Pro-inflammatory cytokine blockade attenuates myeloid expansion in a murine model of rheumatoid arthritis

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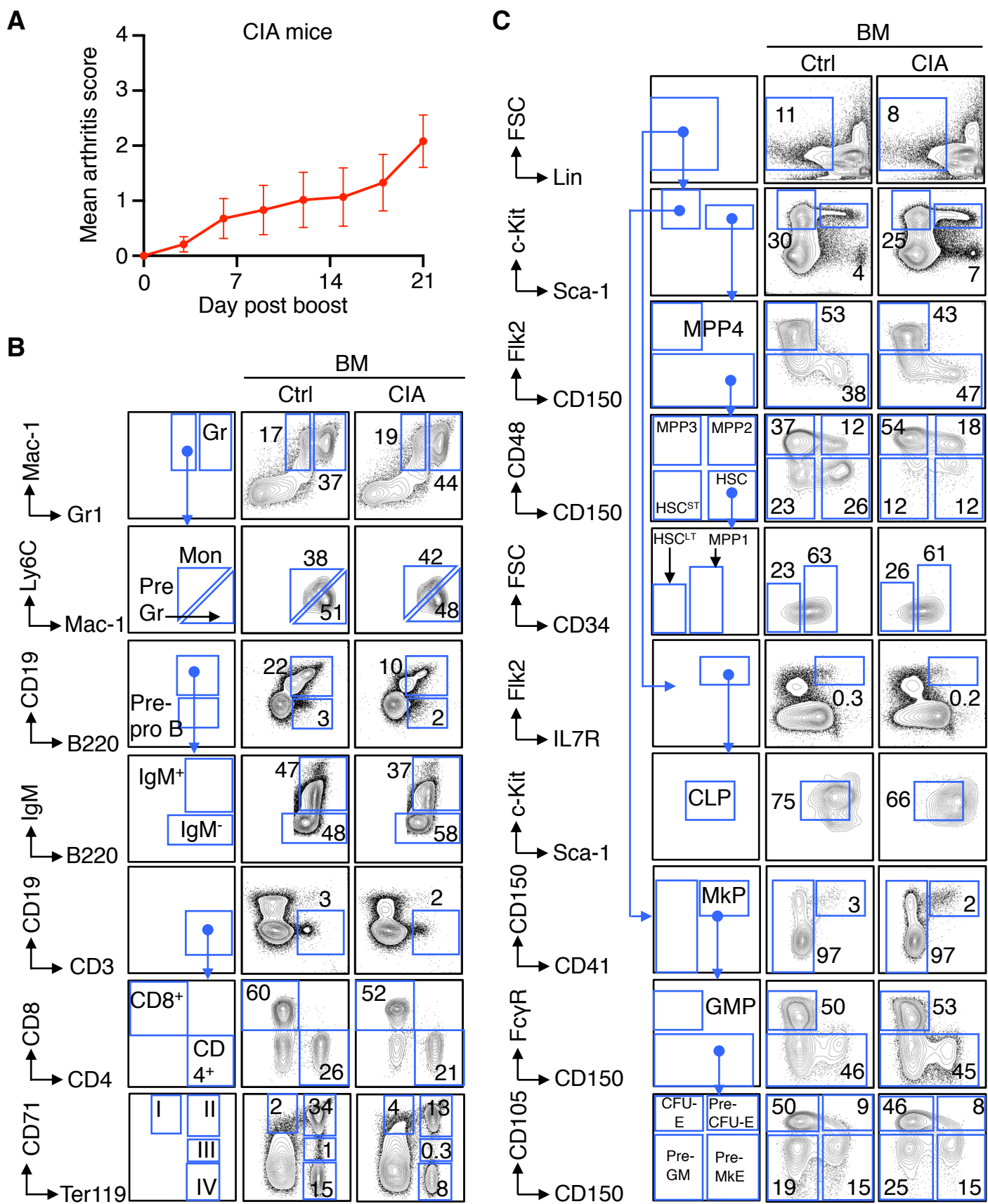
Supplementary Figure S1: Disease score and gating strategies for mature and HSPC BM populations.
(**A**) Clinical arthritis score of CIA mice (n = 11). (**B-C**) Representative FACS plots and frequencies of parent gates for BM (**B**) mature and erythroblast populations, and (**C**) hematopoietic stem and progenitor populations in Ctrl and CIA mice.
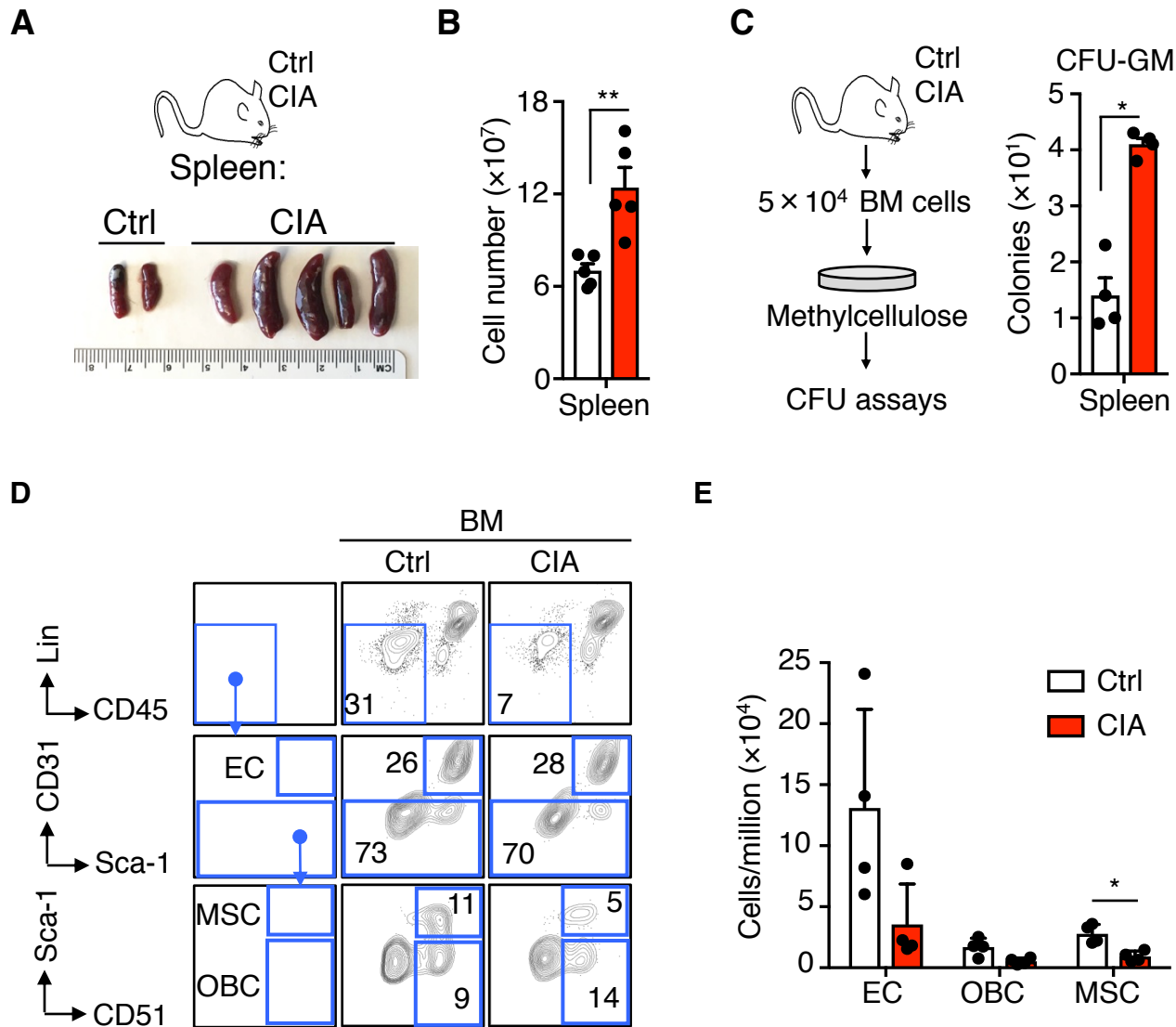
