The pleiotropic benefits of statins include the ability to reduce CD47 and amplify the effect of pro-efferocytic therapies in atherosclerosis

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The pleiotropic benefits of statins may result from their impact on vascular inflammation. The molecular process underlying this phenomenon is not fully elucidated. In the present study, RNA-sequencing designed to investigate gene expression patterns after CD47-SIRPα inhibition identifies a link of statins, efferocytosis and vascular inflammation. In vivo and in vitro studies provide evidence that statins augment programmed cell removal by inhibiting the nuclear translocation of NF-κB1 p50 and suppressing the expression of the critical 'don't-eat-me' molecule, CD47. Statins amplify the phagocytic capacity of macrophages, and thus the anti-atherosclerotic effects of CD47–SIRPα blockade, in an additive manner. Analyses of clinical biobank specimens suggest a similar link between statins and CD47 expression in humans, highlighting the potential translational implications. Taken together, our findings identify efferocytosis and CD47 as pivotal mediators of statin pleiotropy. In turn, statins amplify the anti-atherosclerotic effects of prophagocytic therapies independently of any lipid-lowering effect.

Atherosclerosis is a lipoprotein-driven, inflammatory process underlying heart attack and stroke and is the leading cause of death worldwide1. In the last four decades, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, known as statins, have been established as the drug of first choice for patients with atherosclerotic cardiovascular disease. Striking data from multiple clinical trials have shown that they reduce mortality in both primary and secondary prevention2–4. Statins inhibit the rate-limiting enzyme of cholesterol biosynthesis and reduce serum low-density lipoprotein (LDL)-cholesterol, thus directly addressing one of the most important risk factors for the disease.

However, there is growing evidence that the beneficial properties of statins are not restricted to their influence on LDL. Indeed, the so-called pleiotropic effects of statins are now well described5 and may result from their impact on vascular inflammation6. This possibility has garnered attention due to a series of recent high-profile studies, including CANTOS (which used a monoclonal antibody directed against the interleukin (IL)-1β pathway) and COLCOT (which used colchicine to target tubulin polymerization, microtubule generation and possibly the inflammasome), which helped definitively prove the 'inflammatory hypothesis of atherosclerosis'7. These large clinical trials are spurring the concept of target-engagement in patients8. Although these preliminary observations require confirmation in a prospective trial, pro-efferocytic therapies are hypothesized to represent a new strategy to target inflammation in cardiovascular disease.

In the present study, we describe how an unbiased approach designed to investigate gene expression patterns in response to
Effect of CD47 blockade and atorvastatin on atherosclerosis. The unanticipated crosstalk and the resulting emerging translational potential prompted us to test whether combined treatment of CD47–SIRPα blockade and HMG-CoA reductase inhibition has additive effects on the atherosclerotic plaque activity in vivo. To address this question, high-fat, diet-fed apolipoprotein E-deficient (ApoE<sup>−/−</sup>) mice received therapy with atorvastatin alone or in combination with CD47–SIRPα blockade (Extended Data Fig. 2a–i). The latter was achieved by targeting either CD47 (using anti-CD47 antibodies<sup>11</sup>) or SIRPα’s downstream effector molecule SHP-1 (using SHP1i<sup>13</sup>). Intriguingly, combined treatment not only decreased lesion size but also reduced the necrotic core area (Fig. 2a,b and Extended Data Fig. 2j). This is important to note, given that the necrotic core is thought to be a key driver for plaque vulnerability in lesions and thus for acute vascular events<sup>16</sup>. Of note, there were no notable differences in plasma cholesterol and blood glucose between the cohorts (Fig. 2c). Subsequently, we used the single treatment cohorts to determine additivity/synergy of compounds (Extended Data Fig. 2k,l). Applying the Bliss independence model (formula $E_c = E_a + E_b - E_a \times E_b$, where $E_c$ is the combined effect produced by the combination of compounds $a$ and $b$)<sup>17</sup> on the analyses of lesion area and necrotic core size, we computed...
Fig. 2 | Combined treatment of CD47–SIRPα blockade and atorvastatin showed additive effects on atherosclerotic plaque activity in vivo. 

a, Quantification of atherosclerotic lesion area and cross-sections of aortic roots stained with Oil-red O (n = 10 for statin; n = 13 for anti-CD47 + statin; n = 15 for SHP1i + statin). TVA, total vessel area. Scale bar, 100 µm. 

b, Quantification of necrotic core size and cross-sections of aortic roots stained with Masson’s trichrome (n = 10 for statin; n = 13 for anti-CD47 + statin; n = 15 for SHP1i + statin). Scale bar and scale bar inset, 100 µm. 

c, Quantification of total cholesterol, high-density lipoprotein (HDL), LDL and glucose in the blood (n = 7 for statin; n = 10 for anti-CD47 + statin; n = 12 for SHP1i + statin).

d, Applying the Bliss independence model on the analyses of lesion area and necrotic core size to determine additivity/synergy of compounds (n = 10 for Ecalculated; n = 13 for anti-CD47 + statin Eobserved).

e, Applying the Bliss independence model on the analyses of lesion area and necrotic core size to determine additivity/synergy of compounds (n = 10 for Ecalculated; n = 15 for SHP1i + statin Eobserved). Each datapoint represents a biologically independent animal.

Data and error bars present mean ± 95% CI for parametric and median ± IQR for nonparametric results. Data of a were analyzed using one-way ANOVA with Sidak’s multiple comparisons test. Data of b and c were analyzed using Kruskal–Wallis with Dunn’s multiple comparisons test. Data of d and e were analyzed using a two-tailed, unpaired Student’s t-test and a two-tailed Mann–Whitney U-test.
an additive anti-atherosclerotic effect for both parameters in vivo (Fig. 2d,e). Together, these observations suggested an additive therapeutic effect on combination.  

