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ORIGINAL ARTICLE

Acetylsalicylic acid improves outcome after acute myocardial infarction by reducing thromboinflammation

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Abstract

Background: Inflammation orchestrates an outcome after acute myocardial infarction (AMI). Thromboinflammation, via the CD40- and CD40 ligand (CD40L)–mediated platelet–leukocyte interaction, is involved in post-AMI inflammation.

Objectives: This study hypothesized that acetylsalicylic acid (ASA) exerts pleiotropic cardioprotective effects beyond prevention of reinfarction by reducing thromboinflammation and infarct size.

Methods: A murine AMI model was used to investigate the effects of low-dose ASA, which is applied preischemia or after induction of ischemia (intraischemia), on post-AMI thromboinflammation and the outcome. To investigate the underlying mechanisms, platelet and neutrophil depletion and genetically induced and antibody-induced CD40L deficiency were applied. Thromboinflammation markers were analyzed. Translationally, the outcome after ST-elevation myocardial infarction (STEMI) was measured in ASA-pretreated vs ASA-naive patients (ClinicalTrials.gov ID: NCT03539133).

Results: Both ASA treatment preischemia and intraischemia reduced infarct size and thromboinflammation and improved cardiac function and remodeling. The scar size was smaller with ASA preischemia 21 days after AMI but not with ASA intraischemia. This cardioprotection was blunted in the absence of (a) platelets or (b) neutrophils. Both pharmacologic inhibition or genetic deficiency of CD40L abrogated ASA's protective effect. Accordingly, ASA-pretreated patients with STEMI had improved

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outcome (12.5% vs 23.8%; hazard ratio, 0.50; 95% CI, 0.31-0.80; $P < .001$). This was driven by reduced mortality without differences in recurrent AMI.

Conclusion: Existing ASA therapy shows pleiotropic effects in the reduction of thromboinflammation and improvement of outcome after AMI, independent of its effects on the occurrence of ischemia itself. This should be considered while choosing timing of initiation and the optimal antithrombotic regime post-AMI in patients with coronary artery disease.

KEYWORDS
acetylsalicylic acid, acute myocardial infarction, CD40, CD40L, thromboinflammation

1 | INTRODUCTION

A major cause of morbidity and mortality worldwide is coronary artery disease (CAD) with acute myocardial infarction (AMI) as its major consequence [1,2]. An AMI can present with or without electrocardiographic ST-segment elevation (STEMI and NSTEMI, respectively). Early percutaneous coronary intervention (PCI) is essential for successful coronary reperfusion [3,4]. Thereafter, various processes contribute to myocardial regeneration, which either lead to recovery of cardiac function or heart failure with reduced ejection fraction (EF) [5]. Understanding and optimizing these healing processes is of great socioeconomic importance, as heart failure with reduced EF is accompanied by disability and a significant burden for the health care system [6].

Low-dose acetylsalicylic acid (ASA) has been used as an antithrombotic medication for decades [3,7,8]. For primary prevention of CAD, ASA is generally not recommended, except for patients with a high cardiovascular risk [9]. It has been under debate due to an unfavorable risk–benefit ratio, driven by enhanced bleedings. However, patient selection in terms of age, comedication, and comorbidities might have affected these results [10–12]. Also in secondary prevention, ASA's long-established role has been questioned lately [13–18]. Guidelines already recommend ASA-free regimes for patients with atrial fibrillation, oral anticoagulation, and enhanced risk of bleeding once they have passed the acute phase post-AMI [19]. However, the underlying trials investigated only the first event as the endpoint, such as death, reinfarction, stroke, and bleeding. Further end points beyond the classical major adverse cardiac events (MACEs) have not been addressed so far. Therefore, possible additional effects of ASA—beyond inhibition of thrombus formation—seemed worth investigating.

The main mechanism of ASA is inhibition of predominantly cyclooxygenase 1. Thus, ASA blocks the formation of thromboxane A2 and prevents the activation of platelet aggregation and thrombus formation subsequently. Interaction with comedication [20,21] or coexistence of concomitant diseases [22–24] is known to affect ASA's antiplatelet effects. Platelet function assays can be used to monitor these interactions [25,26]. Besides the antiaggregatory effect, ASA also has analgesic, antipyretic, and anti-inflammatory effects [27,28].

