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Interleukin-1β suppression dampens inflammatory leucocyte production and uptake in atherosclerosis

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Aims Targeting vascular inflammation represents a novel therapeutic approach to reduce complications of atherosclerosis. Neutralizing the pro-inflammatory cytokine interleukin-1β (IL-1β) using canakinumab, a monoclonal antibody, reduces the incidence of cardiovascular events in patients after myocardial infarction (MI). The biological basis for these beneficial effects remains incompletely understood. We sought to explore the mechanisms of IL-1β-targeted therapies.

Methods and results In mice with early atherosclerosis (*ApoE*$^{-/-}$ mice on a high-cholesterol diet for 6 weeks), we found that 3 weeks of NACHT, LRR, and PYD domains-containing protein 3 (NLRP3)-inflammasome inhibition or anti-IL-1β treatment (using either MCC950, an NLRP3-inflammasome inhibitor which blocks production and release of active IL-1β, or a murine analogue of canakinumab) dampened accumulation of leucocytes in atherosclerotic aortas, which consequently resulted in slower progression of atherosclerosis. Causally, we found that endothelial cells from atherosclerotic aortas lowered expression of leucocyte chemoattractants and adhesion molecules upon NLRP3-inflammasome inhibition, indicating that NLRP3-inflammasome- and IL-1β-targeted therapies reduced blood leucocyte recruitment to atherosclerotic aortas. In accord, adoptive transfer experiments revealed that anti-IL-1β treatment mitigated blood myeloid cell uptake to atherosclerotic aortas. We further report that anti-IL-1β treatment and NLRP3-inflammasome inhibition reduced inflammatory leucocyte supply by decreasing proliferation of bone marrow haematopoietic stem and progenitor cells, demonstrating that suppression of IL-1β and the NLRP3-inflammasome lowered production of disease-propagating leucocytes. Using bone marrow reconstitution

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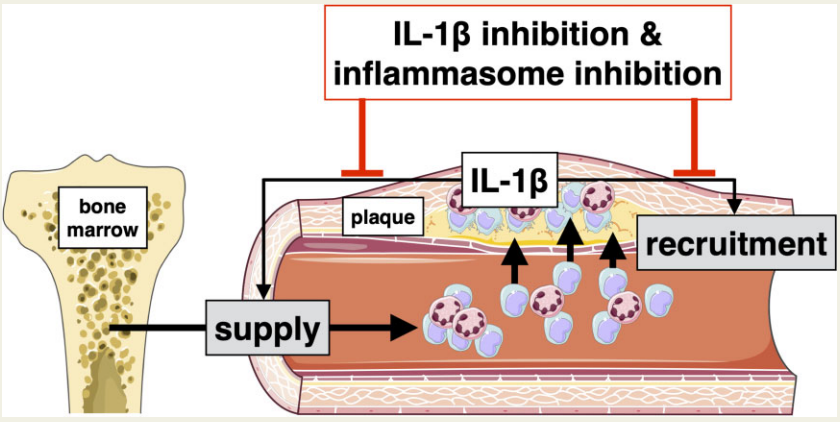
experiments, we observed that haematopoietic cell-specific NLRP3-inflammasome activity contributed to both enhanced recruitment and increased supply of blood inflammatory leucocytes. Further experiments that queried whether anti-IL-1β treatment reduced vascular inflammation also in post-MI accelerated atherosclerosis documented the operation of convergent mechanisms (reduced supply and uptake of inflammatory leucocytes). In line with our pre-clinical findings, post-MI patients on canakinumab treatment showed reduced blood monocyte numbers.

Conclusions Our murine and human data reveal that anti-IL-1β treatment and NLRP3-inflammasome inhibition dampened vascular inflammation and progression of atherosclerosis through reduced blood inflammatory leucocyte (i) supply and

Graphical Abstract



Our data suggest that both anti-IL-1β treatment and NLRP3-inflammasome inhibition beneficially alter the course of atherosclerosis by (i) dampening inflammatory leucocyte production in the bone marrow and consequently suppressing supply of these cells and (ii) de-activating plaque endothelial cells and hence reducing inflammatory leucocyte recruitment from blood to plaque.

and atherogenesis, and into the beneficial effects of NLRP3-inflammasome- and IL-1β-targeted therapies.

Translational perspective

Therapeutic targeting of vascular inflammation represents a promising avenue to reduce complications of atherosclerosis. Neutralizing the pro-inflammatory cytokine interleukin-1β (IL-1β) reduces the incidence of cardiovascular events in patients with prior myocardial infarction. However, the mechanisms underlying these beneficial effects remain incompletely understood. This study explored how IL-1β and NLRP3-inflammasome suppression mitigated plaque progression. Our murine and human data reveal that pharmacological anti-IL-1β treatment and NLRP3-inflammasome inhibition dampened inflammatory leucocyte accumulation in atherosclerotic aortas through (i) decreased blood inflammatory leucocyte supply and (ii) reduced blood inflammatory leucocyte uptake into in atherosclerotic aortas. These data provide additional mechanistic insights into links between haematopoiesis and atherogenesis, and inform future anti-inflammatory interventions in patients with atherosclerosis.

Keywords Inflammasome • Atherosclerosis • Myocardial infarction • Interleukin-1β • Innate immunity

1. Introduction

Atherosclerosis causes myocardial infarction (MI), many strokes, and ischaemic cardiomyopathy. The initiation of this chronic inflammatory disease of the arterial vessel wall involves augmented expression of leucocyte adhesion molecules and release of leucocyte-attracting cytokines and chemokines.[1–3] Consequently, leucocytes—the effector cells of the immune system—adhere to activated endothelial cells and then enter the subintimal space. Once resident in the arterial wall, monocytes mature into tissue macrophages, the most abundant leucocyte subset in plaques.[4] Macrophages engulf lipoprotein particles, become foam cells, and further fuel vascular inflammation. Cholesterol crystals in plaques can co-activate the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome in macrophages, resulting in the release of mature interleukin-1β (IL-1β).[5,6]

Interleukin (IL)-1, the prototypical pro-inflammatory cytokine, contributes to the initiation and progression of atherosclerosis.[7–11] Mature IL-1β is produced by the inflammasome, a multimeric protein complex that assembles in the cytosol after sensing pathogen-associated molecular patterns or danger-associated molecular patterns.[12] The sensing component of the inflammasome is an intracellular protein termed NLRP3, which binds the adaptor protein apoptosis-associated speck-like

protein, and procaspase-1 resulting in the cleavage of procaspase-1 to active caspase 1 (CASP1). CASP1 cleaves pro-IL-1β and pro-IL-18 and allows the extracellular release of active IL-1β and IL-18.[7,13] Of note, there are also CASP1-independent mechanisms for pro-IL-1β cleavage that involve intracellular CASP8 or extracellular proteases from neutrophils or mast cells.[14,15]

Although abundant evidence from preclinical and clinical studies strongly supported the notion that inflammation contributes to the pathogenesis of atherosclerosis, randomized controlled trials only recently confirmed its therapeutic relevance.[16–20] The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), a multinational double-blind phase III study, randomized more than 10 000 patients with previous MI (>30 days before screening) and a heightened residual inflammatory state [defined by high-sensitivity C-reactive protein (hsCRP) levels >2 mg/L] to three different doses of canakinumab or placebo.[7,16,21] Canakinumab, a human monoclonal antibody that neutralizes IL-1β, significantly lowered the risk for the primary (MI, stroke, cardiovascular death) and secondary endpoints. Importantly, treatment did not impact other risk factors, such as lipoprotein or blood pressure levels, but rather was related to the inflammatory response, since patients who experienced the greatest reductions in C-reactive protein levels had profited most from treatment.[22]

Though these results are encouraging, the mechanisms underlying these beneficial effects remain incompletely understood. This study explored how IL-1β suppression mitigated plaque progression. Our data revealed that pharmacological anti-IL-1β treatment and NLRP3-inflammasome inhibition dampen inflammatory leucocyte accumulation in atherosclerotic aortas through (i) decreased blood inflammatory leucocyte supply and (ii) reduced blood inflammatory leucocyte uptake into in atherosclerotic aortas.

2. Methods

2.1 Mice

Male and female *ApoE*$^{-/-}$ mice (B6.129P2-Apoetm1Unc/J, JAX stock #002052, backcrossed at least 10 generations to C57BL/6J inbred mice), *Ubc-GFP* mice [C57BL/6-Tg(UBC-GFP)30Scha/J, JAX stock #004353], and *LDLR*$^{-/-}$ mice (B6.129S7-*Ldlr*^{tm1Her}/J, JAX stock #002207, backcrossed to C57BL/6J mice for 10 generations) were purchased from the Jackson Laboratory and were bred in-house. Wild-type (C57BL/6 J, JAX stock #000664) and *Casp1*$^{-/-}$ mice (B6N.129S2-*Casp1*^{tm1Flv}/J, JAX stock #016621, on a mixed C57BL/6J; C57BL/6N genetic background) were purchased from the Jackson Laboratory and used in experiments directly. For atherosclerosis experiments, 8- to 12-week-old *ApoE*$^{-/-}$ mice were fed a high-cholesterol diet (HCD, 21.2% fat by weight and 0.2% cholesterol, TD.88137, Envigo) for 6 weeks. *ApoE*$^{-/-}$ mice that underwent MI were on HCD for a total of 10 weeks. *LDLR*$^{-/-}$ mice for bone marrow chimera generation were put on HCD for 12 weeks starting 6 weeks after bone marrow irradiation.

Age- and sex-matched littermates that were randomly assigned to groups were used in all experiments. At the end of the experiments, mice were euthanized by cervical dislocation or exsanguination under isoflurane anaesthesia (3%). All experiments were approved by the local authorities (ROB-55.2-2532.Vet_02-16-92) and carried out in accordance with the guidelines from Directive 2010/63/EU.