Supplementary Figure S2: Spleen and BM stroma changes in CIA mice. (**A**) Representative image showing sizes of Ctrl and CIA spleens; (**B**) Total cellularity of RBC-depleted spleens ($N = 5$/grp); (**C**) CFU-GM colony number from splenocytes of Ctrl and CIA mice ($N = 4$/grp). (**D**) Representative FACS plots and (**E**) number of the indicated BM stromal populations in Ctrl and CIA mice, expressed as number per million stromal cells. ($N = 4$/grp); *$p < 0.05$, **$p < 0.01$, as determined by Mann-Whitney u-test. Data are compiled from one experiment.
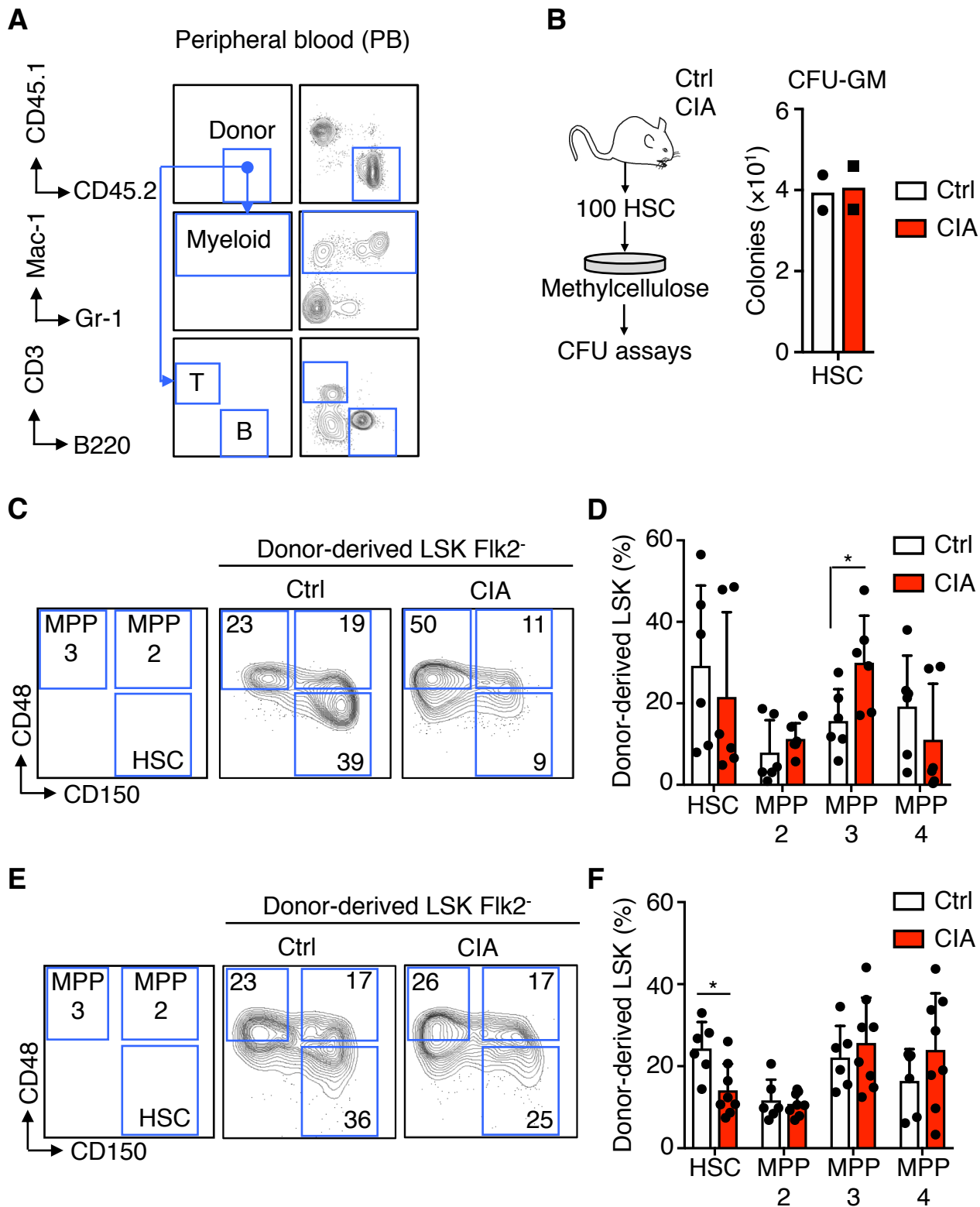
Supplementary Figure S3: CFU assay and HSC and progenitor chimerism in Ctrl and CIA transplant experiments. (**A**) Representative FACS plots showing CD45.2[+] donor chimerism and lineage analysis in PB from recipient mice. (**B**) CFU-GM colony number from HSC of Ctrl and CIA mice ($N = 2$/grp). (**C**) Representative FACS plots and (**D**) composition of donor-derived LSK compartment in recipient mice transplanted with purified HSC from Fig 3A. (**E**) Representative FACS plots and (**F**) composition of donor-derived LSK compartment in recipient mice transplanted with unfractionated BM cells from Fig 3C. Data in C-F are from recipient mice with >5% donor LSK chimerism to ensure sufficient events for analysis. *$p <$ 0.05 as determined by Mann-Whitney u-test. Data are representative of two independent experiments.

