Supplementary Methods

Cell lines and culture conditions

Unless otherwise specified, U937 cells (authentic stock CRL 1593.2 from American type culture collection) were grown in RPMI 1640 medium, 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (all from Gibco, Life Technologies) in a 37°C humidified 5% CO_2 incubator.

Generation of A20 C103A KI and A20 KO cells by CRISPR/Cas9

Supplementary figure 1 represents a schematic outline of the generation of *C103A* KI and A20 KO cells. Targeted knock-in mutation *C103A* was generated by TG-to-GC substitution in human *TNFAIP3* DUB domain (replacing cysteine at position 103 for alanine) using CRISPR/Cas9 in U937 cells. A 6,4 kb genomic region of *TNFAIP3* (containing *C103A* mutation) was cloned into pBSK targeting vector containing a LoxP-flanked Puromycin selection cassette and GFP (Epoch LifeScience). Guide RNAs were designed using the ZiFiT software (http://zifit.partners.org/ZiFiT/) and were cloned into a pCas9-GFP expression vector. To account for possible off-target effects, two different guide-RNAs were used. *TNFAIP3 C103A* targeting vector and pCas9-GFP-guideRNA were co-transfected into U937 cells using electroporation (Neon transfection system, Invitrogen). After 1 week, cells with stable GFP-expression were single-cell sorted into 96-well plates containing 50% conditioned media. After 3 weeks, DNA from GFP-positive clones were analyzed for the *C103A* mutation by restriction fragment length polymorphism (RFLP). Homozygous clones were further analyzed for correct insertion by PCR, using primers binding outside the homology arms as well as in the selection cassette. The amplicon was sequenced by Sanger sequencing and homozygous clones were expanded. The insertion cassette copy number was confirmed to be bi-allelic by droplet digital PCR (BioRad). For removal of the LoxP-flanked selection cassette, cells were transfected with CRE-recombinase expression vector and GFP-negative cells were sorted, expanded, and cryo-preserved. GFP-negative cells were again validated for homozygous insertion of *C103A* by RFLP analysis. Three successfully generated homozygous *C103A* KI

clones (*C103A* KI1-KI3) were confirmed, derived from 2 different guide RNAs. The levels and size of A20 protein was analyzed by western blot (supplementary figure 1B). Control cells were grown in parallel and underwent the same processes as the targeted cells but in absence of plasmids. Generation of A20 knock-out (KO) cells was accomplished by transfecting U937 cells with the Cas9 expression vector coding the same guide RNAs as previously described, but in the absence of targeting vector. 48 hours after transfection, GFP-positive cells were single cell sorted into 96-well plates. Cells were left to proliferate and were screened for the presence or absence of A20 protein by western blot.

Next Generation RNA sequencing

CRISPR/Cas9-generated A20 *C103A* KI (3 clones), A20 KO (4 clones) and WT (7 clones) U937 cells were differentiated into macrophage-like cells using 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 hours as previously described(1). Cells were washed in PBS and after overnight culture in medium without PMA, cells were treated with 10 ng/mL LPS (InvivoGen) or vehicle (H_20) for 6 hours and RNA was extracted using RNeasy Plus Mini kit (Qiagen). RNA integrity was assessed by Bioananalyzer 2100 (Agilent) and RIN>9,2 were obtained for all samples. RNA was diluted to 20 ng/ul and used as input to create mRNA libraries using TruSeq Stranded mRNA kit (Illumina) with dual indexing following standard instructions. Libraries were validated on the Fragment Analyzer platform (AATI) using standard sensitivity NGS fragment analysis kit and the concentration was determined using Quant-iT dsDNA High Sensitivity assay kit on the Qubit fluorometer (Thermo Fisher Scientific). Sample libraries were pooled in equimolar concentrations, diluted and denatured according to Illumina guidelines. Sequencing was performed using a High Output 2 x 76 bp kit on an Illumina NextSeq500. RNA-seq fastq files were processed using bcbio-nextgen (version 0.9.7) where reads were mapped to the human genome build hg38 (GRCh38.79) using hisat2(2) (version 2.0.2-beta), yielding between 18.4-52.5 M mapped reads (>95% mapping frequency) per sample. Gene level quantifications (counts) were generated with Featurecounts(3) (version 1.4.4) within bcbio. ArrayStudio version 9 (OmicSoft)

was used for further data analysis. Differential gene expression were assessed with DESeq2(4), using raw counts as input with a pre-filtering step requiring at least one sample with a count of at least 10. Genes were considered significantly differentially expressed if they had a FDR<0.05 using Benjamini-Hochberg method for multiple testing correction. Transcriptomic data is deposited in NCBI´s Gene Expression Omnibus (GEO Accession GSE116780).