2.2 Anti-IL-1β treatment and NLRP3-inflammasome inhibition

For IL-1β-targeted therapy, we used either (i) isotype-matched control IgG2a [10 mg/kg bodyweight (BW), donated from Novartis, Basel, Switzerland] or anti-IL-1β (01BSUR, a murine analogue of canakinumab, 10 mg/kg BW, donated from Novartis, Basel, Switzerland) injected subcutaneously once a week for 3 weeks[23] or (ii) vehicle (PBS) or MCC950 (10 mg/kg BW, AG-CR1-3615-M010, AdipoGen Life Sciences, USA, dissolved in PBS to generate a 2.5 mg/mL stock solution) injected intraperitoneally every 48 h for 3 weeks.[24]

2.3 Generation of bone marrow chimeras/bone marrow reconstitution

Bone marrow chimeras were generated to analyse the effects of a haematopoietic cell-specific caspase-1 (Casp1) knockout. Here, *LDLR*$^{-/-}$ mice aged 8 weeks underwent lethal bone marrow irradiation by exposure to a single dose of 9 Gy total body irradiation using a Faxitron X-Ray 43855F device. Bone marrow from both wild-type and *Casp1*$^{-/-}$ donor animals was extracted from femurs by flushing the bones and filtering the cells through a 40 µm cell strainer. Irradiated *LDLR*$^{-/-}$ mice were injected intravenously with 3×10^6 bone marrow cells from either wild-type or *Casp1*$^{-/-}$ animals 6 h after irradiation.

2.4 MI surgery

For MI experiments, mice were intubated under MMF anaesthesia (midazolam 5.0 mg/kg BW; medetomidine hydrochloride 1.0 mg/kg BW; fentanyl citrate 0.05 mg/kg BW; intraperitoneally) and thoracotomy was performed in the left intercostal space. The left anterior descending coronary artery was identified and MI was induced by permanent ligation with an 8-0 prolene suture. Atipamezole hydrochloride (5 mg/kg BW) and flumazenil (0.1 mg/kg BW) (AF) injected subcutaneously was used to antagonize MMF anaesthesia. Mice received subcutaneous buprenorphine (0.3 mg/kg BW) as an analgesic every 8 h for 3 days starting at the end of the surgical procedure.

2.5 Tissue processing

Blood samples were obtained by cardiac puncture and subjected to red blood cell lysis in RBC lysis buffer (420302, BioLegend). The reaction was stopped with 1× PBS, centrifuged at 800 *g* for 10 min at 4°C and resuspended in FACS buffer (PBS containing 0.5% bovine serum albumin, A2153, Sigma). Bone marrow single-cell suspensions were obtained from femurs by flushing the bones and filtering the cells through a 40 µm cell strainer. Atherosclerotic aortas were excised removing surrounding tissue and minced in digestion buffer. For flow cytometric analysis of aortic leucocytes, digestion buffer consisted of collagenase I (450 U/mL, C0130), collagenase XI (125 U/mL, C7657), DNase I (60 U/mL, D5319-500UG), and hyaluronidase (60 U/mL, H3506, all Sigma) in 1× PBS, and aortas were digested at 750 rpm for 1 h at 37°C. For flow cytometric analysis of aortic endothelial cells, aortas were digested in 1× PBS containing DNase I (250 U/mL) and collagenase IV (10 mg/mL, LS004212, Cell Systems) at 750 rpm for 40 min at 37°C. Following both digestion protocols, aortas were processed through a 40 µm cell strainer, centrifuged at 800 *g* for 10 min at 4°C, and resuspended in FACS buffer to generate single-cell suspensions.

After single-cell suspensions were generated, a 10 µl aliquot of each sample was taken and total numbers of leucocytes were manually determined using a haemocytometer. Aliquots for cell counting were diluted in trypan blue to distinguish dead from viable leucocytes. Frequencies

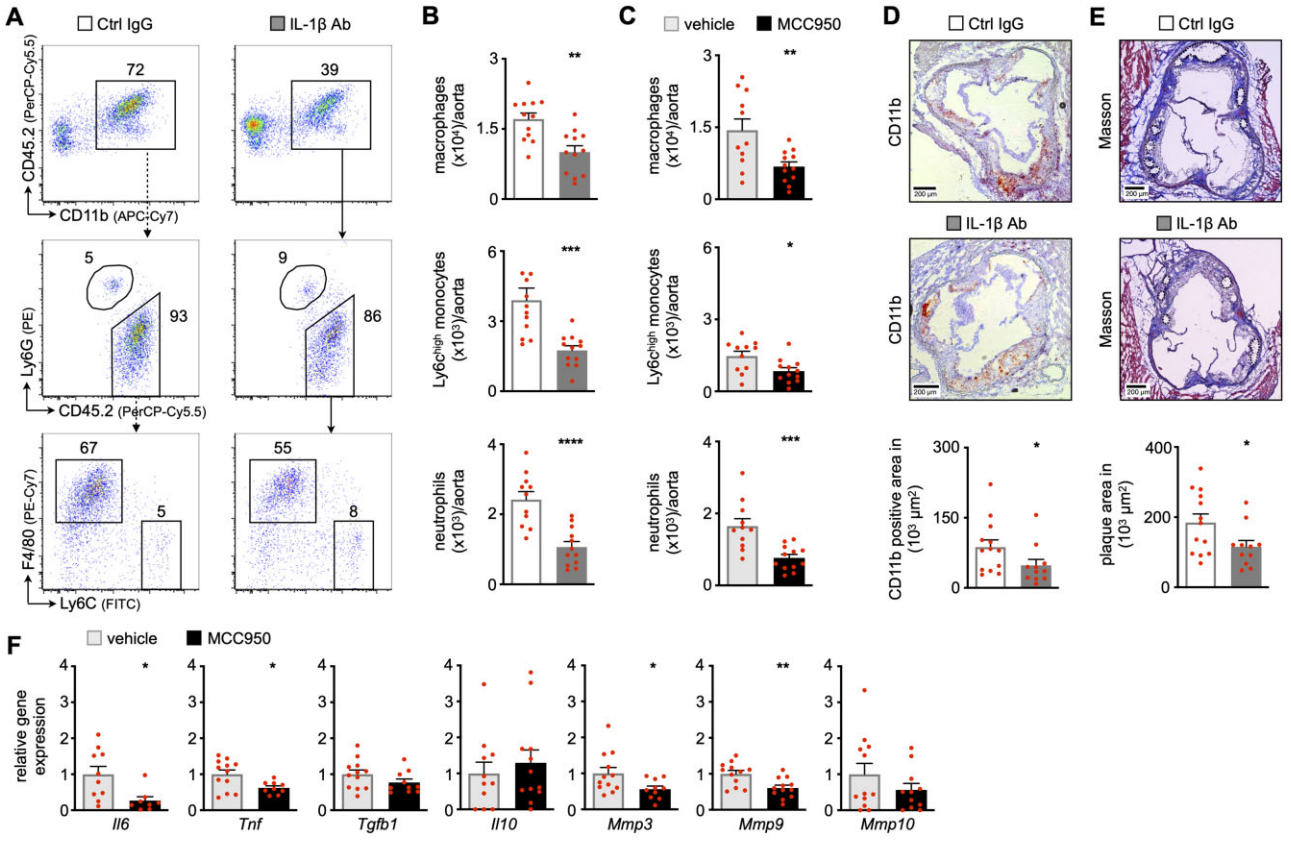


Figure 1 Anti-IL-1β treatment and NLRP3-inflammasome inhibition reduce plaque inflammation. (*A*) Flow cytometric gating and quantification of myeloid cells in atherosclerotic aortas in (*B*) IgG-treated (Ctrl IgG) vs. IL-1β neutralizing antibody-treated (IL-1β Ab) *ApoE*$^{-/-}$ mice ($n = 11$–12 per group, 67% female, Student's *t*-test) and (*C*) vehicle- vs. MCC950-treated (inflammasome inhibitor) *ApoE*$^{-/-}$ mice ($n = 10$–12 per group, 9–17% female, Student's *t*-test). Numbers next to gates indicate population frequencies (%). (*D*) Representative immunohistochemical staining for myeloid cells (CD11b) and quantification of sectioned aortic roots from IgG- vs. IL-1β antibody-treated *ApoE*$^{-/-}$ mice ($n = 11$–13 per group, 46–55% female, Mann–Whitney *U* test). Bar graphs show quantification of positive CD11b area. Scale bars represent 200 µm. (*E*) Representative Masson Trichrome staining and quantification of total plaque area ($n = 11$–13 per group, 46–55% female, Student's *t*-test). Scale bars represent 200 µm. Dotted lines exemplary show necrotic cores within atherosclerotic plaques. (*F*) Quantitative real-time PCR for gene expression quantification of fibrotic and inflammatory genes in aortas of vehicle- vs. MCC950-treated *ApoE*$^{-/-}$ mice ($n = 9$–12 per group, 58% female, Student's *t*-test or Mann–Whitney *U* test as appropriate). IL-6, interleukin 6; IL-10, interleukin 10; MMP-3/MMP-9/MMP-10, matrix metalloproteinase-3/-9/-10; TGFβ1, transforming growth factor beta 1; TNF, tumour necrosis factor. Data are presented as mean + s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

obtained from flow cytometry were applied to the number of total leucocytes to assess the numbers of leucocyte subsets.

2.6 Flow cytometry

All samples were stained at 4°C in 300 µl FACS buffer after generation of single-cell suspensions as described above.

For myeloid cell subset analysis, cells were first incubated with antibodies against murine haematopoietic lineage markers [all labelled with phycoerythrin (PE)] including B220 (103208, clone RA3-6B2); CD90.2 (140308, clone 53-2.1, 1:300 dilution); CD49b (108908, clone DX5, 1:1200 dilution); NK1.1 (108708, clone PK136); Ter-119 (116208, clone TER-119); and Ly6G (127608, clone 1A8). A second staining followed using CD45.2-PerCP/Cy5.5 (109828, clone 104, 1:300 dilution); CD11b-APC/Cy7 (101226, clone M1/70); CD115-BV711 or CD115-biotin (135515 or 135508, clone AFS98); streptavidin-BV510 (405234, 1:300 dilution, used for secondary labelling of CD115-biotin); F4/80-PE/Cy7 (123114, clone BM8); and Ly6C-FITC or Ly6C-BV421 (128006 or 128032, clone HK1.4, 1:600 dilutions unless indicated otherwise, all BioLegend).