A

HSC
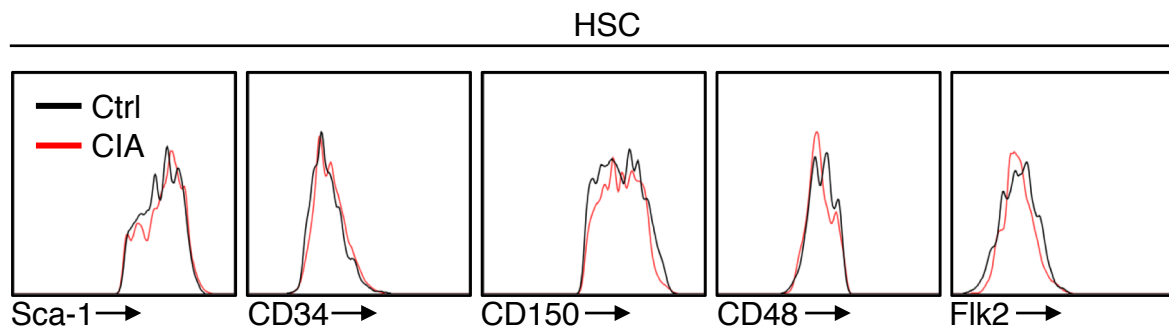
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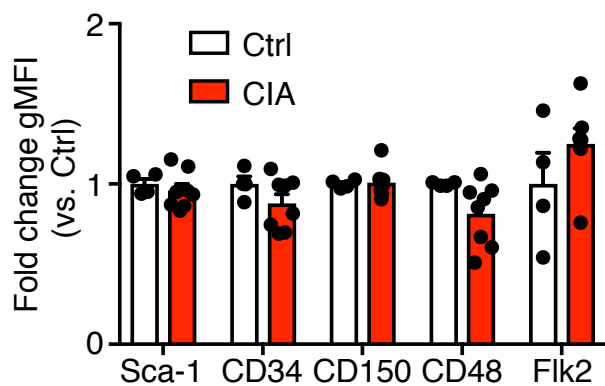
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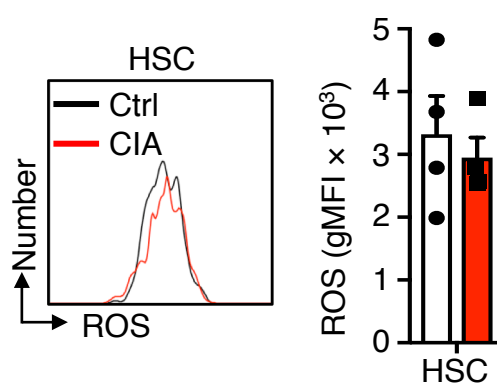
Supplementary Figure S4: **HSC surface markers and ROS levels.** (**A**) Representative FACS plots and (**B**) levels of the indicated surface markers, expressed as geometric mean fluorescence intensity (gMFI) (N = 4 Ctrl and 8 CIA). (**C**) Representative FACS plots and ROS levels in HSC from Ctrl and CIA, expressed as geometric mean fluorescence intensity (MFI) of CellROX dye. (N = 4 Ctrl and 4 CIA). Data are compiled from one experiment.
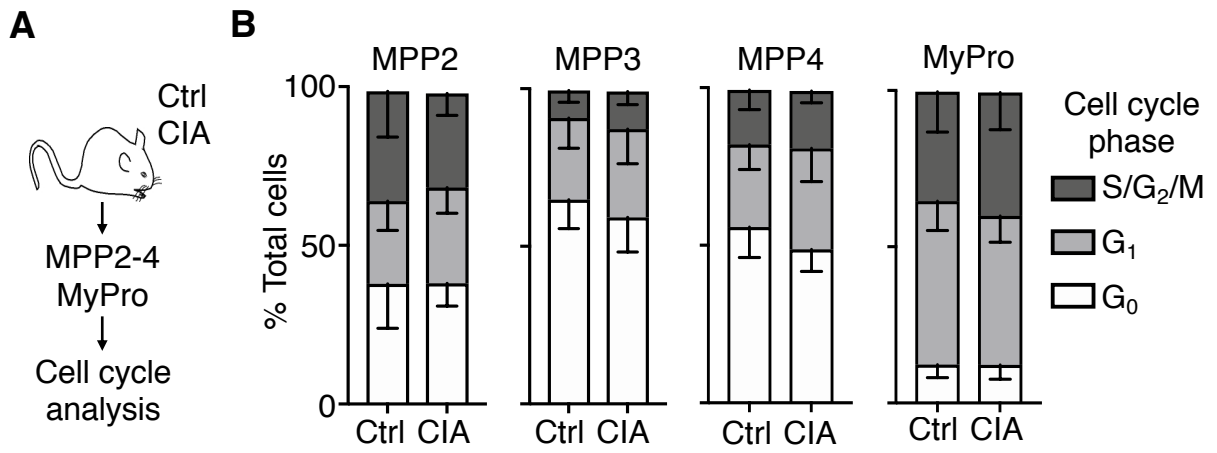
Supplementary Figure S5: **Hematopoietic progenitor cell cycle analysis.** (**A**) Experimental design and (**B**) cell cycle distribution of the indicated populations ($N = 8$ Ctrl and 6 CIA). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as determined by Mann-Whitney u-test. Data are compiled from two independent experiments.