Effect of CD47 blockade and atorvastatin on efferocytosis. Given that the efficient clearance of apoptotic cells, a process called efferocytosis, is impaired in atherosclerosis\(^\text{13}\), we reasoned that atorvastatin might increase the efferocytosis rate and thus beneficially impact lesion development. To test this hypothesis, we first employed an in vitro phagocytosis assay using macrophages as phagocytes and target cells. We observed, using flow cytometry, a relevant increase in the efferocytotic rate of apoptotic cells on combined treatment (atorvastatin + SHP1i) compared with single therapies (Fig. 3a and Extended Data Fig. 3a). Of note, the Bliss independence model confirmed additivity (Fig. 3b). As previously shown, inhibition of the CD47–SIRPα axis (using anti-CD47 antibodies\(^\text{11}\) or SHP1\(^\text{13}\)) did not alter the rate of programmed cell death quantified by caspase-3/7 activity in our cells. Similarly, we did not find an effect on apoptosis by atorvastatin or combined treatment strategies (Fig. 3c and Extended Data Fig. 3b), suggesting an enhancement of efferocytosis without altering apoptosis.

To determine the relevance of these observations in vivo, we also investigated the cleaved caspase-3 activity and the number of ‘free’ apoptotic bodies not associated with an intraplaque macrophage, both reliable measures of accumulation of apoptotic bodies and thus efferocytosis in tissue specimens. In agreement with our in vitro observations, we found a decrease in the number of apoptotic bodies in the lesion with the combined treatment, as suggested by our immunofluorescence studies (Fig. 3d,e and Extended Data Fig. 3c,d). Again, the Bliss independence model demonstrated additivity (Fig. 3f). Taken together, these data suggested that the combination of HMG-CoA reductase inhibition and CD47–SIRPα blockade markedly increased the efferocytosis rate and may thus explain the additive effect on atherosclerotic plaque activity.
Fig. 4 | Atorvastatin inhibited NF-κB p50 nuclear translocation under atherogenic conditions and thus directly regulated gene expression of Cd47. **a**, Cd47 expression by qPCR in smooth muscle cells (n = 12 biologically independent samples per group). **b**, Cd47 expression by flow cytometry in smooth muscle cells (n = 6 biologically independent samples per group). RFI, ratio of median fluorescence intensity. **c**, Overlay presentation of Cd47 expression by flow cytometry in smooth muscle cells. **d**, Cd47 expression by immunofluorescence in smooth muscle cells (n = 10 cells for vehicle and n = 15 cells for TNF-α or TNF-α + statin examined over three biologically independent samples per group). a.u., arbitrary units. SMCs, smooth muscle cells. Scale bar, 10 μm. **e**, Cd47 promoter activity by luciferase assay in smooth muscle cells (n = 18 biologically independent samples per group). **f**, NF-κB p50 nuclear translocation by immunofluorescence in smooth muscle cells (n = 3 biologically independent samples per group). M, mevalonate. Scale bar, 10 μm. **g**, NF-κB p50 nuclear translocation by western blotting in smooth muscle cells (n = 11 biologically independent samples per group). HDAC1, histone deacetylase 1. Lane 1, vehicle; lane 2, TNF-α; lane 3, TNF-α + statin; lane 4, TNF-α + statin + mevalonate. **h**, CD47 expression by qPCR in carotid endarterectomy samples (n = 7 biologically independent samples per group). Each datapoint represents a biologically independent sample, except for **d**, which shows cells examined (mean value per high power field) over three biologically independent samples. Data and error bars present mean ± 95% CI for parametric and median ± IQR for nonparametric results. Data of **a** and **d** were analyzed using one-way ANOVA with Tukey’s multiple comparisons test. Data of **b** were analyzed using a Kruskal–Wallis test with Dunn’s multiple comparisons. Data of **e** were analyzed using a two-tailed, paired Student’s t-test. Data of **g** were analyzed using repeated-measures ANOVA with Tukey’s multiple comparisons test. Data of **h** were analyzed using a two-tailed, unpaired Student’s t-test.
Atorvastatin directly regulated gene expression of Cd47. Having identified the effecrotic rate as a pivotal link for additivity of combined treatment, we sought to elucidate the underlying mechanism. Given the critical emerging role of the key 'don't-eat-me' molecule CD47 in atherosclerosis and efferocytosis, we hypothesized that there might be a direct effect of atorvastatin on CD47 expression. To answer this question, we investigated the Cd47 expression in two of the major cellular components of atherosclerosis, smooth muscle cells and macrophages. Stimulation with tumor necrosis factor-α (TNF-α) increased Cd47 expression, but it is of interest that this effect was more pronounced in smooth muscle cells compared with macrophages. Consequently, treatment with atorvastatin resulted in a larger reduction of Cd47 on both RNA and protein levels in smooth muscle cells (Fig. 4a–d and Extended Data Fig. 4a–c).

To directly link atorvastatin treatment with Cd47 expression in smooth muscle cells, we used a dual luciferase reporter assay, which quantified the relative change to basal values obtained from control-transfected cells. We observed that atorvastatin was able to inhibit the TNF-α-induced Cd47 promoter activity (Fig. 4e). Based on our previous studies, we hypothesized that this treatment might reduce the nuclear translocation of NF-kB1 p50, which is a known key transcriptional factor for CD47. In alignment with this hypothesis, we found that atorvastatin inhibited the nuclear translocation of NF-kB1 p50. Importantly, the effect was eliminated with the addition of mevalonate, an antagonist to atorvastatin (Fig. 4f, g and Extended Data Fig. 4d). Although additional causation studies are warranted, these results suggested that atorvastatin directly reduced the pathological CD47 upregulation in atherosclerosis via inhibition of the proinflammatory factor NF-kB1 p50. These results may provide a mechanistic understanding for statin's pleiotropic benefits through their regulation of efferocytosis.