For each analysis, cells were pre-gated on viable (FSC-A vs. SSC-A) and single (FSC-A vs. FSC-W and SSC-A vs. SSC-W) cells. Myeloid cell subsets were identified as indicated: neutrophils as lineage^{high}CD45.2^{high}CD11b^{high}CD115^{low}Ly6C^{intermediate}, monocytes as lineage^{low}CD45.2^{high}CD11b^{high}F4/80^{low}Ly6C$^{high/low}$ (in aortic cell suspensions) or lineage^{low}CD45.2^{high}CD11b^{high}CD115^{high}Ly6C$^{high/low}$ (in blood), and macrophages as lineage^{low}CD45.2^{high}CD11b^{high}Ly6C$^{low/intermediate}$F4/80^{high}. We performed *in vivo* blood pool labelling using an antibody directed against CD45-BV605 (103140, clone 30-F11, 1:10 dilution in 100 µL PBS, BioLegend) that was intravenously injected 5 min before euthanizing the animals. This technique was carried out in all flow cytometry experiments involving aortic samples and allowed us to exclude contamination from blood cells.

Aortic endothelial cell samples were stained with antibodies against CD54-APC [intercellular adhesion molecule (ICAM)1] (116120, clone YN1/1.7.4, 1:600 dilution); CD102-biotin (ICAM2) (105604, clone 3C4, 1:600 dilution); CD106-PE/Cy7 [vascular cell adhesion molecule (VCAM)1] (105720, clone 429, 1:300 dilution); CD62E-PE (E-Selectin) (553751, clone 10E9.6, 1:300 dilution, BD Bioscience); CD62P-FITC (P-Selectin) (553744, clone RB40.34, 1:300 dilution, BD Bioscience); CD31-BV421 (102424, clone 390, 1:600 dilution); CD107a-APC/Cy7 [lysosomal associated membrane protein (LAMP)1] (121616, clone 1D4B, 1:300 dilution); and CD45.2-PerCP/Cy5.5 (109828, clone 104, 1:300 dilution, all BioLegend unless indicated otherwise). Streptavidin-BV510 (405234, 1:300 dilution, BioLegend) was used as secondary staining for the biotinylated CD102 antibody. Endothelial cells were identified as $CD45.2^{low}CD31^{high}CD107a^{intermediate/high}$ and adhesion molecule expression was quantified using respective histograms. *In vitro* cultured primary murine aortic endothelial cells were stained identically except for CD45.2.

To analyse proliferating $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSKs) and haematopoietic stem cells (HSCs) in the bone marrow, cells were first stained with antibodies against murine haematopoietic lineage markers (all labelled with biotin) including B220 (103204, clone RA3-6B2); CD4 (100404, GK1.5), Gr-1 (108404, RB6-8C5), NK1.1 (108704, clone PK136), Ter119 (116204, clone TER-119), CD11b (101204, M1/70), CD11c (117304, N418), IL-7R (135006, A7R34), and CD8 (100704, 53-6.7). This was followed by a second staining for CD48-PE (103406, HM48-1), CD150-PerCP-Cy5.5 (115922, TC15-12F12.2, 1:300 dilution), sca-1-PE-Cy7 (108114, D7), c-kit-APC (105812, 2B8), and Strepavidin-APC-Cy7 (405208, 1:600 dilutions unless indicated otherwise, all BioLegend) to label biotinylated lineage antibodies. We used the Foxp3/Transcription Factor Staining Buffer Set (00-5523-00, eBioscience) according to the manufacturer's protocol to fix and permeabilize the samples and next performed an intracellular staining with Ki67-FITC (11-5698-82, SolA15, eBioscience, 1:100 dilution). Nuclear stain ('DAPI', FxCycle™ violet stain, F10347, ThermoFisher) was added immediately before flow cytometric analysis and resuspended thoroughly. LSKs were identified as $lineage^{neg}c\text{-}kit^{pos}sca\text{-}1^{pos}$ and HSCs were identified as $lineage^{neg}c\text{-}kit^{pos}sca\text{-}1^{pos}CD48^{neg}CD150^{pos}$.

For compensation, antibodies from the above-mentioned staining protocols were conjugated to OneComp eBeads (01-1111-42, Thermo Fisher). GFP^{high} and $DAPI^{high}$ control samples were used to compensate for GFP and DAPI fluorescence, respectively. To acquire flow cytometry data, an LSRFortessa instrument (BD Bioscience) was used, and files were analysed using FlowJo software (version 9 or 10).

2.7 Blood monocyte counts in anti-IL-1β-treated patients

Monocyte counts were assessed throughout trial follow-up in the CANTOS, which evaluated IL-1β inhibition among stable post-MI patients with residual inflammatory risk (hsCRP levels > 2 mg/L).[16] The CANTOS trial was conducted in accordance to the principles outlined in the Declaration of Helsinki. The study protocol was approved by respective institutional review boards or ethics' committees at participating centres. Repeat monocyte counts over time were available among 3065 trial participants randomly allocated to placebo and among 1974, 2103, and 2094 trial participants randomly allocated to canakinumab at doses of 50 mg, 150 mg, and 300 mg subcutaneously every 3 months, respectively. Monocyte counts were obtained from differential white blood cell counts, which were analysed by respective clinical pathology laboratories. To address for potential dose-response effects over time, the placebo-subtracted mean percent reduction in monocytes was calculated for each dose at 1, 3, 6, 12, 18, 24, and 36 months of follow-up with the statistical significance at each time-point, compared to placebo, estimated by the Wilcoxon 2-sample test.

2.8 Statistical analysis

All statistical analysis was performed using GraphPad Prism version 8. Normality distribution of mouse and cell culture data was checked using the D'Agostino-Pearson omnibus normality test. Two-group comparisons were analysed using two-sided Student's unpaired/paired *t*-test (normally distributed data) or two-sided Mann–Whitney *U* test (non-normally distributed data), as indicated in the figure legends together with sample sizes. When comparing more groups, a one-way ANOVA followed by a Tukey test for multiple comparisons was performed. A two-sided ROUT's test was used to determine statistical outliers. All graphs display data as mean + s.e.m. Statistical significance was assumed if *P*-values were <0.05. Mouse experiments were performed at least twice or with $n \geq 10$. If appropriate, inter-experimental variations were adjusted by normalizing absolute values to one representative experiment.

Detailed methods are provided in the Supplementary material online.

3. Results

3.1 Anti-IL-1β treatment and NLRP3-inflammasome inhibition reduce inflammatory leucocyte accumulation in atherosclerotic aortas

To explore the mechanisms by which anti-IL-1β treatment beneficially alters the course of atherosclerosis, we treated atherosclerosis-prone mice ($ApoE^{-/-}$ mice on a high cholesterol diet for 6 weeks) with an IL-1β neutralizing antibody, the murine version of canakinumab, for 3 weeks.[11,23,25] We found that pharmacological IL-1β neutralization reduced inflammatory leucocyte numbers in atherosclerotic aortas (*Figure 1A and B*, Supplementary material online, *Figure S1A*). In a complementary approach, we inhibited upstream IL-1β production using MCC950, a specific small-molecule inhibitor of the NLRP3 inflammasome.[24,26–29] In accordance with IL-1β-neutralizing antibody treatment, 3 weeks of MCC950 administration also lowered leucocyte numbers in atherosclerotic aortas (*Figure 1C*, Supplementary material online, *Figure S1B*). We corroborated our flow cytometry findings using immunohistochemistry and observed 50% fewer myeloid cells (monocytes/macrophages and neutrophils) in aortic root sections from anti-IL-1β-treated mice stained for the myeloid marker CD11b (*Figure 1D*).

Next, we tested whether reduced plaque inflammation changed the overall plaque character. Histology revealed a smaller total plaque size and smaller necrotic cores in the anti-IL-1β group (*Figure 1E*, Supplementary material online, *Figure S1C*). Furthermore, characterization of plaques using quantitative polymerase chain reaction revealed that MCC950 treatment reduced expression of mRNAs that encode the pro-inflammatory cytokines IL-6 (a cytokine downstream of IL-1β) and tumour necrosis factor (TNF) (*Figure 1F*). Moreover, levels of matrix metalloproteinase (MMP)-3 and MMP-9 decreased in treated mice (*Figure 1F*). MMPs support extracellular matrix degradation, a process that may lead to atherosclerotic plaque destabilization.[30] We also tested whether the effect of murine canakinumab lasts after discontinuation

and found that its beneficial effect on plaque size may be transient and may not be sustained long-term (Supplementary material online, *Figure S1D* and *E*). However, further experiments including additional time-points are needed to fully elucidate a durable, long-term effect of anti-IL-1β therapy. Of note, anti-IL-1β treatment and NLRP3-inflammasome inhibition did not alter body weight, spleen weight, or cholesterol levels in treated vs. non-treated mice (Supplementary material online, *Figure S1F–K*). Taken together, these data indicate that anti-IL-1β treatment and NLRP3-inflammasome inhibition limited plaque inflammation and hence progression of atherosclerosis.

3.2 Anti-IL-1β treatment and NLRP3-inflammasome inhibition dampen leucocyte recruitment

We next addressed how anti-IL-1β treatment reduced leucocyte accumulation in atherosclerotic aortas. To explore whether anti-IL-1β treatment dampened blood inflammatory leucocyte recruitment to atherosclerotic aortas, we performed adoptive transfer experiments. Here, we isolated GFP^{high} myeloid cells (monocytes admixed with neutrophils) from naïve transgenic *Ubc-GFP* mice (all leucocytes express green fluorescent protein, GFP) and injected these cells intravenously into $ApoE^{-/-}$ mice (all cells are $GFP^{negative}$), which were treated with either control or anti-IL-1β for 3 weeks (Supplementary material online, *Figure S2A*). Twenty-four hours after the transfer, we quantitated GFP^{high} myeloid cells inside atherosclerotic aortas using flow cytometry and found that anti-IL-1β treatment lowered GFP^{high} myeloid cell numbers (*Figure 2A*). These data show that anti-IL-1β mitigates myeloid cell uptake from blood to atherosclerotic aortas.

