-κB activation.

a, Wild-type and A20<sup>myel-KO</sup> BMDMs were incubated with 5 μg ml<sup>-1</sup> LPS for the indicated durations before cell extracts were prepared and immunoblotted with the indicated antibodies. 
b, c, Wild-type and A20<sup>myel-KO</sup> BMDMs were stimulated with 5 μg ml<sup>-1</sup> LPS for 3 h, treated with 5 mM ATP or 20 μM nigericin for 60 min, or stimulated with 5 μg ml<sup>-1</sup> LPS for 3 h and then treated with 5 mM ATP or 20 μM nigericin for 60 min. Culture supernatants were analysed for IL-6 secretion (b) and TNF secretion (c). ELISA data are shown as mean ± s.d. of one out of three biological replicates, with three technical replicates each (***P < 0.001; Student’s t-test).
Extended Data Figure 3 | Quantification of ASC, caspase-1 and proIL-18 mRNA expression in wild-type and A20 deficient macrophages. a–c, Wild-type and A20<sup>myel-KO</sup> BMDMs were stimulated with 5 μg mL<sup>–1</sup> LPS for 3 h before mRNA levels of caspase-1 (a), ASC (b) and proIL-18 (c) were analysed by qRT–PCR. Data are shown as mean ± s.d. of one out of three biological replicates, with three technical replicates each (*P < 0.05; ***P < 0.001; Student’s t-test).
Extended Data Figure 4 | Comparison of serum titres of inflammatory cytokines between A20^{myel-KO} mice and A20^{myel-KO}Nlrp3^{−/−} or A20^{myel-KO}Casp1/11^{−/−} mice. a–c, Levels of IL-1α (a), IL-6 (b) and TNF (c) in serum of A20^{fl/fl} (n = 10), A20^{myel-KO} (n = 10), A20^{myel-KO}Casp1/11^{−/−} (n = 12) and A20^{myel-KO}Nlrp3^{−/−} (n = 9) mice between 20 and 35 weeks of age. P values were determined by Mann–Whitney U test (b) and Student’s t-test (c).
Extended Data Figure 5 | The ubiquitin-editing enzyme A20 negatively regulates Nlrp3 inflammasome activation. The NLR member Nlrp3, the inflammatory cytokine proIL-1β and the ubiquitin-editing enzyme A20 are expressed at low levels in resting macrophages. Binding of the TLR4 ligand LPS to its receptor triggers phosphorylation and rapid degradation of IκBα, allowing translocation of NF-κB to the nucleus, and NF-κB-mediated upregulation of proIL-1β, proIL-18, Nlrp3 and A20. A20 prevents excessive Nlrp3 inflammasome activation by dampening basal and LPS-induced NF-κB-mediated upregulation of Nlrp3. As such, A20 reduces the pool of Nlrp3 that is available for inflammasome assembly. In addition, it limits the levels of the inflammasome substrates proIL-1β and proIL-18.
Extended Data Table 1 | Arthritic histopathology of A20<sup>myel-KO</sup>L1R1<sup>+/−</sup> and A20<sup>myel-KO</sup>L1R1<sup>−/−</sup> mice

| Mouse # | Inflammation score | Erosion score |
|---------|-------------------|---------------|
| A20<sup>myel-KO</sup>L1R1<sup>+/−</sup> | | |
| 1 | 1 | 0 |
| 2 | 2 | 1 |
| 3 | 2 | 1 |
| 4 | 4 | 4 |
| 5 | 2 | 1 |
| 6 | 2 | 1 |
| 7 | 2 | 1 |
| 8 | 3 | 2 |
| 9 | 1 | 0 |
| 10 | 4 | 4 |
| A20<sup>myel-KO</sup>L1R1<sup>−/−</sup> | | |
| 1 | 2 | 1 |
| 2 | 1 | 1 |
| 3 | 1 | 0 |
| 4 | 1 | 0.5 |
| 5 | 1 | 0 |
| 6 | 2 | 1 |
| 7 | 1 | 1 |
| 8 | 1 | 0 |

Raw data of histological scores of ankle sections of A20<sup>myel-KO</sup>L1R1<sup>+/−</sup> (n = 10) and A20<sup>myel-KO</sup>L1R1<sup>−/−</sup> mice (n = 8). Arthritic histopathology of ankles was scored on two parameters, calcaneal erosion and inflammation at the synovio-entheseal complex, as described in Methods.
Extended Data Table 2 | Arthritic histopathology of $A20^{myel-KO}$
$Nlrp3^{+/-}$ and $A20^{myel-KO}Nlrp3^{+/-}$ mice

| Mouse # | Inflammation score | Erosion score |
|---------|--------------------|--------------|
| $A20^{myel-KO}Nlrp3^{+/-}$ | 1 | 1 | 0 |
|        | 2 | 3 | 3 |
|        | 3 | 1 | 1 |
|        | 4 | 3 | 2 |
|        | 5 | 1 | 0 |
| $A20^{myel-KO}Nlrp3^{+/-}$ | 1 | 1 | 0 |
|        | 2 | 0 | 0 |
|        | 3 | 0 | 0 |
|        | 4 | 0 | 0 |
|        | 5 | 1 | 0 |

Raw data of histological scores of ankle sections of $A20^{myel-KO}Nlrp3^{+/-}$ ($n = 5$) and $A20^{myel-KO}Nlrp3^{+/-}$ mice ($n = 5$). Arthritic histopathology of ankles was scored on two parameters, calcaneal erosion and inflammation at the synovio-entheseal complex, as described in Methods.