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) tool(5) (http://broad.mit.edu/gsea/) was used to classify gene expression profiles into functional pathways. The gene set for the NF-kB target genes was manually added and described previously(6). Gene expression profiles from A20 KO or A20 *C103A* KI U937 cells were compared to their respective WT samples using a t-test to create a ranked gene list for GSEA analysis. False discovery rate (FDR) <0.05 was considered significant.

Generation of C103A mice

The targeting vector constructed to generate the *C103A* mutation in ES cells is represented in supplementary figure 12. The required regions of homology were generated by PCR using an appropriate BAC clone as a template. All PCR clones were fully sequenced to ensure there were no PCR generated mutations and then assembled in to the targeting vector via DNA ligation. The full sequence of the vector is available on request. The vector was used to generate knock-in mice via standard techniques. Briefly the vector was electroporated into C57/Bl6 ES cells and colonies isolated following positive and negative selection with G418 and ganclivoir. Colonies were screened by Southern analysis of Bam HI digested genomic DNA using probes external to the targeting vector. Correctly targeted ES cells were injected into blastocysts to generate chimeric mice. Selected chimeras were then crossed onto Flpe transgenic mice on a C57/Bl6 background and pups analyzed for germline transmission. The

use of the Flpe transgenic mice resulted in the excision of the neomycin selectable marker. Routine genotyping of the mice was carried out by PCR of ear biopsies. Primer sequences are listed in table S4. This PCR spanned the region were the 3' loxP site is inserted and gives a 216 bp fragment for wild type and 331 bp fragment for C103S knockin alleles. Use of tissue for *ex-vivo* functional and mechanistic studies (bone marrow and blood) were approved by the local ethics committee in Gothenburg.

Bone marrow-derived macrophage (BMDM) differentiation and stimulation

Bone marrow cells from 8 week old female WT and *C103A* KI mice were differentiated into macrophages in the presence of 50 ng/mL mouse recombinant M-CSF (R&D Systems) for 7 days. Macrophage differentiation was assessed by surface staining of CD11b (clone M1/70, BD Biosciences) and F4/80 (clone BM8, eBiosciences) by FACS on day 7. On day 8, BMDMs were stimulated with 10 ng/mL LPS (derived from E-coli, InvivoGen), 10 ng/mL Tumor necrosis factor (Upstate GF027), 100 ng/mL R848 (InvivoGen) or 50 ng/mL Pam3CSK4 (InvivoGen). Cells were harvested for protein extraction at different time-points and supernatants were taken 24 hours after stimulation for analysis of cytokine expression.

Immunoblotting

Protein was extracted from cells using RIPA buffer (Pierce, Thermo Fischer Scientific) supplemented with Complete protease-inhibitor cocktail (Roche Diagnostics) and PhoStop phosphatase-inhibitor cocktail (Roche). For intracellular histone detection, histones were extracted using the EpiQuick total histone extraction kit (EpiGentek). Protein concentrations were measured with BCA assay (Pierce, Thermo Fischer Scientific) and equal amounts were separated on 4–12% gradient NuPage Bis-Tris gels and transferred to nitrocellulose membranes (Life Technologies) and blocked in 5% dry milk in Tris-buffer saline-Tween 0.05%. The following primary antibodies were used: human A20 (Abcam,

ab13597, clone 59A426), IκBα (Cell Signaling, 9242), α-tubulin (Abcam, ab4074 and Sigma T6199), p-IκBα (Cell Signaling, 9246), citrullinated histone H3 (Abcam, ab5103), histone H3 (Cell Signaling 3638, clone 96C10), mouse A20 (Cell Signaling 5630), mouse p100/p52 (Cell Signaling, 4882), GAPDH (Cell Signaling 2118), p-JNK (Cell Signaling 4668), p-ERK (Cell Signaling 4376), p-p38 (Cell Signaling, 4511), p105/p50 (Cell Signaling 13586) and PAD4 (Abcam, ab128086). IRDye 800CW and 680LT goat anti-mouse or anti-rabbit secondary antibodies (Li-Cor) were used for detection with Odyssey CLx imaging system (Li-Cor) and antibody signal intensities were quantified using Image Studio v4 (Li-Cor).