To investigate how anti-IL-1β treatment limited leucocyte recruitment from blood to atherosclerotic aortas, we used flow cytometry to assess levels of leucocyte adhesion molecules on endothelial cells from atherosclerotic aortas. Our data revealed that NLRP3-inflammasome inhibition lowered protein levels of adhesion molecules ICAM-1, ICAM-2, VCAM-1, E-Selectin, and P-Selectin (*Figure 2B and C*). In parallel, we also found reduced gene expression of leucocyte-attracting chemokines C-X-C motif chemokine ligand 1 (*Cxcl1*), C-X-C motif chemokine ligand 12 (*Cxcl12*), and C-C motif chemokine ligand 7 (*Ccl7*) in atherosclerotic aortas of treated mice (*Figure 2D*). Incubation of cultured murine aortic endothelial cells with recombinant IL-1β increased levels of leucocyte adhesion molecules VCAM-1, E-Selectin, and P-Selectin (Supplementary material online, *Figure S2B and C*). In sum, these data demonstrate that IL-1β enhances blood leucocyte recruitment into atherosclerotic aortas by activating aortic endothelial cells, an effect which was mitigated by anti-IL-1β treatment and NLRP3-inflammasome inhibition.

3.3 Anti-IL-1β treatment and NLRP3-inflammasome inhibition suppresses blood leucocyte supply

The bone marrow continuously produces leucocytes derived from haematopoietic stem and progenitor cells (HSPC).[31] We next assessed whether decreased supply of blood inflammatory leucocytes also contributes to the observed reduction in leucocyte numbers in atherosclerotic aortas after anti-IL-1β treatment and NLRP3-inflammasome inhibition. IL-1β and NLRP3-inflammasome suppression indeed lowered blood inflammatory leucocyte numbers (*Figure 3A–C*). Thus, we next tested this mechanistic hypothesis. Upon anti-IL-1β treatment and NLRP3-inflammasome inhibition, HSC and LSK progenitor cells—both precursor cells of mature leucocytes—proliferated less and their numbers declined in the bone marrow (*Figure 4A–C*), indicating that anti-IL-1β treatment and NLRP3-inflammasome inhibition lowered blood leucocytes by suppressing leucocyte production in the bone marrow.

Parenchymal cells frame HSPCs in the bone marrow and regulate haematopoiesis by secreting factors that signal to HSPCs to control their proliferative capacity and motility.[32] In that context, niche factors angiopoietin-1, osteopontin, stem cell factor, and C-X-C motif chemokine 12 control haematopoietic progenitor proliferation, while vascular cell adhesion molecule 1 promotes HSPC retention in the haematopoietic niche.[33] We next asked how NLRP3-inflammasome inhibition suppresses HSPC proliferation: does NLRP3-inflammasome suppression affect haematopoietic cells directly or act on niche cells regulating HSC activity? To this end, we found no difference in niche cell factor expression levels in the bone marrow after NLRP3-inflammasome suppression, indicating that IL-1β may elevate proliferation by directly interfering with HSPCs (*Figure 4D*), although further experiments are needed to fully elucidate that link.

3.4 Haematopoietic cell-specific NLRP3-inflammasome activity contributes to vascular inflammation

Since different cells inside plaques can produce IL-1β (e.g. leucocytes, endothelial cells, and vascular smooth muscle cells),[34] we next determined the contribution of haematopoietic cell-specific NLRP3-inflammasome activity to enhanced leucocyte accumulation in atherosclerotic aortas. Here, we performed bone marrow reconstitution experiments in which we lethally irradiated $Ldlr^{-/-}$ mice that were subsequently reconstituted with bone marrow from either wild-type or $Casp1^{-/-}$ mice to generate bone marrow chimeras. This experimental set-up enabled us to generate a scenario in which non-haematopoietic cells are able to produce mature IL-1β, while no haematopoietic cells can activate IL-1β NLRP3-inflammasome-dependent since they lack Casp1, an integral part of the inflammasome (*Figure 5A*). We first determined leucocyte numbers in atherosclerotic aortas and found fewer inflammatory leucocytes in atherosclerotic aortas from mice with haematopoietic cell-specific NLRP3-inflammasome depletion (*Figure 5B and C*).

We next addressed whether haematopoietic cell-specific NLRP3-inflammasome activity contributes to blood inflammatory leucocyte recruitment. To explore this, we adoptively transferred GFP^{high} myeloid cells retrieved from naïve transgenic *Ubc-GFP* mice into the bone marrow chimeric mice described above (*Figure 5A*). We found reduction of GFP^{high} myeloid cells in atherosclerotic aortas from mice with haematopoietic cell-specific Casp1 depletion (*Figure 5D*), indicating that haematopoietic cell-specific NLRP3-inflammasome activity substantially supports leucocyte recruitment to atherosclerotic aortas. Furthermore, we explored whether haematopoietic cell-specific NLRP3-inflammasome activity takes part in blood leucocyte supply and found reduced leucocyte production in the bone marrow of mice with haematopoietic cell-specific NLRP3-inflammasome depletion (*Figure 5E*). Consequently, blood inflammatory leucocyte numbers were lower in these mice (*Figure 5F*). These experiments indicate that haematopoietic cell-specific NLRP3-inflammasome activity substantially contributed to both leucocyte recruitment and leucocyte supply.

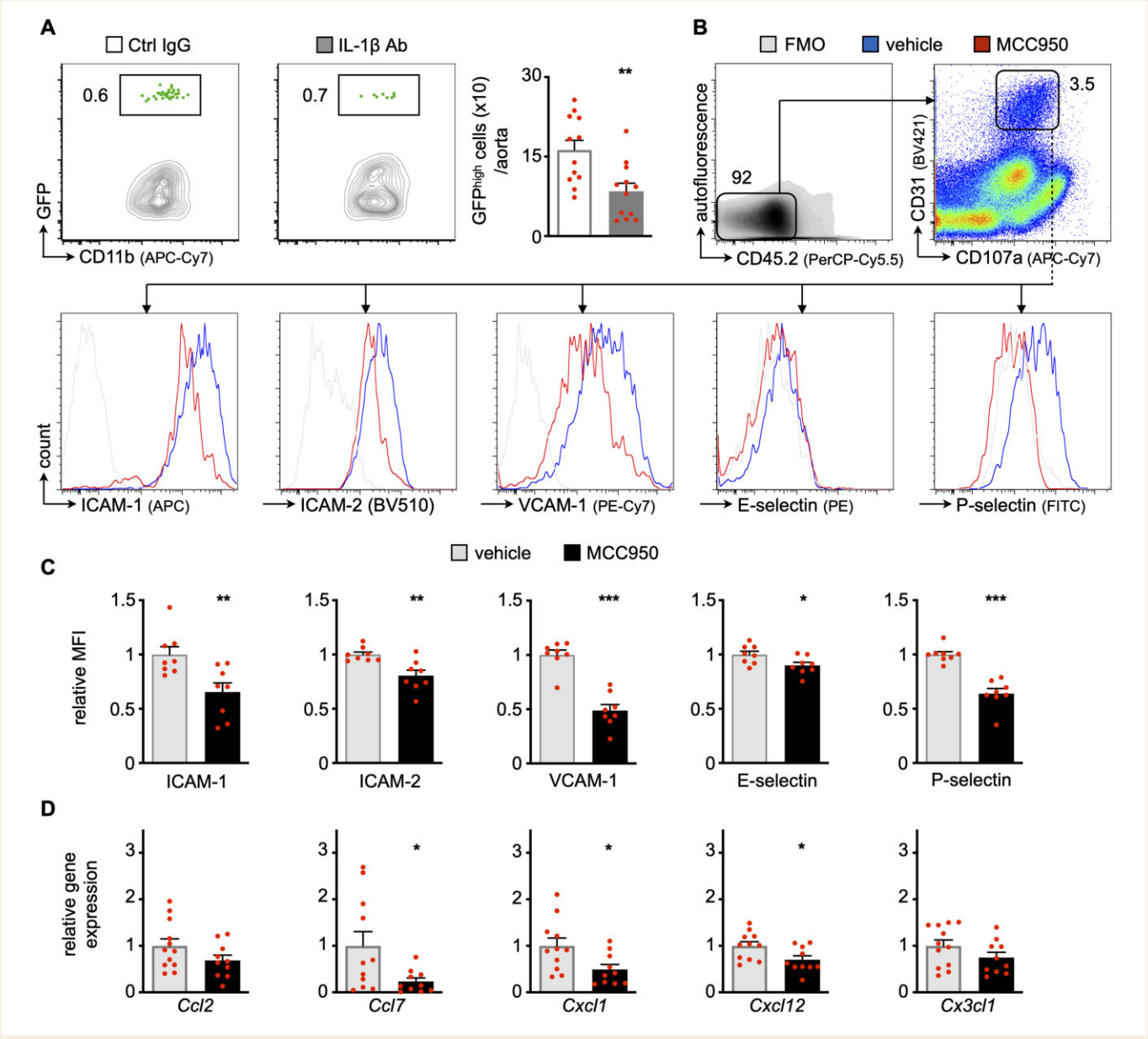


Figure 2 Anti-IL-1β treatment and NLRP3-inflammasome inhibition dampen leucocyte recruitment to atherosclerotic aortas by reducing adhesion molecule expression on aortic endothelial cells. (*A*) Flow cytometric gating and quantification of GFP^{high} myeloid cells in atherosclerotic aortas 24 h after adoptive transfer of GFP^{high} monocytes and neutrophils into IgG- vs. IL-1β antibody (IL-1β Ab)-treated *ApoE*$^{-/-}$ mice ($n = 12$ per group, 67% female, Student's *t*-test). (*B*) Gating strategy and histograms of leucocyte adhesion molecules on aortic endothelial cells from vehicle- vs. MCC950-treated *ApoE*$^{-/-}$ mice. (*C*) Quantification of mean fluorescent intensities (MFI, representing relative protein levels) of adhesion molecules expressed by aortic endothelial cells from vehicle vs. MCC950-treated *ApoE*$^{-/-}$ mice ($n = 8$ per group, 38% female, Student's *t*-test or Mann–Whitney *U* test as appropriate). Bar graphs indicate relative change of MFI standardized to controls. FMO control, Fluorescence Minus One (respective antibody omitted). (*D*) Quantitative real-time PCR for gene expression quantification in aortas of vehicle- vs. MCC950-treated *ApoE*$^{-/-}$ mice ($n = 10$–12 per group, 58% female, Student's *t*-test or Mann–Whitney *U* test as appropriate). ICAM-1, intercellular adhesion molecule 1; ICAM-2, intercellular adhesion molecule 2; VCAM-1, vascular cell adhesion protein 1; *Ccl2*/*Ccl7*, C-C motif chemokine ligand 2/7; *Cxcl1*/*Cxcl12*, C-X-C motif chemokine ligand 1/12; *Cx3cl1*, C-X3-C motif ligand 1. Data are presented as mean + s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Numbers next to gates indicate population frequencies (%).