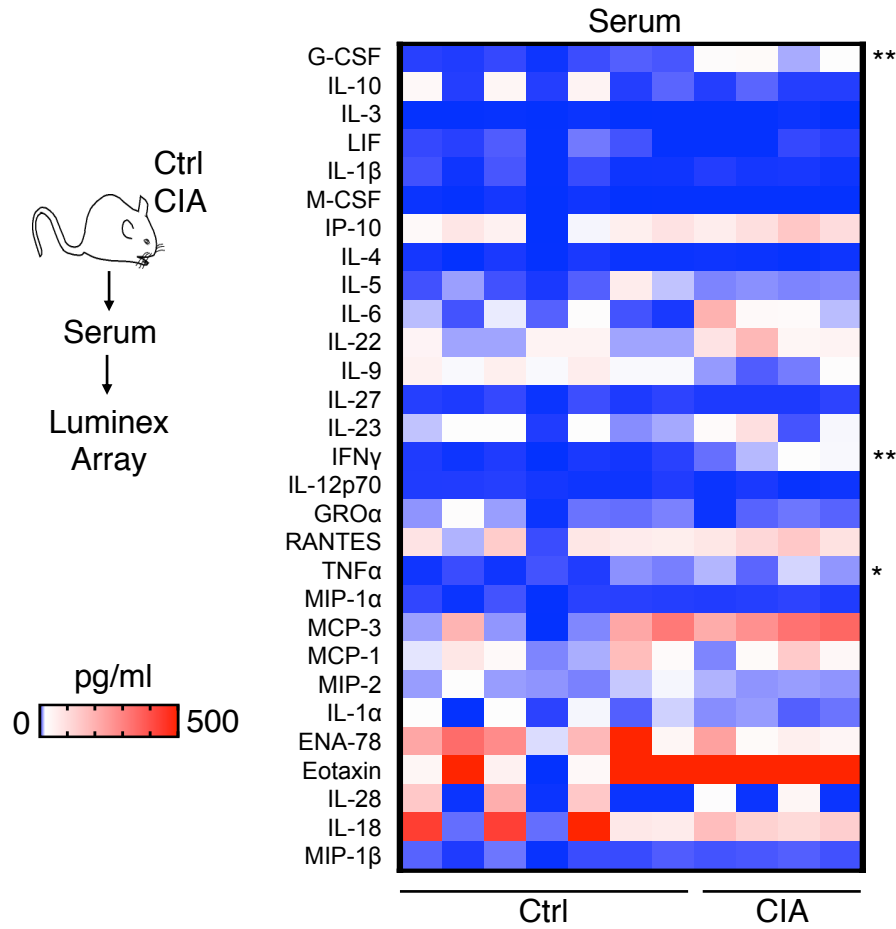
Supplementary Figure S6: Cytokine array analysis of serum. Experimental strategy and heatmaps from Luminex cytokine arrays showing cytokine levels for serum ($N = 7$ Ctrl and 4 CIA) collected from mice from two independent experiments. Statistically significant differences are indicated to the right of each row in the heatmaps. *$p < 0.05$; **$p < 0.01$ as determined by Mann-Whitney u-test.
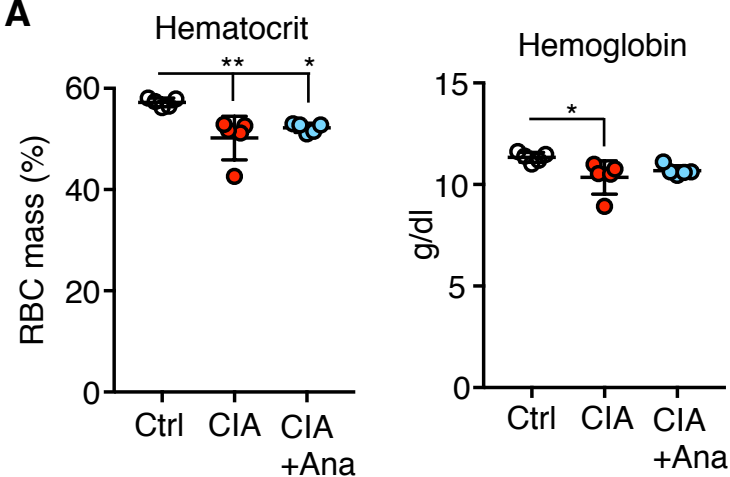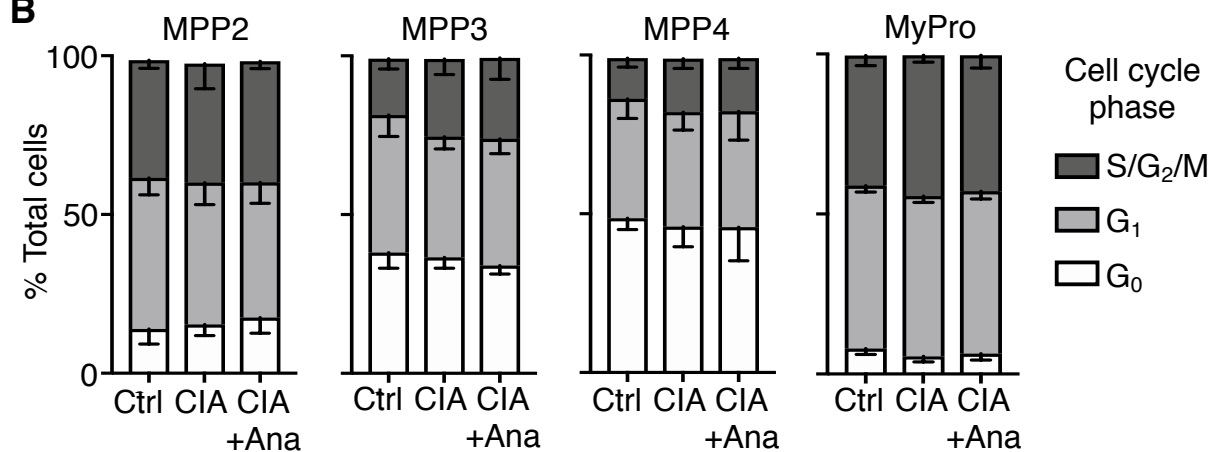
Supplementary Figure S7: Impact of IL-1 blockade on RBC and hematopoietic progenitor cell cycle distribution. (**A**) Complete blood count (CBC) analysis. (**B**) cell cycle analysis of MPP and MyPro populations in Ctrl, CIA and CIA +Anakinra (Ana) mice. (N = 5 Ctrl, 5 CIA and 5 CIA + Ana for all assays). **p < 0.05, **p < 0.01 as determined by one-way ANOVA with Tukey's test for multiple comparisons. Data are representative of two independent experiments.