Inflammation contributes to myocardial recovery postinfarction [29,30]. The interplay of platelets and neutrophils is crucial in thromboinflammation. Neutrophil extracellular trap (NET) formation is a known defense mechanism in infections [31] but also contributes to sterile inflammation. NET formation is increased in patients with acute coronary syndrome (ACS) and has been investigated as a marker for the diagnosis of ACS [32–34]. Cluster of differentiation (CD) 40 (CD40) on neutrophils and platelet CD40 ligand (CD40L) are an important crosslink in thromboinflammation [35]. Neutrophil CD40 enhances platelet's release of soluble CD40L [36]. Platelet CD40L interacts with CD40 on neutrophils, forms platelet–neutrophil aggregates, and activates the endothelium, thus contributing to atherosclerosis [37–39]. Moreover, a modulating role of ASA in CD40/CD40L-mediated platelet activation has been investigated [40,41]. Of note, CD40L can also be expressed on neutrophils [42]; however, CD40L expression on platelets is likely to be more relevant for the underlying mechanisms.

It is uncertain whether ASA affects these thromboinflammatory processes. Therefore, in this study, we hypothesized that ASA improves outcome by reducing CD40/CD40L-mediated thromboinflammation.

2 | METHODS

A more detailed description of all methods can be found in the Supplementary material.

2.1 | Experimental murine model of AMI

Wild-type C57BL/6J mice were anesthetized and underwent baseline echocardiography (see Supplementary Section S1.2). Thirty minutes