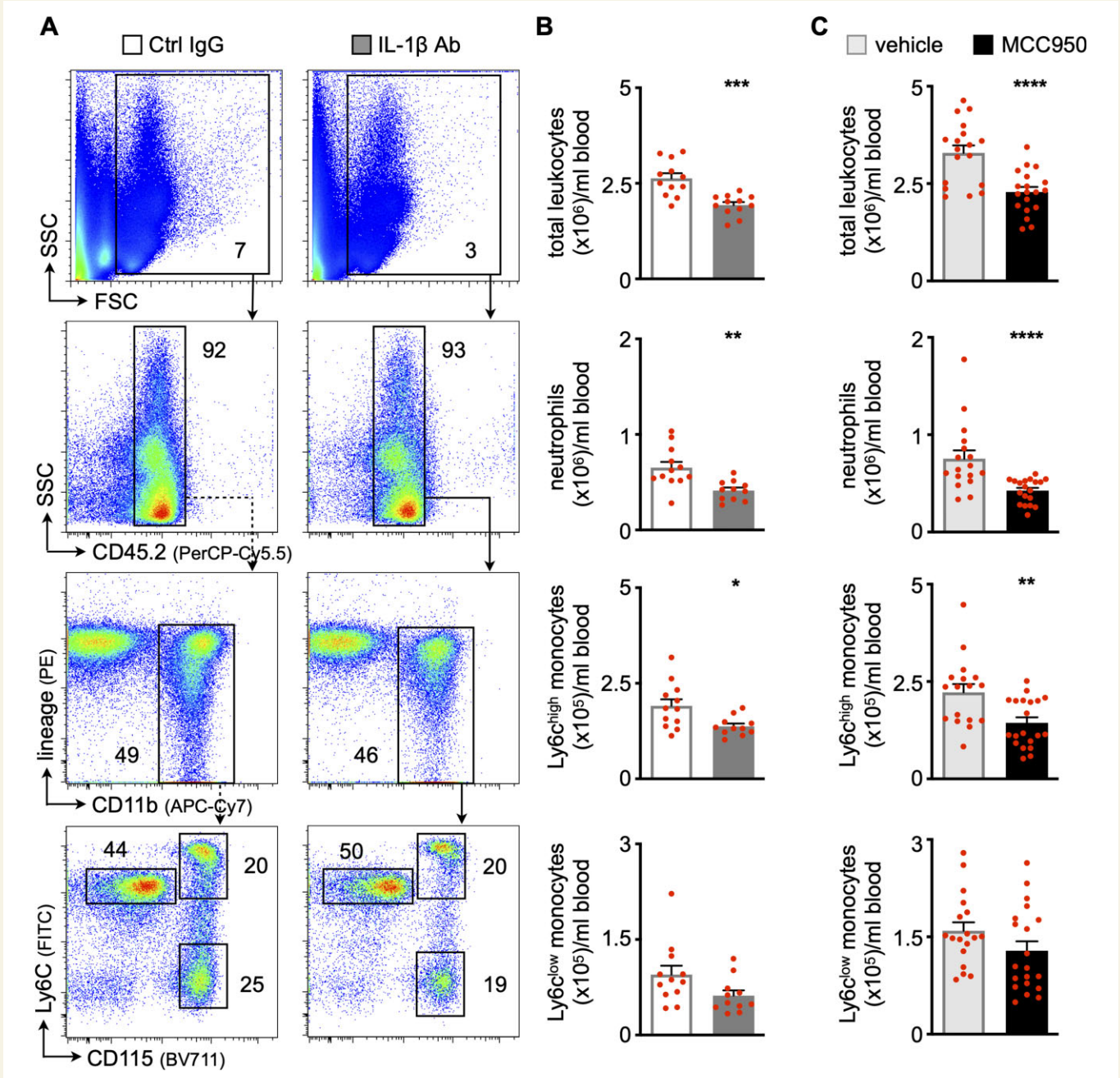


Figure 3 Anti-IL-1β treatment and NLRP3-inflammasome inhibition reduce blood leucocyte numbers. (*A*) Flow cytometric gating and quantification of blood leucocytes in (*B*) IgG (Ctrl IgG)- vs. IL-1β-neutralizing antibody (IL-1β Ab)-treated *ApoE*$^{-/-}$ mice ($n = 11$–12 per group, 67% female, Student's *t*-test or Mann–Whitney *U* test as appropriate) and (*C*) vehicle- vs. MCC950-treated *ApoE*$^{-/-}$ mice ($n = 17$–20 per group, 21–25% female, Student's *t*-test or Mann–Whitney *U* test as appropriate). Data are presented as mean + s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Numbers inside/next to gates indicate population frequencies (%).

3.5 Anti-IL-1β treatment also curtails leucocyte accumulation in atherosclerotic aortas in post-MI accelerated atherosclerosis in mice and humans

The above experiments elucidated the benefit of anti-IL-1β treatment in early atherosclerosis (atherogenesis), i.e. primary prevention. We next asked whether these mechanisms also affect secondary prevention, i.e. in the setting of previous MI as investigated in the CANTOS trial. We therefore started feeding *ApoE*$^{-/-}$ mice HCD and infarcted these mice 2 weeks thereafter (permanent left anterior descending coronary artery ligation) (*Figure 6A*). Four weeks after MI, we initiated treatment with either control IgG or anti-IL-1β for 4 weeks. We assessed inflammatory leucocyte numbers in atherosclerotic aortas and found that treatment significantly reduced the leucocyte content (*Figure 6B and C*). In accord, histology revealed smaller total plaque sizes in the anti-IL-1β group (*Figure 6D*).