Supplementary Table Legends

Supplementary Table S1: Up-and downregulated genes in CIA HSC. Data are expressed as log2 fold change in CIA HSC versus Ctrl HSC. Genes highlighted in bold are significantly differentially expressed based on p_{adj} <0.05.

Supplementary Table S2: Ingenuity Pathway analysis of CIA HSC. Upstream regulator analysis of significantly differentially expressed genes in CIA HSC versus Ctrl HSC, including activation z-score, p-value and pathway target molecules significantly enriched in each pathway. Items highlighted in bold are shown in Figure 4.

Supplementary Table S3: Gene ontology (GO) analysis of downregulated genes in CIA HSC. GO analysis of significantly differentially expressed genes in CIA HSC versus Ctrl HSC, showing p-value, number and percentage of genes enriched in each GO category, p-value and genes significantly enriched in each category. Items highlighted in bold are shown in Figure 4.

Supplementary Table S4: Gene ontology (GO) analysis of upregulated genes in CIA HSC. GO analysis of significantly differentially expressed genes in CIA HSC versus Ctrl HSC, showing p-value, number and percentage of genes enriched in each GO category, p-value and genes significantly enriched in each category. Items highlighted in bold are shown in Figure 4.

Supplementary Table S5: GSEA analysis downregulated genes in CIA HSC. Gene set enrichment analysis (GSEA) of RNA-seq data showing gene set names, gene set sizes, enrichment scores and p-values for each enriched gene set.

Supplementary Table S6: GSEA analysis upregulated genes in CIA HSC. Gene set enrichment analysis (GSEA) of RNA-seq data showing gene set names, gene set sizes, enrichment scores and p-values for each enriched gene set.

Supplementary Table S7: Antibodies used in this study. Antibody list includes clones, manufacturer, fluorochrome and dilution.

Supplementary methods

Collagen emulsion preparation, CIA induction and clinical scoring

CIA induction protocols are based on published, detailed methodologies for induction in C57BL/6J mice[1] and in B10.RIII mice[2]. Due to differences in strain susceptibility to CIA and disease course, protocols for C57BL/6 and B10.RIII mice are distinct. For C57BL/6 mice, collagen was prepared by making a 2mg/ml solution of chicken collagen in 0.01N acetic acid (Sigma) and mixed with an equal volume of Complete Freund's adjuvant (Sigma). CFA/collagen emulsions also contained 2mg/ml heat-killed *Mycobacterium tuberculosis* strain H37Ra (InVivoGen). For B10.RIII mice, a 2mg/ml solution of bovine collagen in 0.01N acetic acid (Sigma) was mixed with an equal volume of Complete Freund's adjuvant without additional heat-killed *Mycobacterium tuberculosis*. Both emulsions were produced by hand mixing components in 5 ml glass emulsifying syringes joined with 22G micro-emulsifying needles (Thomas Scientific). Syringes were kept partially submerged in ice water for 10 minutes, and complete emulsion was verified by dropping a bead of the material in water; completely emulsified beads hold their shape in water. 150 μl of emulsion was injected intradermally near the base of the tail in mice anesthetized with isoflurane. Booster injections were performed at day 15 (B10.RIII) or day 21 (C57BL/6J).