Cytokine arrays

For detection of secreted cytokines from WT and *C103A* KI BMDMs, the Mouse Cytokine Array Panel A (Proteome Profiler, R&D systems), including printed antibodies for 40 cytokines was used. Supernatants (500 uL) were collected 24 hours after stimulation the manufacturer's protocol was followed. Signal intensities were quantified with Image Studio v4 (Li-Cor) and normalized to reference spots.

RNA isolation and reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA was extracted using RNeasy Plus Mini kit (Qiagen), according to the manufacturer's instructions. 1000 ng of RNA from U937 cells or 100-200 ng of RNA from PBMC or PBMC-derived cells were reverse-transcribed using the High capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific). qPCR was conducted with QuantStudio 7 Flex real-time PCR system (Applied Biosystems) using the following TaqMan assays: *PADI4* Hs01057483, *TNFAIP3* Hs01568116, *GUSB* Hs00939627, *ACTB* Hs01060665, *SRXN1* HS00607800, *NFE2L2* HS00975961, Padi4 Mm01341658, Actb Mm02619580. Reactions were run in triplicate and Cycle thresholds (Ct values) were normalized to

those of housekeeping genes GUSB and ACTB. Relative quantity calculations were performed using the 2^{-ddCt} method using the average of the control samples as reference sample.

Isolation of peripheral blood mononuclear cells (PBMC) and immune cell subsets from healthy donors

Peripheral blood collected from 26 age-matched healthy female donors with or without the risk variant of rs2230296 (13 individuals in each group) from UB were used for preparation of PBMC. The number of genotyped healthy blood donors to be included in the study was calculated to be 13 per genotype using a power calculation test (80% power, p<0,05 in one-sided t-test) based on a pilot experiment. The PBMC were isolated from buffy coats using Ficoll-Paque gradient (GE Healthcare) centrifugation and frozen immediately in liquid nitrogen at a density of 10 million cells/mL in 10% DMSO (Sigma-Aldrich) diluted in FBS (Gibco). T-cells, B-cells and NK-cells were isolated from cryo-preserved PBMC using cell sorting (supplementary figure 13) by FACS Aria III (BD Biosciences). Anti-CD3-PerCP (clone SK7), anti-CD19-BV785 (clone SJ25C1) and anti-CD56-PE (MY31) were used for positive selection and were purchased from BD Bioscience. Due to upregulation of PADI4 when using CD14 antibodies for cell sorting, monocytes were instead enriched using negative selection with the Pan monocyte isolation kit (Miltenyi). Samples with >70% monocyte purity were included in the analysis.

SNP analysis of SLE-patients and healthy controls

Genomic DNA was obtained from whole blood samples using QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). Genotyping of SLE-patients and healthy controls was performed with the ImmunoChip (an Illumina iSelect custom array designed by the ImmunoChip Consortium)(7). Quality control (QC) was performed sample-wise and SNP-wise. Samples and SNPs with call rate below 98% were excluded. Samples with abnormal autosomal heterozygosity rate with more than 5SD from the mean of Wright's inbreeding coefficient F were excluded. A check for mislabeled gender was

performed using Wright's inbreeding coefficient F, calculated from X chromosome data. Annotated females with F close to one or annotated males with F close to zero were excluded. Cryptic relatedness was analyzed using KING software(8) and one sample from each related pair (up to 2nd degree of relatives) was excluded. Furthermore, PCA was performed on 1000 Genomes Project and then used to project and exclude samples with more than 5SD from each of 5 principal components of European population. SNPs with MAF < 1% or with HWE-p within FDR < 5% (based on controls only) were excluded. 137,213 SNPs remained for analysis after QC. 982 SLE-patients and 1980 healthy controls were analyzed for the rs2230926 risk allele.