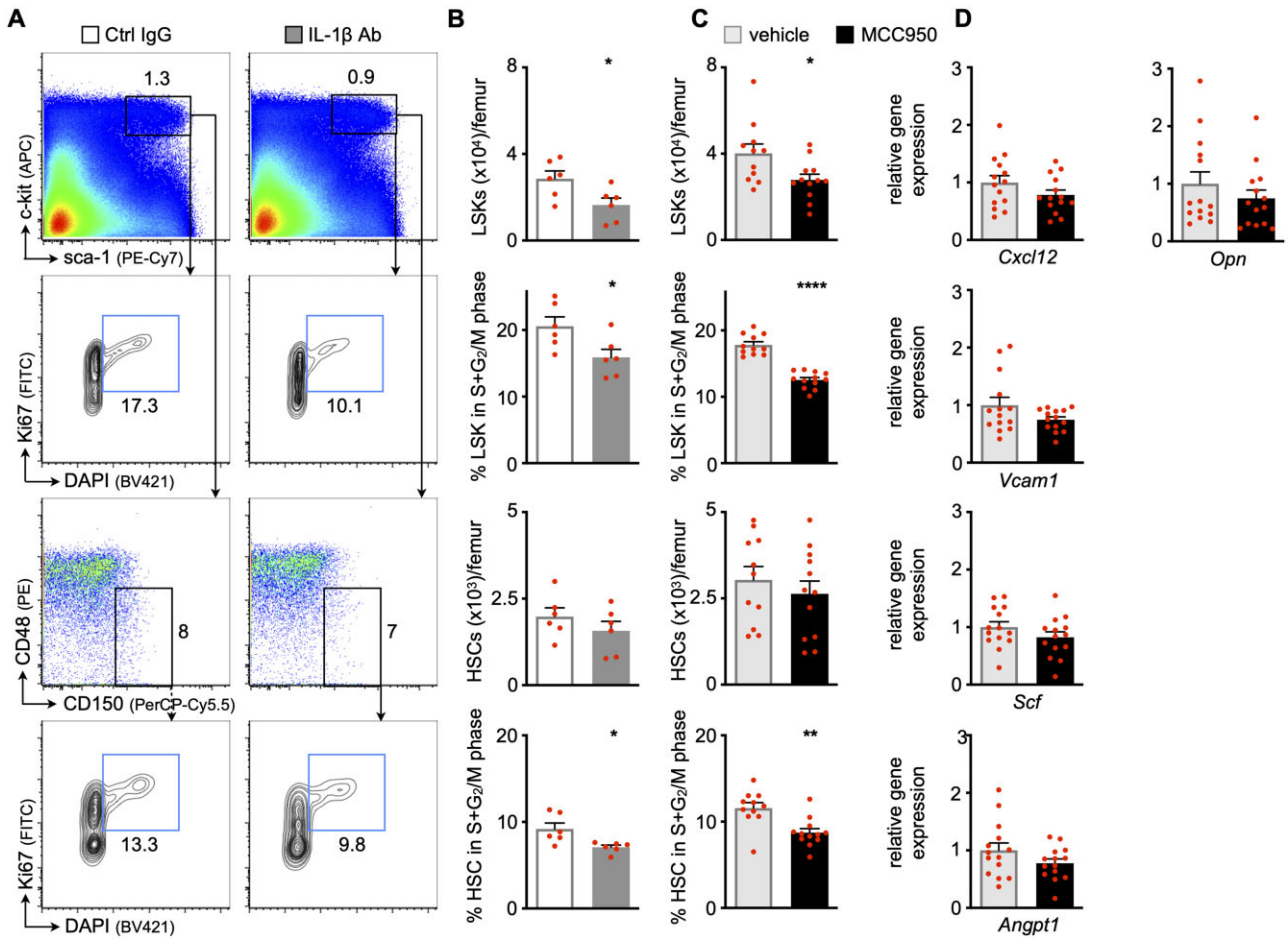


Figure 4 Anti-IL-1β treatment and NLRP3-inflammasome inhibition reduce bone marrow haematopoietic stem and progenitor cell proliferation. (*A* and *B*) Flow cytometric gating (pre-gated on lineage$^-$ cells) and quantification of lineage^{neg}sca-1^{pos}c-kit^{pos} progenitor cells (LSKs) and haematopoietic stem cells (HSCs) in the bone marrow of IgG (Ctrl IgG)- vs. IL-1β-neutralizing antibody (IL-1β Ab)-treated *ApoE*$^{-/-}$ mice ($n = 6$ per group, 83% female, Mann–Whitney *U* Test). Bar graphs compare total numbers of LSKs and HSCs as well as percentages of LSKs and HSCs in the S+G_2/M phase of the cell cycle (indicating proliferating cells). (*C*) Quantification of lineage^{neg}sca-1^{pos}c-kit^{pos} progenitor cells (LSKs) and haematopoietic stem cells (HSCs) in the bone marrow of vehicle- vs. MCC950-treated *ApoE*$^{-/-}$ mice ($n = 11$–12 per group, 36–42% female, Student's *t*-test or Mann–Whitney *U* test as appropriate). Bar graphs compare total numbers of LSKs and HSCs as well as percentages of LSKs and HSCs in the S + G_2/M phase of the cell cycle (indicating proliferating cells). (*D*) Quantitative real-time PCR for gene expression quantification of bone marrow niche factors *Cxcl12* (C-X-C motif chemokine 12), *Vcam1* (vascular cell adhesion molecule 1), *Scf* (stem cell factor), *Angpt1* (angiopoietin-1), and *Opn* (osteopontin) in bone marrow of vehicle- vs. MCC950-treated *ApoE*$^{-/-}$ mice ($n = 14$ per group, 50% female, Student's *t*-test or Mann–Whitney *U* Test as appropriate). Numbers next to gates indicate population frequencies (%). Data are presented as mean + s.e.m., $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

Using adoptive transfer, we tested whether treatment also lowered blood leucocyte uptake (*Figure 6A*). As in regular atherosclerosis (*Figure 2A*), we found fewer GFP^{high} myeloid cells inside atherosclerotic aortas in the treatment group in post-MI accelerated atherosclerosis (*Figure 6E*).

We next determined whether this treatment reduced leucocyte supply and indeed found lower numbers of leucocyte subsets in atherosclerotic aortas post-MI (*Figure 6F*). These data indicate that anti-IL-1β treatment limited inflammatory leucocyte uptake and supply in post-MI accelerated (*Figure 6*) as well as primary atherosclerosis (*Figures 1–5*).

Finally, we addressed whether the phenotype of decreased blood leucocyte numbers also occurred in canakinumab-treated patients after MI. As shown in *Figure 7*, dose-dependent and statistically significant reductions in monocyte counts were observed among participants in the CANTOS trial who were randomly allocated to IL-1β inhibition with canakinumab at doses of 50 mg, 150 mg, and 300 mg subcutaneously given once every 3 months.[16] Effects were evident as early as 1 month following trial initiation and were maintained over time, particularly for the 150 mg and 300 mg doses. Of interest, these were also the two doses of canakinumab that achieved statistically significant reductions in major adverse cardiovascular events in the main CANTOS trial.

4. Discussion

The prototypic proinflammatory cytokine IL-1β and its blood levels strongly associate with plaque progression and destabilization.[7] Pharmacological depletion of IL-1β reduced recurrent events in patients with previous MI and residual inflammation in a large phase III clinical

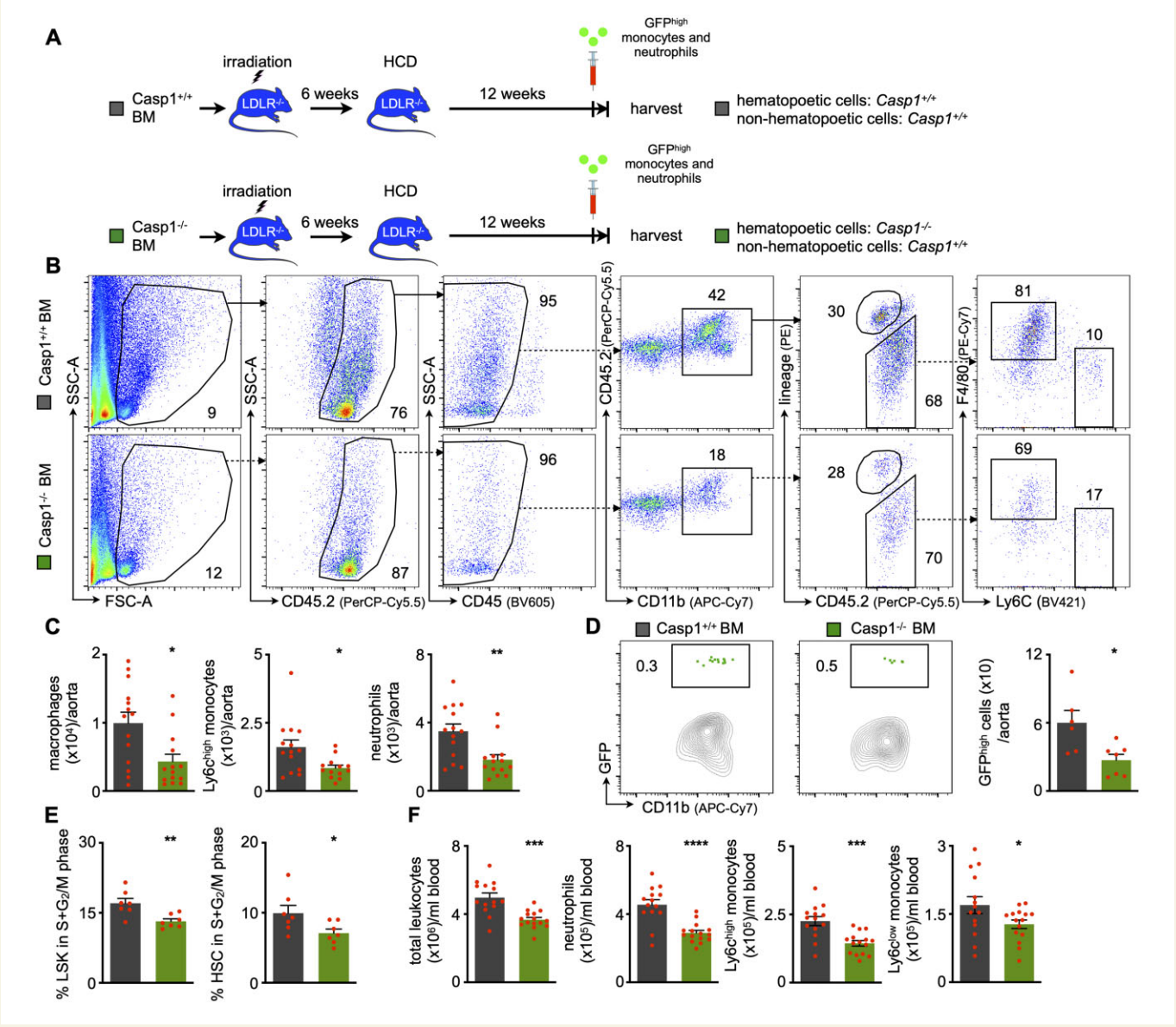


Figure 5 Haematopoietic cell-specific NLRP3-inflammasome depletion attenuates inflammatory leucocyte accumulation in atherosclerotic aortas. (*A*) Experimental scheme for *Figure 5B–F*. In brief, *LDLR*$^{-/-}$ mice were lethally irradiated and reconstituted with bone marrow (BM) from either wild-type (*Casp1*$^{+/+}$) or Casp1 knock-out (*Casp1*$^{-/-}$) mice. After 6 weeks of regeneration, *LDLR*$^{-/-}$ mice were fed a high-cholesterol diet (HCD) for 12 weeks to induce plaque formation. (*B* and *C*) Flow cytometric gating and quantification of aortic myeloid cells in *LDLR*$^{-/-}$ mice reconstituted with either *Casp1*$^{+/+}$ or *Casp1*$^{-/-}$ BM ($n = 13–14$ per group, 50–62% female, Mann–Whitney *U* test). (*D*) Flow cytometric gating and quantification of GFP^{high} myeloid cells in atherosclerotic aortas 24 h after adoptive transfer of GFP^{high} monocytes and neutrophils into *LDLR*$^{-/-}$ mice reconstituted with either *Casp1*$^{+/+}$ or *Casp1*$^{-/-}$ BM ($n = 6–7$ per group, 50–57% female, Mann–Whitney *U* test). (*E*) Quantification of proliferating lineage^{neg}sca-1^{pos}c-kit^{pos} progenitor cells (LSKs) and haematopoietic stem cells (HSCs) in the bone marrow of *LDLR*$^{-/-}$ mice reconstituted with either *Casp1*$^{+/+}$ or *Casp1*$^{-/-}$ BM ($n = 7$ per group, 57% female, Mann–Whitney *U* test). (*F*) Flow cytometric gating and quantification of blood leucocytes in *LDLR*$^{-/-}$ mice reconstituted with either *Casp1*$^{+/+}$ or *Casp1*$^{-/-}$ BM ($n = 14–15$ per group, 50–60% female, Student's *t*-test). Data are presented as mean + s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Numbers next to gates indicate population frequencies (%).

trial.[16] However, the mechanisms underlying this beneficial effect remain incompletely understood.