Induced C57BL/6J animals developed disease with approximately 40-80% penetrance. Animals failing to develop disease following the booster injection according to clinical scoring criteria were not included in final experimental cohorts. Induced B10.RIII animals developed disease with 100% penetrance. Arthritis was scored using a 5-point scale: 0 = normal; 1 = 1 hind or fore paw joint affected or minimal diffuse erythema and swelling; 2 = 2 hind or fore paw joints affected or mild diffuse erythema and swelling; 3 = 3 hind or fore paw joints affected or moderate diffuse erythema and swelling; 4 = marked diffuse erythema and swelling, or 4 digit joints affected;

and 5 = severe diffuse erythema and severe swelling of entire paw, unable to flex digits. Animals were analyzed 21 days after the second collagen type II injection. Peripheral blood parameters were determined via post-euthanasia retro-orbital bleeding and complete blood count (CBC) analysis using a HemaVet analyzer (Drew Scientific).

Detailed flow cytometry methods

Antibody clones and dilutions used are listed in **Supplementary Table 7**. All surface staining was performed as previously described[3] on ice in staining medium (SM; HBSS +2% heat-inactivated FBS). Following RBC depletion, cellularity was determined using a ViCell automated counter (Beckman Coulter) To identify HSPC, RBC-depleted cells were incubated on ice for 30 min. with the following antibodies: PE-Cy5-conjugated anti-CD3, CD4, CD5, CD8, B220, Gr-1, Mac-1 and Ter119 (Lineage), plus CD34-FITC, CD150-PE, FcγR-PerCP/eFluor710, IL-7R-PE/Cy7, Sca-1-BV421, CD41-BV510, Flk2-Biotin, CD105-BV786, ESAM-APC, CD48-AlexaFluor 700, and c-Kit-APC/Cy7 (Biolegend). 50ug/ml Rat IgG (Sigma) was included in all live staining incubations to block nonspecific antibody interactions. Cells were subsequently washed in SM, spun at 1200 × g, stained with streptavidin-BV605, and dead cells counterstained with SM containing 1μg/ml propidium iodide (PI). Mature BM and spleen populations were identified by staining with the following antibodies: CD4-FITC, CD8-PE, Mac-1-PE/Cy7, Gr-1-Pacific Blue, Ly6C-BV605, B220-BV786, IgM-APC, CD3-A700 and CD19-APC/Cy7 (Biolegend). For analysis of intracellular ROS, cells were surface stained as above, followed by incubation for 30 min in HBSS without FBS at 37°C with 5μM CellROX Deep Red dye (Thermo Fisher) according to manufacturer's instructions. Following incubation, cells were washed in SM, counterstained with PI and analysed by flow cytometry. BM stroma were analyzed as previously described[3,4]. Briefly, leg bones were crushed in HBSS without FBS, bone chips were collagenase-

I digested at 37°C while shaking for 1hr, and stained with Lineage-PE/Cy5, Sca-1 Pacific Blue, CD45-APC/Cy7, CD31-FITC, and CD51-Biotin, washed and stained by SA-APC. Cells were analyzed using a FACSCelesta or FACS LSRII (Becton-Dickenson). For cell cycle analysis, Ki67/DAPI staining was performed as previously described[5,6]. BM cells were stained with the aforementioned lineage cocktail, plus CD34-FITC, Flk2-PE, Sca-1-PE/Cy7, ESAM-APC, CD48-A700, c-Kit APC/Cy7, and CD150-BV785. Following staining, cells were washed in SM, fixed at room temperature (RT) for 30 min. with Cytofix/Cytoperm (BD Biosciences), washed and spun down at 1200 x g for 5 min. in 1x PermWash Buffer (BD Biosciences), permeablized in Cytoperm Plus (BD Biosciences) for 10 min. at RT, washed and spun again in 1x PermWash, and re-fixed in Cytofix/Cytoperm for 5 min. Fixed and permeablized cells were subsequently stained with anti-Ki67-PerCP/Cy5.5 for 30 min at RT, washed in 1x PermWash Buffer, and resuspended in SM containing 2µg/ml DAPI. For IL-1β intracellular staining, 1×10^6 BM cells were stained with the aforementioned mature cell panel, with CD8-BV510. Cells were subsequently fixed as described above, and stained with anti-IL-1β-PE for 30 min. at RT. Positive staining was determined using fluorescence minus one (FMO) controls. For cell sorting, c-Kit enriched BM cells were subsequently stained with the aforementioned lineage cocktail, plus CD150-PE, CD48-AlexaFluor 700, c-Kit-APC/Cy7, Sca-1-BV421, and Flk2-Biotin followed by SA-BV605. Cells were then counterstained with SM containing PI before sorting.