**Human atherosclerosis.** To determine whether HMG-CoA reductase inhibitors result in lower CD47 expression during human atherogenesis, we evaluated carotid endarterectomy samples from the Munich Vascular Biobank. Of note, we found that patients receiving statin treatment had lower CD47 expression than a propensity score (age, gender, medication, symptom and physical status)-matched cohort without such a medication (Fig. 4h). Although the potential for residual confounding exists, these data suggested that HMG-CoA reductase inhibitors reduce the pathological upregulation of CD47 in human atherosclerosis and thus may have additive effects on the efferocytosis rate on combined treatment (Fig. 5).

**Discussion**

The present study provides new insights that may help explain the pleiotropic effects of statins. We show that statins augment efferocytosis by inhibiting the nuclear translocation of NF-kB1 p50 and suppressing expression of the key 'don't-eat-me' molecule CD47. We demonstrate that statins amplify the anti-atherosclerotic effects of two recently described pro-efferocytic therapies, and do so independent of any lipid-lowering effect. Analyses of clinical biobank specimens suggest a similar link between statins and CD47 expression in humans, highlighting the potential translational implications of these findings. Together, these results provide a possible mechanism for how statins provide benefit beyond their well-described effect on cholesterol metabolism, highlighting the possibility that they may also reduce atherosclerosis by exerting a prophagocytic and anti-inflammatory effect directly in the vessel wall.

For decades, researchers have had difficulty attributing the outsized clinical benefit associated with statins solely to their impact on LDL levels. For example, patients randomized to intensive statin therapy on hospital admission for an acute coronary syndrome experience a reduction in clinical event rates in the first 16 weeks (MIRACL study) or as early as 30 d after the start of therapy (PROVE IT-TIMI 22). This early time–benefit window may be ascribed to a wide range of potential anti-inflammatory
mechanisms, including reduced C-reactive protein, T-cell activation and endothelial function modulation via Krüppel-like factor 2 (refs. 31,32), leukocyte–endothelial cell adhesion33 and/or inhibition of prenylation of small G proteins14,15. The fact that atorvastatin restores dysfunctional efferocytosis in atherosclerosis is therefore interesting, given that efferocytosis signaling is thought to occur independently of traditional risk factors, and has been directly linked to inflammation downstream of TNF-α34. As evidence continues to accrue showing that anti-cytokine agents such as canakinumab (IL-1β, CANTOS)35 and ziltiavimab (IL-6, RESCUE)36 may be promising targets in cardiovascular disease, it is tempting to speculate whether the disproportionately powerful effects of statins on atherosclerosis may be mediated, in part, via the clearance of inflammatory cells within the necrotic core.

Although previous studies have suggested that statins can increase the rate of phagocytosis, the mechanism has been elusive to date37,38. In the present study, we demonstrate that atorvastatin is directly linked to the critical ‘don’t-eat-me’ molecule CD47 and thus to the removal of apoptotic debris, supporting a causal relationship. Signaling studies have demonstrated that statins inhibit the nuclear translocation of the inflammatory transcription factor NF-κB p50 in vascular cells39. As statins are being repurposed for use in other systemic inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus40,41, it is interesting to speculate that any benefit they might provide could be primarily mediated through effects on the TNF-α–CD47 axis, given that these are lipid-independent diseases.

New therapeutic options are urgently needed in cardiovascular medicine. The finding that pro-efferocytic therapies amplify the pleiotropic benefits of statins in mice is therefore interesting, particularly given our translational observation that statin usage is associated with lower vascular CD47 expression in humans. It is also important to note that this observed benefit occurs independent of classic risk factors such as hypertension, glucose and lipid levels, which is consistent with previous reports studying these agents in vascular models32,33. As pro-efferocytic therapies reduce risk irrespective of traditional risk pathways (which can already be addressed with currently available medicines), we hypothesize that reactivating intraplaque efferocytosis is an auspicious target for the residual inflammatory risk in atherosclerosis. Our approach involves targeting either CD47 or SIRPα’s downstream effector molecule, SHP-1. Although we have recently provided evidence that an anti-CD47 antibody may reduce vascular inflammation in humans39, additional dose-ranging studies are needed to address the expected transient anemia induced by anti-CD47 therapy40,41.

Given the observed additive effect of dual treatment, we anticipate that combination therapy with lower doses of anti-CD47 antibodies might maintain full efficacy, while hopefully providing a method to avoid induction of erythropoietic effects. Although CD47 is ubiquitously expressed (including on red blood cells), its receptor, SIRPα, is largely restricted to myeloid cells, such as macrophages in the atherosclerotic plaque. For this reason, the benefit observed after blockade of SIRPα’s downstream signaling molecule, SHP-1, provides proof of concept for more precise targeting of this axis, and could spur future clinical investigations on anti-SIRPα antibodies that theoretically should also have few, if any, off-target toxicities. Finally, it is worth noting that statins have much more potent lipid-lowering effects in humans than in mice34,35,38. Accordingly, it is tempting to speculate whether the additive benefits of combination therapy observed in the current preclinical study may actually become synergistic when advanced to clinical trials, where concomitant LDL reduction can be expected.