This study explores the biological basis of IL-1β- and NLRP3-inflammasome-targeted therapies and provides evidence that anti-IL-1β treatment and NLRP3-inflammasome inhibition dampened vascular inflammation (i.e. more numerous plaque inflammatory leucocytes) and hence progression of atherosclerosis. Our pharmacological treatment strategies aimed at two different targets in the same signalling cascade: MCC950 inhibits upstream NLRP3-inflammasome formation, while the murine equivalent of canakinumab provides downstream IL-1β neutralization.

Specifically, we found that aortic endothelial cells decreased the expression of leucocyte chemoattractants and adhesion molecules upon NLRP3-inflammasome inhibition. These two molecule groups participate crucially in leucocyte recruitment, a process that involves leucocyte capture, rolling, adhesion, and intraluminal crawling and finally leads these cells to transmigrate across the endothelial layer to enter the subintimal space/plaque.[35] Since a decrease in these molecules does not rigorously reflect reduced

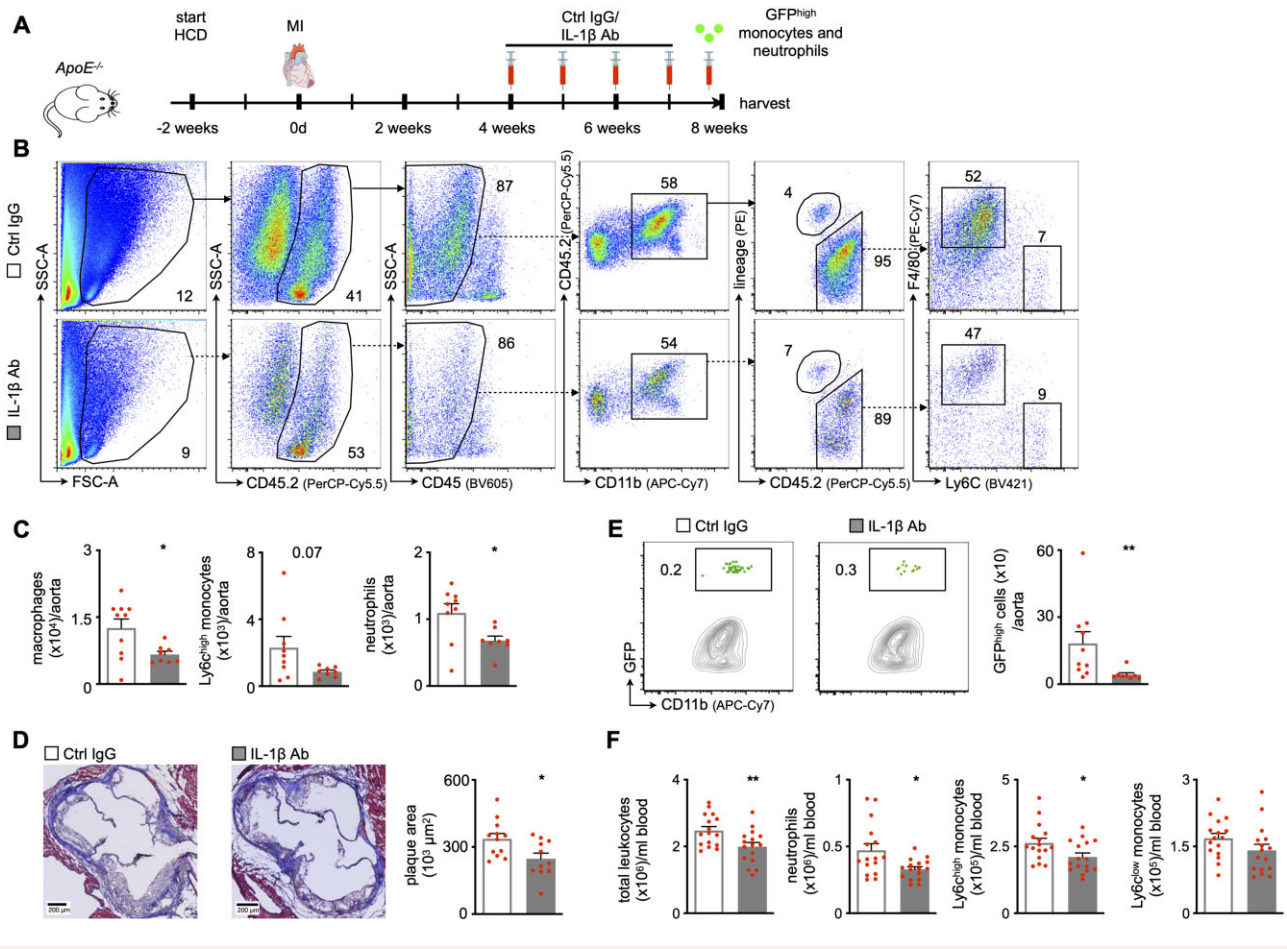


Figure 6 Anti-IL-1β treatment also reduces vascular inflammation in post-myocardial infarction accelerated atherosclerosis. (*A*) Experimental scheme for *Figure 6B–E*. In brief, *ApoE*$^{-/-}$ mice underwent permanent coronary artery ligation after being fed a high-cholesterol diet (HCD) for 2 weeks. HCD was continued for another 8 weeks with mice being treated with either IgG (Ctrl IgG) or IL-1β neutralizing antibody (IL-1β Ab) for the last 4 weeks of the experiment. (*B* and *C*) Flow cytometric gating and quantification of aortic myeloid cells in infarcted *ApoE*$^{-/-}$ mice treated with IgG vs. IL-1β antibody ($n = 8–10$ per group, 70–100% female, Student's *t*-test or Mann–Whitney *U* test as appropriate). (*D*) Representative Masson Trichrome staining and quantification of plaque area of sectioned aortic roots from infarcted *ApoE*$^{-/-}$ mice treated with IgG vs. IL-1β antibody ($n = 11–12$ per group, Student's *t*-test). Scale bars represent 200 µm. (*E*) Flow cytometric gating and quantification of GFP^{high} myeloid cells in atherosclerotic aortas 24 h after adoptive transfer of GFP^{high} monocytes and neutrophils into infarcted *ApoE*$^{-/-}$ mice treated with IgG vs. IL-1β antibody ($n = 8–10$ per group, 70–100% female, Mann–Whitney *U* test). (*F*) Quantification of blood leucocytes in infarcted *ApoE*$^{-/-}$ mice treated with IgG vs. IL-1β antibody ($n = 16$ per group, 100% female, Mann–Whitney *U* test). Data are presented as mean + s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$. Numbers next to gates indicate population frequencies (%).

adhesion/migration, we tested directly whether treatment mitigates leucocyte uptake from blood to atherosclerotic aortas. To that end, we adoptively transferred GFP^{high} inflammatory monocytes and neutrophils into atherosclerotic mice, which received either a control or an IL-1β neutralizing antibody. We tracked the GFP^{high} myeloid cells inside atherosclerotic aortas using flow cytometry 24 h after the intravenous transfer and found that IL-1β neutralization indeed lowered GFP^{high} myeloid cell numbers inside atherosclerotic aortas, indicating that these cells were recruited less.

Apart from this reduced inflammatory leucocyte uptake into atherosclerotic lesions, we further report that anti-IL-1β treatment and NLRP3-inflammasome inhibition also limited inflammatory leucocyte supply. In that light, we found that anti-IL-1β treatment and NLRP3-inflammasome inhibition lowered inflammatory leucocyte numbers in the blood. When we investigated how blood cell numbers dropped, we found that HSPCs—the precursor cells of leucocytes—proliferated less. Myeloid cells are continuously produced in the bone marrow (and in some cases also at extramedullary sites) in a process termed myelopoiesis and develop through a series of well-defined stages including HSCs and multipotent progenitors, followed by common myeloid progenitors, and granulocyte-macrophage progenitors that finally differentiate into granulocytes and monocytes, which are released into the circulation.[36,37] Our data indicate that anti-IL-1β treatment and NLRP3-inflammasome inhibition modulate immune cell supply by decreasing output of disease-propagating neutrophils and inflammatory monocytes.

Finally, we performed an experiment that resembled the CANTOS trial set-up to probe whether anti-IL-1β treatment reduced vascular inflammation not only in early atherosclerosis (atherogenesis) but also in post-MI accelerated atherosclerosis. Our data provide evidence that the same mechanisms, i.e. reduced supply and uptake of inflammatory leucocytes, occur in both primary (atherogenesis) and secondary prevention (previous MI). In line with our pre-clinical findings, we also found reduced blood monocytes numbers in individuals on canakinumab in the

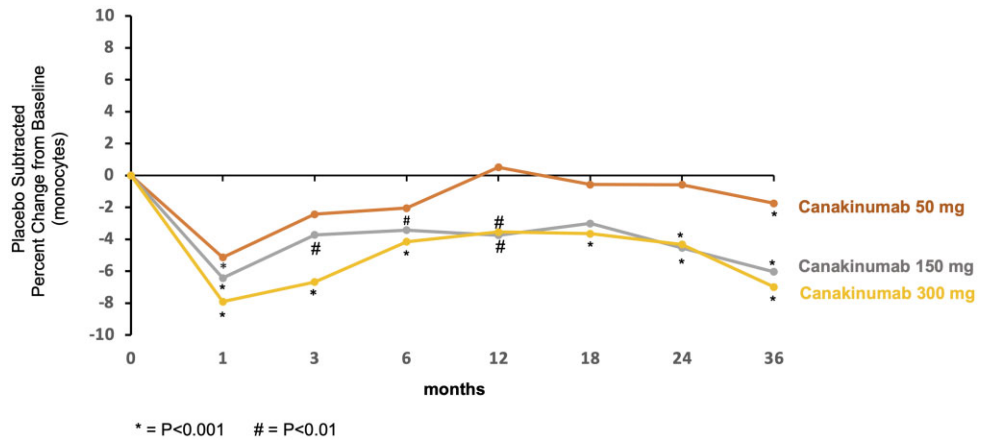


Figure 7 Anti-IL-1β treatment reduces blood monocyte numbers in patients after MI. Placebo-subtracted mean percent change in monocyte counts over time in the CANTOS trial among participants randomly allocated to IL-1β inhibition with canakinumab at doses of 50, 150, and 300 mg subcutaneously every 3 months. *P*-values refer to the percent change in monocyte count at each dose compared to placebo at that point in time. *$P < 0.001$, #$P < 0.01$.