Detection of antibodies in SLE-patients

IgG-class ACPA was analyzed in serum of 195 SLE-patients as previously described(9) by enzyme-linked immunosorbent assays (ELISA) with 2nd generation cyclic citrullinated peptides (CCP) as antigen. Antibody levels ≥25 units/mL were defined as positive for ACPA as recommended by the manufacturer, and this cut-off has been previously used in clinical cohorts of rheumatoid arthritis patients(10). IgG antibodies against non-citrullinated control peptides (cyclic arginine peptides, CAP), were analyzed as previously described(9). IgG antibodies against cardiolipin (aCL, IgG and IgM), β_2-glycoprotein-I (anti-β2GPI, IgG) and prothrombin (PT, IgG) were analyzed in serum of 490 SLE-patients as previously described(11) by ELISA (Orgentec, Mainz, Germany).

Genetic association analysis

The genetic association analysis (case-control) was performed using a logistic regression model with PLINK(12) (v1.07). Logistic, linear or ordinal regression models using PLINK or R software were used to test the association between rs2230926 and clinical manifestations in SLE-patients. P-values<0.05 were considered significant.

Flow Cytometry

Flow cytometric analysis of protein citrullination: WT and *C103A* KI U937 cells were pretreated with PAD4 inhibitor GSK484 (1 and 10 μM) or vehicle (DMSO) for 30 minutes before overnight-stimulation with 4 μM Ionomycin and 2 mM $CaCl_2$. Cells were then washed in PBS, stained with LIVE/DEAD fixable aqua stain kit (Molecular Probes) and fixed in BD Cytofix/Cytoperm buffer according to manufacturer's protocol. Cells were then stained with anti-peptidyl-citrulline antibody (clone F95, MerckMillipore, 1:4000 dilution) and secondary goat anti-mouse IgG/IgM (H+L) Alexa Fluor 488 antibody (ThermoFisher Scientific). Measurement of protein citrullination was performed on gated live cells. Cell viability was measured with LIVE/DEAD aqua stain kit (supplementary figure 7).

Analysis of intracellular IL-6 and TNF expression: Cryo-preserved PBMCs from 14 healthy individuals with or without the rs2230926 risk variant were thawed in RPMI medium complemented with 10% FBS and 1% Penicillin/Streptomycin and rested overnight. The next day, cells were stimulated with vehicle (H_20) or 20 ng/mL LPS (InvivoGen) for 5 hours. BD GolgiStop (BD Biosciences) was added 1 hour after LPS addition. Following stimulation, cells were collected, washed in PBS and stained with LIVE/DEAD fixable aqua stain (Molecular Probes) and antibodies against human CD3 (Alexa Fluor 488, clone UCHT1), CD19 (Alexa Fluor 488, clone H1B19), CD56 (Alexa Fluor 488, clone B159), HLA-DR (BV-786, clone L243) from BD Biosciences, and CD1c (PerCp5.5, clone L161), CD11c (BV-421, clone B-lyt) and CD14 (BV-605, clone M5E2) from BioLegend. CD14 monocytes were identified as CD3-/CD19-/CD56-/CD14+, CD16-monocytes were identified as CD3-/CD19-/CD56-/CD14-/CD11c+/HLA-DR+/CD1c- and myeloid DC identified as CD3-/CD19-/CD56-/CD14-/CD11c+/HLA-DR+/CD1c+. For expression of intracellular cytokines, cells were fixed in BD Cytofix/Cytoperm buffer according to manufacturer's protocol (BD Biosciences) and stained with anti-TNF (BUV-395, clone MAb11) and anti-IL-6 (APC, clone MQ213A5) from BD Biosciences. Gating strategy is outlined in supplementary figure 3.

A five laser BD LSR Fortessa instrument (BD Biosciences) was used for data collection and the data was analyzed with FlowJo software.

Detection of secreted citrullinated histone H3 and dsDNA

WT and *C103A* U937 cells were plated at a density of 1 million cells/mL in RPMI-medium complemented with 2% FBS and 1% Penicillin/Streptomycin. Human neutrophils were plated at a density of 1 million cells/mL in RPMI medium complemented with 10% FBS. PAD4 inhibitor GSK484 (10 μM) or vehicle alone (DMSO) were added to the cells 30 minutes before stimulation with 4 μM Ionomycin + 2 mM $CaCl_2$. The supernatant from U937 cells and primary neutrophils was collected after 6 hours and 1 hour of treatment, respectively. Double-stranded DNA (dsDNA) was measured in the cell supernatant using Quant-iT PicoGreen dsDNA reagent (Invitrogen), according to the manufacturer's protocol. For detection of citrullinated histone H3, the proteins in the supernatant were precipitated with 20% trichloroacetic acid (Sigma-Aldrich), resuspended in 2,5x LDS loading buffer (Invitrogen) and analyzed by western blotting.