In conclusion, our data show that atorvastatin promotes efferocytosis via a reduction in CD47, leading to a lipid-independent anti-atherosclerotic effect. In addition, the combination of CD47–SIRPα blockade and HMG-CoA reductase inhibition amplifies the phagocytic capacity of macrophages and thus prevents necrotic core expansion in an additive manner. An important area of future research will be to understand which target, CD47 or SIRPα, is the most suitable in humans and which provides the most favorable therapeutic window. If successful, pro-efferocytic strategies could become a new orthogonal therapy on top of guideline-directed medications to further reduce the complications of atherosclerotic vascular disease.

Methods

Preparation of single-walled carbon nanotubes. Single-walled carbon nanotubes were prepared as previously reported27. Briefly, raw, high-pressure, catalytically decomposed carbon nanotubes (diameter 0.8–1.2 nm, Unidym) were added to an aqueous solution of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycolamine-diacetylated (DSPE-PEG, Nanosense, catalog no. 2828-5000), sonicated for at least 1 h and then centrifuged at 100,000g for 1 h to obtain PEGylated single-walled carbon nanotubes. Unbound surfactant was washed away by repeated filtration through 100-kDa filters (Millipore Sigma-Aldrich, catalog no. UFC910024). For conjugation of Cy5.5 Mono NHS Ester (GE Healthcare, catalog no. GEPA15601), Cy5.5 Mono NHS Ester was incubated with PEGylated single-walled carbon nanotubes solution (10:1 mole ratio) for 2 h. Excess Cy5.5 dye was removed by five to six rounds of centrifugal filtration until the filtrate became clear (PEGylated single-walled carbon nanotubes loaded with fluorescent Cy5.5 probes are referred to hereafter as SWNT). SWNT concentrations were determined spectrophotometrically with an extinction coefficient of 7.9×10⁻¹⁴ M⁻¹ cm⁻¹ at 808 nm. The small-molecule inhibitor NSC-87877 (Sigma-Aldrich, catalog no. NSC-87877) of SIRPα’s downstream effector molecule SHP-1 was added to stirred SWNT at 4°C (pH 7.4) overnight to form SWNT and the small-molecule inhibitor bafilomycin A (bafilomycin A is referred to hereafter as SHPI1). After 24 h, SWNT were dialyzed for another 24 h next to phosphate-buffered saline (PBS) (to remove unbound NSC-87877 molecules. The concentration of the loaded small-molecule inhibitor NSC-87877 was measured using a NanoDrop One (Thermo Fisher Scientific) at its absorption of 320 nm.

Bone marrow-derived macrophages and RNA-seq. Bone marrow cells were isolated from male C57BL/6J mice (The Jackson Laboratory) at the age of 6–8 weeks and differentiated ex vivo to macrophages in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml⁻¹ of penicillin and 100 μg ml⁻¹ of streptomycin (HyClone GE Healthcare, SV30010), and 10 ng ml⁻¹ of murine macrophage colony-stimulating factor (M-CSF; Peprotech, catalog no. 315-02, lot no. 0518245) for 7–10 d. After differentiation, the primary mouse macrophage cell line M1.2.1 (ATCC) was added to the culture medium (10% FBS–PBS, macrophages were sorted using a FACSAria cell sorter equipped with FACSdiva 6 software (BD Life Sciences, Stanford Shared FACS Facility). Channel compensation was performed using single-stained UltraComp eBeads (Thermo Fisher Scientific, catalog no. 01-2222-41) or control macrophages. In addition, macrophages were stained with STYTOX Blue (Invirotigen, catalog no. S34837) to discriminate and exclude nonviable cells. Viable cells (STYTOX Blue negative) were sorted with a 100-μm nozzle into populations that were Cy5.5 positive and Cy5.5 negative and collected in 2% FBS–PBS. Then RNA was extracted using the mirNeasy Mini Kit (QIAGEN, catalog no. 217004). In each group (SWNT and SHPI1), three biological replicates were sequenced. The RNA samples were sent to Novogene Co. (Sacramento, CA, USA) for sample quality control, preparation and sequencing. All samples passed quality control. Subsequently, complementary DNA library construction and sequencing were performed for each sample on an Illumina Novaseq 6000 platform with paired-end 150-bp reads. The data quality report is provided in Source Data Fig. 1a. The sequencing data were uploaded to the Galaxy web platform and we used the public server at usegalaxy.org to further analyze the data (v.2.0.1)39. Briefly, quality control of sequencing data was performed using FastQC (v.0.11.8). HISAT2 (v.2.1.0) was used to map the reads to the reference genome (built-in mm10). FeatureCounts was then used to count the number of reads mapped and DESeq2 (v.1.22.1) to generate the list of differentially regulated genes. P values were adjusted for multiple testing using the Benjamini–Hochberg FDR. Pathway and upstream regulator analyses were performed using IPA (QIAGEN). Of note, the overlap P value in Fig. 1b and Extended Data Table 2 was calculated whether there is an overlap between the dataset genes generated by the RNA-seq and the genes that are regulated by statins (analysed by Fisher’s exact test, IPA).