CANTOS trial indicating that the beneficial effect in these patients may be partially mediated by reduced supply of disease-propagating monocytes.

In summary, these data demonstrate that targeting IL-1β- and NLRP3-inflammasome-induced leucocyte supply and plaque leucocyte recruitment reduced vascular inflammation in atherosclerosis. Our major findings are summarized in the *Graphical Abstract*.

IL-1β, the prototypical proinflammatory cytokine, can be produced at inflammatory sites (e.g. atherosclerotic lesions) and can derive from various cell types. Within atherosclerotic plaques, lesional macrophages likely comprise the major source of IL-1β production, while endothelial cells and vascular smooth muscle cells may also release IL-1β to some extent.[34] Upon engulfment of cholesterol crystals, inflammasome formation occurs in macrophages, leading to the release of mature IL-1β and IL-18 into the extracellular space.[5] To distinguish better the cellular source of IL-1β, we generated mice in which haematopoietic cells lacked Casp1, the machinery proteinase of the inflammasome, using bone marrow reconstitution experiments. Our data showed that haematopoietic cell-specific NLRP3-inflammasome activity contributes substantially to activation of both plaque endothelial cells and bone marrow HSPCs.

The observation that IL-1β can directly activate endothelial cells agrees with *in vivo* studies that showed better leucocyte adherence to endothelial cells previously primed with IL-1.[34] In line, other studies using genetically and/or pharmacologically disrupted IL-1β signalling showed a decrease in adhesion molecule and/or chemokine levels as surrogates of dampened leucocyte recruitment.[28,38,39]

IL-1β is known to drive emergency haematopoiesis[40–43]: that IL-1β impacts inflammatory cell output in the bone marrow concurs with an observation that we made after induction of acute inflammation. Upon experimental MI, active IL-1β rises in the ischaemic heart and enters the circulation resulting in elevated haematopoietic stem and progenitor proliferation in the bone marrow and consequently in increased leucocyte production.[23] These leucocytes in turn are deployed to the ischaemic wound where they remove necrotic debris.

Our findings have direct clinical relevance, as they provide insights into the mechanisms whereby anti-IL-1β treatment and NLRP3-inflammasome inhibition may reduce atherosclerotic complications. The notion that blood monocyte numbers decreased upon canakinumab treatment may indicate that anti-IL-1β therapy—similar to what we found in our pre-clinical experiments—also reduces the production of disease-propagating monocytes. Of note, CANTOS subgroups that benefitted most from treatment also showed the most pronounced and sustained drop in blood monocyte numbers. Furthermore, a recent analysis of the CANTOS trial showed a reduction also for blood neutrophils in the canakinumab treatment groups,[44] which align with our blood neutrophil data in the anti-IL-1β group.

New mechanistic clues on IL-1β-targeting drugs' actions provide novel insight into CANTOS and might aid the identification of subgroups of patients that respond best to anti-inflammatory treatment strategies. For example, strong responders might exhibit reduced blood leucocyte numbers upon initiation of treatment. In contrast to CRP, which represents a biomarker of inflammation without pathophysiological relevance,[45] a reduction in monocyte counts may directly mediate the beneficial effects. Apart from its favourable effects in secondary prevention, we provide data that therapeutically targeting IL-1β and NLRP3-inflammasome may also induce beneficial changes in the setting of primary prevention with early-stage atherosclerosis (atherogenesis, i.e. development of atherosclerosis). Moreover, our results provide further experimental endorsement of the interruption of the inflammasome/IL-1β pathway in the setting of recent acute coronary syndrome. Further trials are warranted to also test these propositions in a clinical setting.

4.1 Study limitations

Our study has several limitations. First, the experiments in which the inflammasome inhibitor MCC950 was used focused on NLRP3-inflammasome-dependent mechanism of active IL-1β release. However, pro-IL-1β can also be cleaved to active IL-1β, independent of the NLRP3-inflammasome by, for instance, extracellular proteases.[14,15] In contrast, murine canakinumab depletes IL-1β regardless of its upstream activation. Secondly, we used bone marrow from Casp1 knock-out (*Casp1*$^{-/-}$) mice in our bone marrow reconstitution experiments. Apart from the Casp1 deficiency, these mice are known to also have an incidental caspase 4 (Casp4, also known as caspase 11) deficiency. Hence, we cannot rule out that Casp4 depletion may also have contributed to

the phenotype that we observed in mice that were transplanted with bone marrow from these double knock-out mice. Furthermore, *Casp1*$^{-/-}$ mice are on a mixed C57BL/6J; C57BL/6N genetic background, while wild-type mice are on a C57BL/6J background. We cannot exclude that these differences in genetic backgrounds may have biased the results from our bone marrow chimera experiments.

Thirdly, we found reduced blood monocytes numbers in individuals on canakinumab in the CANTOS trial. Whether this reduction is caused by decreased production of these cells in the bone marrow—as we observed it in our pre-clinical experiments—remains to be investigated. Moreover, whether canakinumab also reduced uptake of blood inflammatory leucocytes in patients enrolled in CANTOS is also yet unclear.

Finally, our data show a treatment-induced reduction in leucocyte recruitment. Whether reduced leucocyte recruitment is mediated via treatment-induced phenotypic changes of endothelial cells with reduction of adhesion molecules and/or chemokines—as our data suggest—or other mechanisms remains to be fully investigated.

5. Conclusion

Taken together, our data indicate that pharmacological as well as genetic suppression of IL-1β and the NLRP3-inflammasome reduced vascular inflammation in primary (pre-MI) and secondary (post-MI) prevention through (i) reducing blood inflammatory leucocyte supply and (ii) dampening blood inflammatory leucocyte recruitment. Our data shed new light on the mechanisms through which anti-IL-1β treatment and NLRP3-inflammasome inhibition reduce cardiovascular complications and inform future anti-inflammatory interventions in patients with atherosclerosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

H.B.S. and T.K. generated the hypothesis and conceived the project. H.B.S., T.K., J.He., and J.Hi. designed the experiments. H.B.S., J.He., J.Hi., B.M., C.M., and Q.B. performed experiments. H.B.S., T.K., J.He., and J.Hi. analysed and interpreted data. M.-A.D. and X.L. performed MI surgeries. N.H.A, P.R., and P.L. analysed and provided human data from the CANTOS trial. H.G., A.A.B.R., M.A.C., O.G., M.K., and O.S. provided intellectual input and techniques. P.L., H.S., P.R., W.K., and C.W. provided intellectual input and edited the manuscript. H.B.S., T.K., J.He., and J.Hi. made the figures. H.B.S. and T.K. wrote the manuscript, which was approved by all authors.

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Conflict of interest: H.G. is a full time Novartis employee. H.S. reports personal fees from MSD Sharp & Dohme, AMGEN, Bayer Vital GmbH, Boehringer Ingelheim, Daiichi-Sankyo, Novartis, Servier, Brahms, Bristol-Myers Squibb, Medtronic, Sanofi Aventis, Synlab, Pfizer, grants and personal fees from Astra-Zeneca, and personal fees from Vifor outside the submitted work. H.S. and T.K. are named inventors on a patent application for prevention of restenosis after angioplasty and stent implantation outside the submitted work. T.K. received lecture fees from Bayer AG, Pharmaceuticals. H.B.S. reports grants from the European Research Council, the 'Else-Kröner-Fresenius-Stiftung', the 'Deutsche Herzstiftung', and the 'Deutsche Forschungsgemeinschaft' during the conduct of the study. A.A.B.R. is inventor on several licensed patents on novel NLRP3 inhibitors. P.L. is an unpaid consultant to, or involved in, clinical trials for Amgen, AstraZeneca, Esperion Therapeutics, Ionis Pharmaceuticals, Kowa Pharmaceuticals, Novartis, Pfizer, Sanofi-Regeneron, and XBiotech, Inc. He is a member of the scientific advisory boards for Amgen, Corvidia Therapeutics, DalCor Pharmaceuticals, IFM Therapeutics, Kowa Pharmaceuticals, Olatec Therapeutics, Medimmune, Novartis, and XBiotech, Inc and also serves on the Board of XBiotech, Inc. P.L.'s laboratory has received research funding in the last 2 years from Novartis, and he has a financial interest in Xbiotech, a company developing therapeutic human antibodies. P.L.'s interests were reviewed and are managed by Brigham and Women's Hospital and Partners HealthCare, Boston, USA, in accordance with their conflict of interest policies. W.K. reports personal fees from AstraZeneca, Novartis, Pfizer, The Medicines Company, DalCor, Kowa, Amgen, Corvidia, Daiichi-Sankyo, Berlin-Chemie, Sanofi, Bristol-Myers Squibb, and grants and non-financial support from Singulex, Abbott, Roche Diagnostics, and Beckmann, all outside the submitted work. P.R. has received research grant support from Novartis, Kowa, Amarin, Pfizer, and the NHLBI; and has served as a consultant to Corvidia, Novartis, Flame, Agepha, Inflazome, AstraZeneca, Janssen, Civi Biopharm, SOCAR, Novo Nordisk, Uptton, Omeicos, Health Outlook, and Boehringer-Ingelheim. All other authors have nothing to disclose.

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Data availability

All data are incorporated into the article and its online supplementary material.

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