Methylcellulose cultures

For granulocyte-macrophage (GM) colony forming assays, 1×10^4 BM cells, 1×10^5 splenocytes or 100 purified HSC were cultured in a 3cm plate containing 1ml Methocult (Stemcell Technologies, M3231) supplemented with penicillin (50U/ml), streptomycin (50 µg/ml) (Life Technologies), SCF (25 ng/ml), IL-11 (25ng/ml), Flt3L (25ng/ml), GM-CSF (10ng/ml), IL-3

(10ng/ml), Epo (4U/ml), and Tpo (25ng/ml) (Peprotech). For CFU-E assays, 1×10^5 BM cells were plated in 1ml Methocult supplemented with recombinant human insulin, transferrin and EPO (Stemcell Technologies, M3334). For CFU-B assays, 1×10^4 BM cells were plated in 1ml Methocult supplemented with IL-7 (Stemcell Technologies, M3630). Plates were incubated in a humidified air-jacket incubator (Thermo Fisher) with 5% CO_2 at 37°C. Colonies were enumerated 2 (CFU-E), 7 (CFU-B) or 7-10 days (CFU-GM) after plating.

RNA-seq sample preparation, quality control and analysis

RNA was isolated from cell pellets using an RNEasy Micro kit (Qiagen) per manufacturer's recommendations. 150pg of cDNA was used to generate Illumina compatible sequencing libraries with the NexteraXT library preparation kit (Illumina) per manufacturer's protocols. RNA concentration was determined with the NanopDrop 1000 spectrophotometer (NanoDrop) and RNA quality assessed with the Agilent Bioanalyzer 2100 (Agilent). 1ng of total RNA was pre-amplified with the SMARTer Ultra Low Input kit v4 (Clontech) per manufacturer's recommendations. The quantity and quality of the subsequent cDNA was determined using the Qubit Flourometer (Life Technnologies) and the Agilent Bioanalyzer 2100 (Agilent). The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina). Single end reads of 100nt were generated for each sample using an Illumina HiSeq2500v4 high-throughput sequencer. Raw reads generated from the Illumina HiSeq2500 sequencer were demultiplexed using bcl2fastq version 1.8.4. Quality filtering and adapter removal were performed using Trimmomatic version 0.32 with the following parameters: "LEADING:13 TRAILING:13 SLIDINGWINDOW:4:20 ILLUMINACLIP:adapters.fasta:2:30:10 MINLEN:15". Processed/cleaned reads were then mapped to the GRCm38.p4 (mm10/mg38) with STAR_2.4.2a

with the following parameters: "--twopassMode Basic --runThreadN ${CPUS} --runMode alignReads --genomeDir ${STARREF}--readFilesInclt_${SAMPLE}_R1.fastq-- outSAMtypeBAM SortedByCoordinate—outSAMstrandFieldintronMotif --outFilterIntronMotifs RemoveNoncanonical --outReadsUnmapped Fastx". Raw read counts were obtained using htseq-count v0.6.1 and Gencode-M12 gene annotations. Additional analysis was performed using Ingenuity Pathway Analysis software with default user settings (Qiagen) and the GSEA tool (Broad Institute). GO analyses of significantly up- or downregulated genes were performed using DAVID 6.8 (https://david.ncifcrf.gov/).

Fluidigm gene expression analysis

Sample preparation and data analysis using the Fluidigm platform was performed as previously described[3,7]. Briefly, eight pools of 100 cells per well were sorted into 96-well PCR plates containing 5μl CellsDirect buffer (Invitrogen) and subjected to 18 rounds of specific target amplification using DeltaGene assay primer pairs commercially designed by Fluidigm. Genomic DNA contamination was removed using exonuclease 1 (New England Biolabs) digestion. Samples were mixed with SsoFast EvaGreen Supermix (BioRad), and sample and DeltaGene primer mixes were loaded and run on Fluidigm 96.96 or 48.48 IFC gene expression arrays in a Biomark instrument (Fluidigm). Ct values were obtained using Fluidigm Biomark analysis software and exported for expression was using the ΔCt method, normalized to *Actb*.

Serum collection for cytokine analysis

For serum collection, blood was obtained from mice via cardiac puncture with a 28.5G needle and 0.5ml syringe (Becton Dickenson). Blood was allowed to coagulate in a 1.5ml microcentrifuge tube (Eppendorf) at room temperature for 30 minutes. Samples were subsequently

spun at 10,000 × g for 10 minutes to separate blood cells and serum was stored frozen at -80ºC until analysis. Cytokine arrays were analyzed on a MAGPIX analyzer running xPONENT 4.2 software (Luminex).

Supplementary Methods References

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