Animals and diet. All mice were housed in a pathogen-free animal facility and maintained on a 12-h light:dark cycle at 22°C with free access to food and water. Mice were acclimatized for 1–2 weeks before experimentation. A total of 100 male ApoE⁻⁻ mice (B6.129P2-Apoetm1Unc/J, 002052) on a C56BL/6J background (The Jackson Laboratory) aged 8 weeks were used for the present study. The animals were assigned to the experimental groups by complete randomization. For earlier

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power analysis, the following parameters were used based on previous experience with the therapeutic agents $1, 2$: $\alpha = 0.05$; power of $0.80$; expected attrition of $10\%$; and effect size of $1.5$ for atorvastatin and $1.2$ for anti-CD47 antibodies and SHPI1. Ten animals were allocated to the PBS and atorvastatin groups as well as at least $13$ animals to the immunoglobulin (IgG), anti-CD47, anti-CD47 + atorvastatin, SWNT, SHPI1 and SHPI1 + atorvastatin cohorts. Four animals were not included in the results: two were euthanized due to skin lesions and two were excluded owing to poor quality of histopathology. Consequently, $9$ animals in the PBS group, $10$ in the atorvastatin group, $13$ in the IgG group, $13$ in the anti-CD47 group, $13$ in the anti-CD47 + atorvastatin group, $12$ in the SWNT group, $11$ in the SHPI1 group and $15$ in the SHPI1 + atorvastatin group were finally analyzed. Of note, the lesion area of SHPI1 animals compared with SWNT-treated animals was published in our previous analysis $17$. After blood sample collection, mice were perfused with PBS via cardiac puncture and then perfusion fixed with $4\%$ paraformaldehyde (PFA). Blotted tissues were then incubated for the ensuing $9$ weeks ($1$ PBS by daily gavage versus atorvastatin (Liptor, Pfizer, prescription formulation) at a dose of $10$ mg per kg body weight per day by daily gavage; $2$ $200\mu g$ of the inhibitory anti-CD47 antibody (BioXCell, MIAP410, catalog no. BE0283, lot no. 705318N1) intraperitoneally every other day versus $200\mu g$ of the IgG1 isotype control (BioXCell, MOPC-21, catalog no. BE0083, lot no. 6919916O1B) intraperitoneally every other day $3$; or (3) SWNT at a dose of $200\mu l$ of $400 nM intravenously once weekly versus SHPI1 at a dose of $200\mu l$ of $400 nM intravenously once weekly$4$. Animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care (protocol no. 27279) and conformed to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

**Tissue preparation and histological analyses.** Tissue preparation and histological analyses were performed as previously described $17$. After blood sample collection, mice were perfused with PBS via cardiac puncture and then perfusion fixed with $4\%$ paraformaldehyde (PFA). Blotted tissues were then incubated for the ensuing $9$ weeks ($1$ PBS by daily gavage versus atorvastatin (Liptor, Pfizer, prescription formulation) at a dose of $10$ mg per kg body weight per day by daily gavage; $2$ $200\mu g$ of the inhibitory anti-CD47 antibody (BioXCell, MIAP410, catalog no. BE0283, lot no. 705318N1) intraperitoneally every other day versus $200\mu g$ of the IgG1 isotype control (BioXCell, MOPC-21, catalog no. BE0083, lot no. 6919916O1B) intraperitoneally every other day $3$; or (3) SWNT at a dose of $200\mu l$ of $400 nM intravenously once weekly versus SHPI1 at a dose of $200\mu l$ of $400 nM intravenously once weekly$4$. Animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care (protocol no. 27279) and conformed to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

**Apoptosis assay.** The apoptosis assay was performed as previously described $1$. To evaluate apoptosis, the luminesimetric Caspase-Glo 3/7 Assay System (Promega, catalog no. G8091) was used. Cell viability was assessed using an iD3 luminometer (Molecular Devices) was used. RNA isolation and qPCR. To measure Cd47 expression, mouse smooth muscle cells and murine bone marrow-derived macrophages were exposed to DMSO, $10\mu M$ atorvastatin, $50 ng/ml$ of TNF-α + DMSO or $50 ng/ml$ of TNF-α + atorvastatin (Atv, 50 ng/ml). TNF-α (50 ng/ml) was used. To measure Cd47 expression in bone marrow-derived macrophages, bone marrow-derived macrophages were exposed to DMSO or $10\mu M$ atorvastatin for $48 h$. RNA was extracted from cell lysates using the miNeasy Mini Kit (QIAGEN, catalog no. 217004) according to the manufacturer’s protocol or the TRIzol method (Invitrogen, catalog no. 1596026). Then, RNA was quantified using a NanoDrop One (Thermo Fisher Scientific). RNA was reverse transcribed using the High-Capacity RNA-to-cDNA Synthesis Kit (Applied Biosystems, catalog no. 4387406). Then, qPCR of the cDNA samples was performed on a Viia7 Real-Time PCR system or a QuantStudio 5 (both Applied Biosystems). Gene expression levels were measured using TaqMan Universal Master Mix II Applied Biosystems, catalog no. 4400447, lot no. 06762278 and commercially available TaqMan probes. Data were quantified using the $2^{-\Delta\Delta C_{t}}$ method and normalized to Gapdh as an internal control. The following TaqMan Primers were used: Cd47 (Mm00495011_m1), Apoe (Mm01307193_g1), Gpx3 (Mm00492427_m1), Rhob (Mm01250721_m1), Rhob (Mm0045902_m1), XIAP (Mm0111594_m1) and Gapdh (Mm99999915_g1).
In vitro immunofluorescence. Mouse smooth muscle cells were seeded in Millicell EZ Slides (Sigma-Aldrich, catalog no. PZEGS0416 or PZEGS0816). For CD47 staining, cells were first treated with DMSO, 50 ng/ml of TNF-α or DMSO or 50 ng/ml of TNF-α for 48h. For NF-κB1 p50/p105 staining, cells were first treated with DMSO, 10 µM atorvastatin or 10 µM atorvastatin + 100 µM mevalonate for 24h and then exposed to 50 ng/ml of TNF-α for 45 min. After stimulation/treatment, cells were rinsed with PBS and fixed with 4% phosphate-buffered paraformaldehyde. For CD47 staining (BioXCell, MIA410, 25 µg ml⁻¹), vector-on-mouse fluorochrome Immunodetection Kit (Thermo Fisher Scientific, catalog no. NC9801950) was used according to the manufacturer’s instructions. For NF-κB1 p105/p50 staining, cells were blocked with 5% goat serum (Sigma-Aldrich, catalog no. G9023) for 30 min, then incubated with NF-κB1 p105/p50 (Cell Signaling Technology, catalog no. 13586S, D4P4D, 1:200) overnight at 4°C. After extensive washing, cells were incubated with Alexa Fluor-594 goat anti-mouse (Thermo Fisher Scientific, catalog no. A-11005, lot no. 1696463.1, 1:300) and Alexa Fluor-647 goat anti-rabbit (Thermo Fisher Scientific, catalog no. A-21244, lot no. 56897A, 1:300) and DAPI. Images were captured using a Leica DMi8 microscope (equipped with a Leica DMC4500 color camera and a Leica K5 camera for fluorescence imaging).