Neutrophil isolation and NETosis induction

Isolation of murine neutrophils: Whole blood from 8-12 week old sex-matched WT and A20 *C103A* KI mice was collected in heparin tubes. Blood from 3-5 mice per group were pooled together in each experiment. Erythrocytes were cleared from the samples by using BD Pharm Lyse lysing solution (BD Bioscience) and neutrophils were enriched using EasySep™ Mouse Neutrophil Enrichment Kit (Stemcell Technologies) according to manufacturer's protocol.

Isolation of human neutrophils: Blood from SLE-patients was collected in vacutainer tubes with heparin (BD Bioscience) and mixed 1:1 with 2% dextran (Sigma) After sedimentation of erythrocytes, PBMCs and granulocytes were separated by density gradient centrifugation in LymphoPrep (StemCell

Technologies). Remaining erythrocytes were lysed in distilled water and the neutrophils were collected and resuspended in RPMI medium supplemented with 10 % FBS (Gibco). Neutrophil purity was >95% as assessed by Sysmex XT 1800i (Sysmex Kobe Japan).

NETosis induction: Equal number of neutrophils were plated in 8-well chamber glass slides (Lab-Tek II Chamber Slide, NUNC) pre-coated with Poly-L-lysine (Sigma-Aldrich). PAD4 inhibitor GSK484 (10 µM) or vehicle alone (DMSO) were added to the cells 30 min before stimulation with 1 or 4 µM Ionomycin + 2mM $CaCl_2$. After 1 hour and 45 min for murine neutrophils, or after 1 hour for human neutrophils, cells were fixed for immunofluorescence staining and confocal analysis of NET structures.

Immunofluorescent staining of citrullinated histone H3 and NETs

Neutrophils were plated and stimulated in Poly-L-Lysine coated chamber glass slides, fixed in 4% paraformaldehyde (VWR Chemicals) for 30 minutes, permeabilized with 0.25% Triton X-100 (Sigma) for 10 min and blocked in 1% BSA/2 % goat serum overnight at 4°C. Next, the cells were incubated for 2 h at room temperature with antibodies against citrullinated histone H3 (ab5103; Abcam). The cells were washed in PBS and stained with Goat anti-Rabbit IgG (H+L) secondary antibody (Alexa Fluor 568; Thermo Fisher Scientific) for 1 h. For myeloperoxidase staining, cells were co-incubated with anti-Myeloperoxidase antibody (clone 2C7, ab25989; Abcam) for 2 h and secondary Goat anti-Mouse IgG (H+L) secondary antibody (Alexa Fluor 633; Thermo Fisher Scientific) for 1h. Nuclei and DNA were visualized by Hoechst (Life technologies) and SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific) according to the manufacturer's protocols. After washing with PBS, VECTASHIELD Mounting Media (Vector Laboratories) was added on top of dried cells. Fluorescence microscopy was performed using Carl Zeiss LSM 880 confocal microscope and the images were analyzed by ZEN v2.3 image software. The researcher was blinded to the patient genotype when performing the experiment and analysis. Manual quantification of the numbers of cells with NET extrusions in neutrophils from SLE patients

were performed independently by 3 researchers in a blinded manner (counting >1000 cells/patient). For analysis H3cit expression in U937 cells, cells were plated and differentiated into macrophage-like cells with PMA on a 96-well plate (Black, clear bottom, BD Falcon). Cells were fixed and stained as described above. The cells were analyzed by ImageXpress reader micro XL and MetaXpress 5.1.0.41 software (Molecular Devices) was used for quantification. The multi wavelength cell scoring (MetaXpress) was used for quantification which enabled us to create a mask covering the H3cit positive areas within the cytoplasm. Fluorescence intensity was measured within the masks and the cells where the staining was higher than the threshold were classified as H3cit positive.

References to Supplementary Methods

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