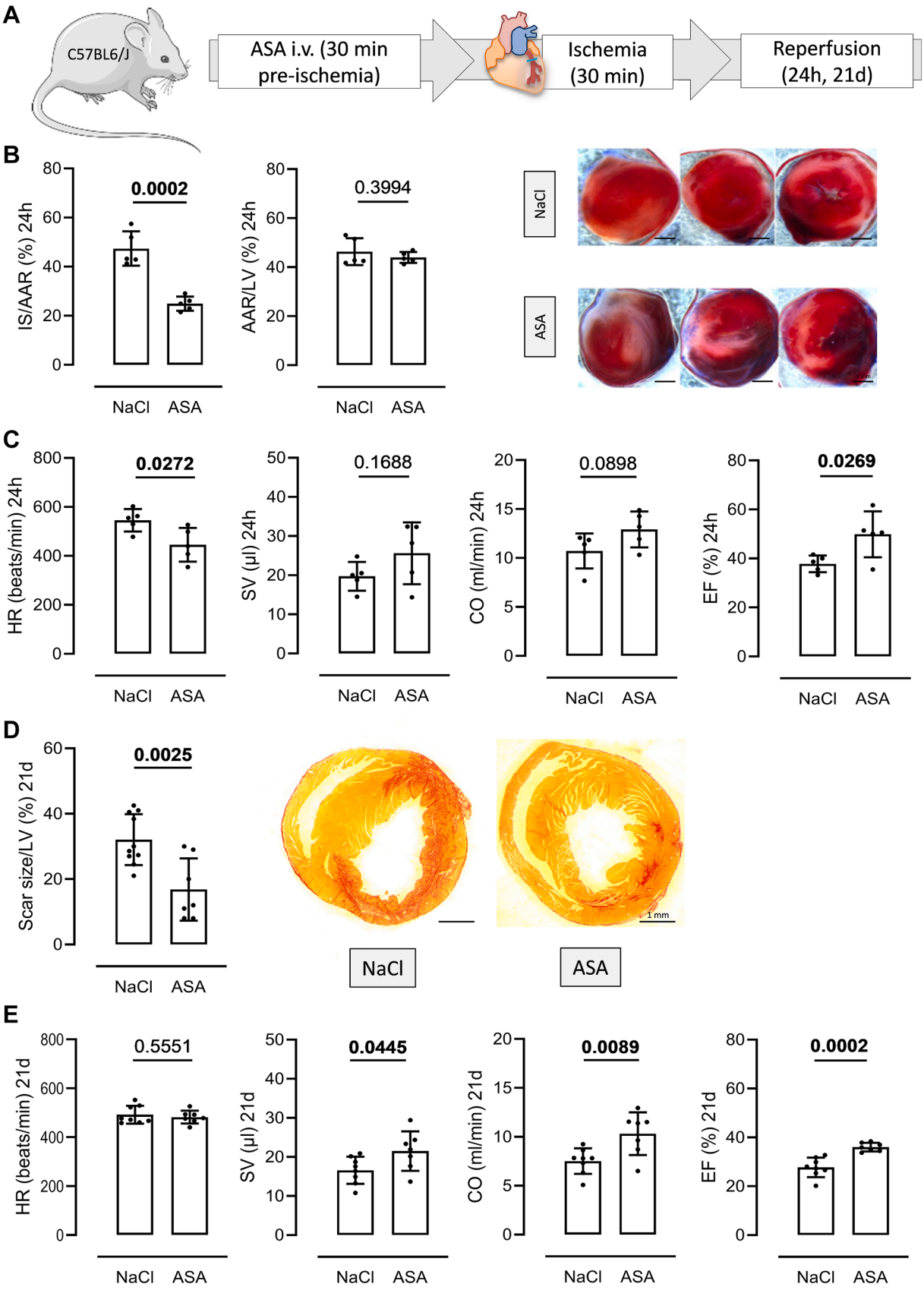

FIGURE 1 Preischemic ASA improves cardiac outcome post-AMI. (A) C57BL/6 wild-type mice were injected i.v. with ASA 30 minutes prior to ischemia. Myocardial ischemia was induced surgically via an open-chest access by ligating the LAD coronary artery with a suture for 30 minutes. Afterward, reperfusion was initiated by opening the suture. Outcome was analyzed either 24 hours (short-term phase [B, C]) or 21

prior to AMI induction, one group of mice (Figure 1A–E) received an intravenous injection of 100 μL (1 mg/mL) low-dose [43] ASA or 0.9% sodium chloride (NaCl) as control. Afterward, AMI was performed via transient ligation of the left anterior descending (LAD) coronary artery. This was achieved by the help of a small piece of tube in the loop of the suture, pressing down on the LAD. The other group was injected with ASA 15 minutes intraischemia (Figure 2A–E). The ligating suture was reopened after 30 minutes and followed by 24 hours (short-term phase), 3 or 5 days (intermediate phase), or 21 days (long-term phase) of coronary reperfusion in different groups for further analyses. Echocardiography was performed (Supplementary Section S1.2), and blood was taken via the retrobulbar plexus for flow cytometry analyses (Supplementary Section S1.9) before euthanasia at each timepoint. Afterward, the thorax was opened under anesthesia, the heart was extracted for histologic analysis of either infarct size (IS) by triphenyl tetrazolium chloride (TTC) after 24 hours (Supplementary Section S1.5), neutrophils in platelet-depleted hearts by Ly6G immunofluorescence staining (Supplementary Section S1.6), NET formation by immunofluorescence (1, 3, and 5 days; Supplementary Section S1.7), or scar size formation by Sirius Red staining after 21 days (Supplementary Section S1.8).

2.2 | Antibody-induced platelet depletion in mice

To test the role of platelets in ASA-mediated cardioprotection during AMI, we excluded platelets from the system. Therefore, C57BL/6J mice were injected with a platelet-depletion antibody (2 μg/g of body weight; #R300, antimouse GPIb, 0.5 mg/mL, Emfret Analytics) or isotype control (2 μg/g of body weight, Wistar Rat IgG, AB C301, Emfret Analytics) 24 hours [44] before ischemia (Supplementary Section S1.3). This was followed by a preischemic injection of ASA and induction of AMI (see Section 2.1 and Supplementary Section S1.1 for details). In the 21-day group, the antibody injection was repeated on days 2 and 4 post-AMI, as platelet levels start to rise again 48 hours after injection according to the company's instructions (data sheet #R300, antimouse GPIb, Emfret Analytics) [45] (Figure 3A).

2.3 | Langendorff isolated perfused heart model

The Langendorff heart model was used to examine ASA's cardioprotective effects on endo- and myocardial cells, excluding potential influence from circulating blood cells [46]. This is achieved by rinsing hearts of C57BL/6J wild-type mice with the perfusion buffer Krebs–Henseleit buffer (KHB) to eliminate blood from the coronary arteries and cavities. Mice were anesthetized; hearts were explanted and placed in 4 °C cold cardioplegic buffer. The ascending aorta was cannulated and fixed in the apparatus. The heart was perfused retrogradely with a constant buffer flow pressure. The left ventricle (LV) pressure gradient was measured (Supplementary Section S1.4). Hearts were loaded with either ASA or control via a syringe pump. To induce global ischemia, the flow pump was stopped for 40 minutes. Reperfusion with KHB was recommenced for 120 minutes (Figure 3H). Hemodynamic parameters were registered 60 minutes after the return of circulation (Figure 3J). Hearts were frozen at −20 °C overnight, and IS was determined by TTC staining (Figure 3I, Supplementary Section S1.5).