Luciferase reporter assay. The luciferase reporter assay was performed as previously described. CD47 LightSwitch Promoter Reporter GoClones (RenSP, 710450) and Cypridina TK Control constructs (pTKE-Cuc, SN0322S) were obtained from SwitchGen Gearcomics. The RenSP reporter, 45 ng, and the pTKE-Cuc reporter, 5 ng, of the pTKE multiwells were reconstituted into mouse smooth muscle cells using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, catalog no. L3000-008) and Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, catalog no. 31985062). After 48h, the medium was changed to fresh medium and cells were then exposed to DMSO, 50 ng/ml of TNF-α or DMSO or 50 ng/ml of TNF-α + 10 µM atorvastatin. The cell lysate and supernatant were harvested 24h after stimulation/treatment and dual luciferase activity was measured with the LightSwitch Luciferase Assay Kit (Active Motif, catalog no. 32031, NC0999256) and Pierce Cypridina Luciferase Glow Assay Kit (Thermo Fisher Scientific, catalog no. P16170) using an iD3 luminometer (Molecular Devices). Relative luciferase activity (RenSP/Cypridina ratio) was quantified as the percentage change relative to the baseline value obtained from control-transfected cells.

Protein extraction and western blotting. To measure NF-κB1 p50 nuclear translocation, mouse smooth muscle cells were first treated with DMSO, 10 µM atorvastatin or 10 µM atorvastatin + 100 µM mevalonate for 24h and then exposed to 50 ng/ml of TNF-α for 45 min. Total protein was isolated from mouse smooth muscle cells using a subcellular protein fractionation kit (Thermo Fisher Scientific, catalog no. 78840) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, catalog no. 78442). The protein concentration in each sample was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, catalog no. 232252). Equal amounts of protein were loaded and separated on precast gels (BioRad, catalog no. 456-1084) and thereafter transferred on to EZ Slides (Sigma-Aldrich, catalog no. PEZGS0416 or PEZGS0816). Further, the authors state that all data supporting the findings of the present study are available within the paper, its supplementary information files or publicly available. Raw RNA-seq data are available from the National Center for Biotechnology Information under accession no. PRJNA733740. Source data are provided with this paper.

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Author contributions

K.-U.J., J.Y., Z.Y., H.G., L.L., S.S., M.L., N.L. and A.V.E. conducted experiments and collected and analyzed data. K.-U.J., J.Y., Z.Y., H.G., L.L., R.A.B., B.R.S., I.L.W., L.M. and N.J.L. conceptualized and designed experiments, discussed results and interpreted data. K.-U.J. and N.J.L. designed figures and wrote the manuscript. K.-U.J. directed the study. N.J.L. supervised the study.

Competing interests

L.W. and N.J.L. are cofounders and directors of Bitterroot Bio Incorporated, a cardiovascular company studying macrophage checkpoint inhibition. K.-U.J., Y.K., I.L.W. and N.J.L. have filed a provisional patent (US Application serial no. 63/106,794): ‘CD47 Blockade and Combination Therapies Thereof For Reduction Of Vascular Inflammation’. The remaining authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | RNA sequencing revealed HMG-CoA reductase inhibitor as one of the top upstream regulators of SHP-1 inhibition in macrophages. a, Flow cytometry gating strategy for cell sorting to isolate Cy5.5-positive bone marrow-derived macrophages in each group (SHP1i versus SWNT), which were then subjected to RNA sequencing. b, Rbl1, Xiap, ApoE, Rhob, and Gpx3 expression by quantitative polymerase chain reaction in bone marrow-derived macrophages upon atorvastatin treatment (n = 6 biologically independent samples per group). c, Functional pathways enriched among all differential expressed genes (false-discovery rate < 0.10) as determined by pathway analysis (p-value of overlap). d–e, Samples from vascular smooth muscle cells, bone marrow-derived macrophages, and RAW 264.7 macrophages were collected to rule out mycoplasma contamination by polymerase chain reaction and/or biochemical detection (n = 3 biologically independent samples per group). Each datapoint represents a biologically independent sample. Data and error bars present mean ± 95 % CI for parametric results. Data of b were analyzed using a two-tailed, unpaired Student’s t-test. Data of c were analyzed using a Fisher’s Exact Test, calculated by IPA.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Combined treatment of CD47-SIRPα blockade and atorvastatin showed additive effects on atherosclerotic plaque activity in vivo. a, Quantification of atherosclerotic lesion area (n=9 for PBS; n=10 for statin). TVA, total vessel area. b, Quantification of necrotic core size (n=9 for PBS; n=10 for statin). c, Quantification of total cholesterol, HDL, LDL, and glucose in the blood (n=9 for PBS; n=7 for statin). d, Quantification of atherosclerotic lesion area (n=13 for IgG; n=13 for anti-CD47; n=13 for anti-CD47 + statin). e, Quantification of necrotic core size (n=13 for IgG; n=13 for anti-CD47; n=13 for anti-CD47 + statin). f, Quantification of total cholesterol, HDL, LDL, and glucose in the blood (n=10 for IgG; n=11 for anti-CD47; n=10 for anti-CD47 + statin). g, Quantification of atherosclerotic lesion area (n=12 for SWNT; n=11 for SHP1; n=15 for SHP1i + statin). h, Quantification of necrotic core size (n=12 for SWNT; n=11 for SHP1; n=15 for SHP1i + statin). i, Quantification of total cholesterol, HDL, LDL, and glucose in the blood (n=11 for SWNT; n=10 for SHP1; n=12 for SHP1i + statin). j-l, Quantification of atherosclerotic lesion area and necrotic core size (n=10 for Statin; n=13 for anti-CD47; n=13 for anti-CD47 + statin; n=11 for SHP1; n=15 for SHP1i + statin). Each datapoint represents a biologically independent animal. Data and error bars present mean ± 95% CI for parametric and median ± IQR for nonparametric results. Data of a were analyzed using a two-tailed, unpaired Student’s t-test. Data of b–c were analyzed using a two-tailed Mann–Whitney U test. Data of d,g, and j–l were analyzed using one-way ANOVA with Sidak’s multiple comparisons test. Data of e–f and h–l were analyzed using Kruskal–Wallis with Dunn’s multiple comparisons test.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Combined treatment of CD47-SIRPα blockade and atorvastatin showed additive effects on efferocytosis rate in vitro and in vivo. a, Flow cytometry plots depicting the staining controls for the conditions. b, Apoptosis assay to quantify the rate of programmed cell death in vitro in the presence or absence of atorvastatin, SHP1i, and dual treatment after staurosporine (STS) stimulation (n = 5 biologically independent samples per group). c, Immunofluorescence images depicting cleaved caspase-3 activity (n = 9 for PBS; n = 10 for Statin; n = 11 for SHP1i; n = 15 for SHP1i + statin). White line depicts intima. Scale bar, 50 μm; scale bar inset, 10 μm. d, Immunofluorescence images depicting the ratio of free to macrophage associated cleaved caspase-3 activity (n = 9 for PBS; n = 10 for Statin; n = 11 for SHP1i; n = 15 for SHP1i + statin). White line depicts intima. *free cleaved caspase-3. #macrophage-associated cleaved caspase-3. Scale bar, 50 μm; scale bar inset, 10 μm. Each datapoint represents a biologically independent sample. Data and error bars present mean ± 95% CI for parametric results. Data of b were analyzed using one-way ANOVA test.
Extended Data Fig. 4 | Atorvastatin inhibited NF-κB1 p50 nuclear translocation under atherogenic conditions and thus directly regulated gene expression of Cd47. a, Cd47 expression by quantitative polymerase chain reaction in bone marrow-derived macrophages (n = 6 biologically independent samples per group). TNF-α, tumor necrosis factor-α. b, Cd47 expression by flow cytometry in bone marrow-derived macrophages (n = 4 biologically independent samples per group). RFI, ratio of median fluorescence intensity. c, Cd47 expression by immunofluorescence in smooth muscle cells (n = 10 cells for vehicle and n = 15 cells for TNF-α or TNF-α + Statin examined over three biologically independent samples per group). SMC, smooth muscle cells. Scale bar, 10 µm. d, NF-κB1 p50 nuclear translocation by immunofluorescence in smooth muscle cells (n = 3 biologically independent samples per group). M, mevalonate. Scale bar and scale bar inset, 10 µm. Each datapoint represents a biologically independent sample. Data and error bars present mean ± 95 % CI for parametric and median +/− IQR for non-parametric results. Data of a were analyzed using one-way ANOVA test. Data of b were analyzed using Kruskal-Wallis test.
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- GraphPad Prism 9
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- BD FACSDiva v6 software
- Applied Biosystems QuantStudio 5 and 3
- Illumina NovaSeq 6000
- Molecular Devices ID3
- Thermo Scientific iBright 1500 Imaging system
- Leica DMi8 microscope and Leica LAS-X
- Thermo Scientific NanoDrop One

Data analysis

- GraphPad Prism 9
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- Excel for Mac version 16.16.27
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Life sciences study design

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| Sample size | Sample sizes for in vivo experiments were determined based on prior experience with the therapeutic agents in mouse models of vascular disease. For a priori power analysis, the following parameters were used: α of 0.05; power of 0.80; expected attrition of 10% and effect size of 1.5 for atherosclerosis and 1.2 for anti-CD47 antibodies and SHP1. In atherosclerosis studies, a group size of 10 to 20 is considered sufficient to detect changes and is a common sample size in the field (scientific statement from the American Heart Association). Sample sizes for in vitro experiments were approximated based on prior experiments and available data without statistical predetermination. |
| Data exclusions | No data were excluded from the analysis. |
| Replication | All experiments were successfully repeated at least once. |
| Randomization | Mice were selected and allocated at random. Human cardiac endarterectomy samples were age-, gender-, medication-, symptom-, and physical status matched as described in Methods and Source Data. In vitro samples were selected and allocated at random. |
| Blinding | For in vivo experiments, investigators were generally not blinded to group allocation. However, the analyses were performed in a blinded fashion. For in vitro experiments, investigators were blinded where appropriate. |