2.4 | Analysis of myocardial NET formation and circulating thromboinflammation

In each time series, mice with ASA/control treatment preischemia or intraischemia were anesthetized, blood was taken via the retrobulbar plexus for flow-cytometric measurements of circulating markers of thromboinflammation (Figure 4), and moreover, citrullinated histone 3 (CitH3) enzyme-linked immunosorbent assay for circulating NET formation (Figure 5). Afterwards, mice were sacrificed 1, 3, or 5 days after AMI (Figure 5A). Previous steps are explained in Section 2.1 and Supplementary Section S1.1. They were perfused with cold phosphate-buffered saline, and the hearts were removed and fixed in 4% paraformaldehyde overnight. Hearts were then dehydrated, embedded in paraffin, and cut into 5-μm sections. Afterward, they underwent different steps such as deparaffinization, rehydration, washing, antigen retrieval, and

days (long-term phase [D, E]) after AMI. Statistical test: *t*-test for graphs B–E. (B) IS/AAR was lower (24.92 ± 2.90 vs 47.38 ± 7.01%, $P = .0002$) in mice 24 hours after AMI, treated with ASA pre-ischemia [$n = 5$], than that of the control group (injected with NaCl [$n = 5$]). AAR/LV did not differ in mice 24 hours after AMI, treated with ASA, compared with that of the control group (43.98% ± 2.23% vs 46.34% ± 5.50%, $P = .3994$). Exemplary TTC stainings are shown on the right (scale bar, 1 mm). Vital tissue is marked by Evans blue, AAR is stained red by TTC, and nonviable infarcted tissue remains white. (C) Functional parameters were measured in echocardiography 24 hours post-AMI. HR was lower in the ASA than in the control group (445.60 ± 69.21 vs 545.70 ± 45.86 beats/min, $P = .0272$). SV and CO tended to be higher in the ASA-treated mice but did not reach statistical significance (SV: 25.62 ± 7.89 vs 19.73 ± 3.70 μL, $P = .1688$; CO: 12.92 ± 1.83 vs 10.71 ± 1.78 mL/min, $P = .0898$). Systolic function in terms of EF was higher (37.84% ± 3.39% vs 49.86% ± 9.35%, $P = .0269$) with ASA than with control treatment. (D) Scar size formation in the LV 21 days after AMI was lower in mice with ASA medication ($n = 7$) than in NaCl-treated mice ($n = 8$; 16.86% ± 9.53% vs 33.63% ± 7.98%, $P = .0026$). Exemplary Sirius Red stainings are shown on the right, scar tissue; ie, collagen fibers are dyed red (scale bar, 1 mm). (E) After 21 days, functional parameters such as SV, CO, and EF were higher with ASA than with control treatment (SV: 21.51 ± 5.05 vs 16.60 ± 3.47 μL, $P = .0445$; CO: 10.32 ± 2.18 vs 7.51 ± 1.30 mL/min, $P = .0089$; EF: 36.07% ± 1.78% vs 27.75% ± 3.98%, $P = .0002$). HR was not different between the 2 groups in the long-term phase after AMI (482.0 ± 26.40 vs 492.10 ± 36.71 beats/min, $P = .5551$). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. AAR, area at risk; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; CO, cardiac output; EF, ejection fraction; GP, glycoprotein; HR, heart rate; IS, infarct size; i.v., intravenously; LAD, left anterior descending; LV, left ventricle; SV, stroke volume; TTC, 2,3,5-triphenyl tetrazolium chloride.