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| n/a | Involved in the study |
|----------------------------------|------------------------|
| Antibodies | |
| Eukaryotic cell lines | |
| Palaeontology and archaeology | |
| Animals and other organisms | |
| Human research participants | |
| Clinical data | |
| Dual use research of concern | |

| Methods | n/a | Involved in the study |
|----------------------------------|------------------------|
| | | Chip-seq |
| | | Flow cytometry |
| | | MRI-based neuroimaging |

Antibodies

Antibodies used for flow cytometry:
- anti-CD47 antibody (BD Life Sciences, Catalogue# 561890, FITC, MIAP301, 0.5 mg/ml); isotype control antibody (BD Life Sciences, Catalogue# 553929, FITC, R35-95, 0.5 mg/ml); Fc receptor blockade (BD Biosciences, Catalogue# 553142, anti-mouse CD16/CD32).
- Antibodies used for Western Blot:
  - NFκB p105/p50 (Cell Signaling Technology, Catalogue# 135865, D4P4D, 1:1000); HDAC1 (Cell Signaling Technology, Catalogue# 53565, 102, 1:1000); Alexa Fluor 647 goat anti-mouse (Invitrogen, Catalogue# 37278, Lot# TA252659, 1:10,000); Alexa Fluor 488 goat anti-rabbit (Thermo Scientific, Catalogue# A11034, Lot# 2110499, 1:10,000).
  - Antibodies used for immunofluorescence:
    - Mac3 (BD Life Sciences, Catalogue# 530292, 1:100); cleaved caspase-3 (Cell Signaling Technology, Catalogue# 9661, 1:200); NFκB p105/p50 (Cell Signaling Technology, Catalogue# 135865, 04P4D, 1:200); anti-CD47 antibody (BioXCell, MIAP410, Catalogue# BE0283, Lot# 72924051, 25 μg/ml); IgG1 control (BioXCell, MOPC-21, Catalogue# BE0083, Lot# 11291962, 25 μg/ml); Alexa Fluor 647 goat anti-rat (Thermo Scientific, Catalogue# A-21247, Lot# 2119156, 1:250); Alexa Fluor 488 goat anti-rabbit (Thermo Scientific, Catalogue# A11034, Lot# 2110499, 1:250); Alexa Fluor 594 goat anti-mouse (Thermo Scientific, Catalogue# A-11005, Lot# 1696463, 1:300); Alexa Fluor 647 goat anti-rabbit (Thermo Scientific, Catalogue# A 21244, Lot# 56897A, 1:300).
    - Antibodies used for in vivo experiments:
      - anti-CD47 antibody (BioXCell, MIAP410, Catalogue# BE0283, Lot# 705318N1); IgG1 control (BioXCell, MOPC-21, Catalogue# BE0083, Lot# 61991018).

Validation

All antibodies used for flow cytometry, immunofluorescence, and in vivo application were previously validated for the respective
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) Mouse aortic vascular smooth muscle cells were purchased from Cell Biologics (Catalogue# C57-6080) and mouse macrophage cells (RAW 264.7, ATCC No. TIB-71) were purchased from American Type Culture Collection.

Authentication Authentication was performed by immunofluorescence (alpha-smooth muscle actin for smooth muscle cells) or PCR (for RAW 264.7).

Mycoplasma contamination Cell lines were tested negative for mycoplasma contamination by the supplier and experimenters (Extended Data Figure 1).

Commonly misidentified lines (See ICLAC register) No commonly misidentified cell lines were used in the study.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines, recommended for reporting animal research

Laboratory animals Male apolipoprotein E-deficient (ApoE-/-) mice (B6.129P2-Apoetm1Unc/J, 002052) on a C57Bl/6j background (The Jackson Laboratory) at the age of 8 weeks were used for the in vivo experiments. Bone marrow-derived macrophages were isolated from male C57Bl/6j mice (The Jackson Laboratory) at the age of 6-8 weeks.

Wild animals This study did not involve wild animals.

Field-collected samples This study did not involve field-collected samples.

Ethics oversight Animal studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care (protocol# 27279) and conformed to the NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize suffering.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics The Munich Vascular Biobank contains human atherosclerotic plaques and plasma samples, along with clinical data obtained from patients receiving carotid endarterectomy. The authors state that their study complies with the Declaration of Helsinki, that the locally appointed ethics committee has approved the research protocol and that informed consent has been obtained from the subjects.

Recruitment In this study, a total of 14 human carotid endarterectomy samples were used as follows: age-, gender-, medication-, symptom-, and physical status-matched samples from 7 patients with statin medication were compared with 7 patients without such a medication as described in Methods and Source Data.

Ethics oversight Study protocol was approved by the Institutional Review Board of the Technical University Munich, Germany.

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Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation RNA sequencing experiment with bone marrow-derived macrophages: Channel compensations were performed using single-stained UltraComp eBeads (Thermo Scientific, Catalogue# 01-2222-41) or control macrophages. In addition, macrophages were stained with SYTOX Blue [Invitrogen, Catalogue# S34837] to discriminate and exclude non-viable cells. Viable cells (SYTOX Blue negative) were sorted with a 100 µm nozzle into populations that were Cy5.5-positive and Cy5.5-negative and collected in 2 % FBS-PBS. Then RNA was extracted.
| Instrument          | FACSARia cell sorter (BD Life Sciences) and LSRII (BD Life Sciences). |
|--------------------|------------------------------------------------------------------------|
| Software           | FACSDiva and FlowJo10.7.1 (BD Life Sciences).                         |
| Cell population abundance | Post sort, cell abundance was sufficient for downstream applications. |
| Gating strategy    | The gating strategy is described in Extended Data Figure 1.            |

*Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.*