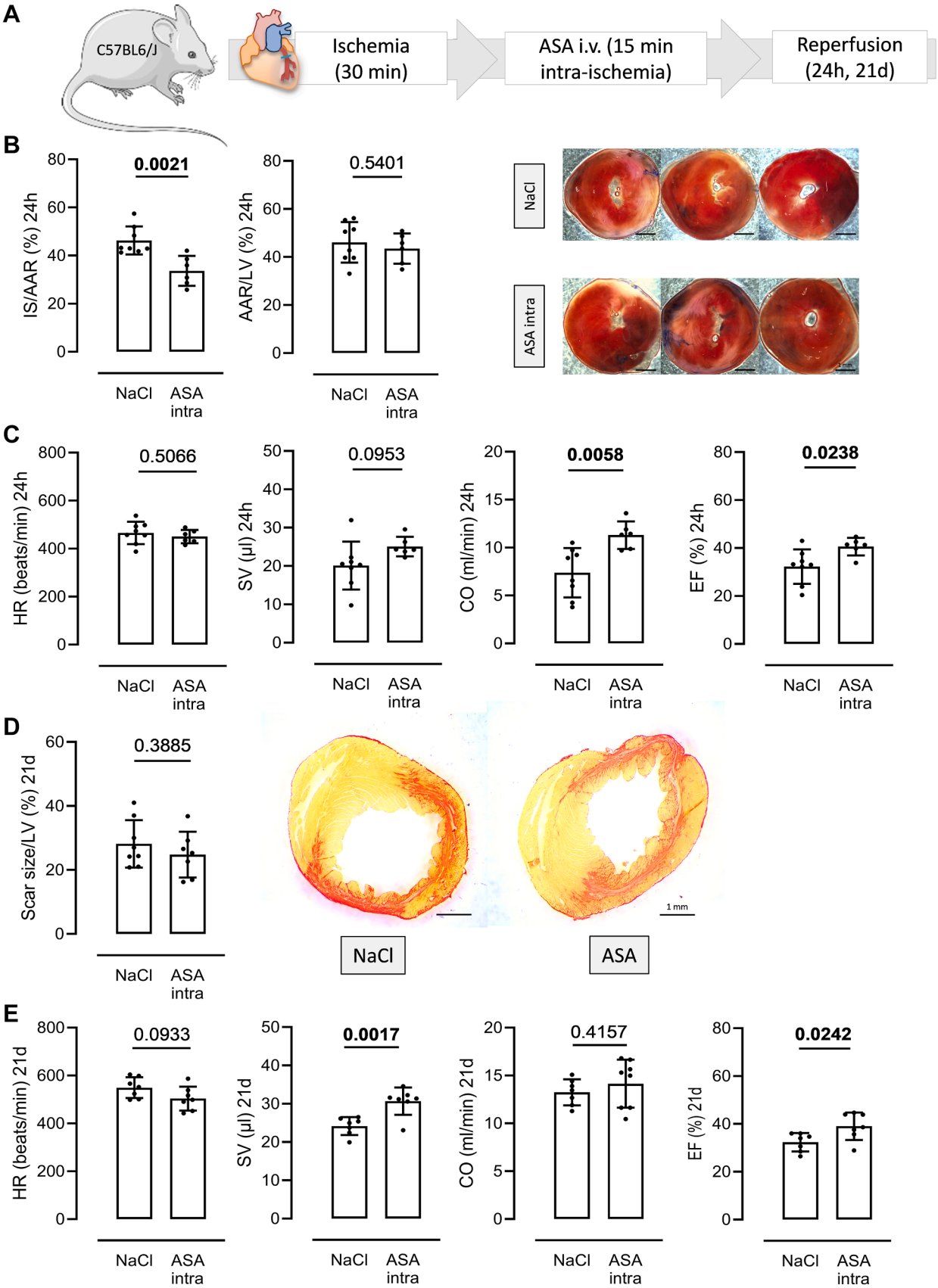

FIGURE 2 Intraischemic ASA improves cardiac function and infarct size post-AMI. (A) Myocardial ischemia was induced via an open-chest access by ligating the LAD coronary artery with a suture for 30 minutes. After 15-minute intraischemia, mice were injected i.v. with ASA. Afterward, reperfusion was initiated by opening the suture. Outcome was analyzed either 24 hours (short-term [B, C]) or 21 days (long-term

antibody incubation (Supplementary Section S1.7). Images of stained sections were captured by using a fluorescence microscope. Cell nuclei appeared blue (DAPI staining), neutrophils (Ly6G) appeared green, and histones (CitH3) appeared red. Five randomly chosen regions of interest of 100 μm × 100 μm each were imaged with the LASX software (Leica Microsystems) of one heart section and analyzed by using ImageJ software. The investigator was blinded to the treatment vs control groups while counting the number of Ly6G-/CitH3-positive cells (ie, NETs).

For the analysis of circulating NET formation at the different timepoints, murine blood was centrifuged to plasma and CitH3 enzyme-linked immunosorbent assay was performed according to the manufacturer's instructions (Nordic Biosite). Circulating markers of thromboinflammation were analyzed in flow cytometry as described in Supplementary Section S1.9.

2.5 | Antibody-induced neutrophil depletion

C57BL/6J wild-type mice received a neutrophil depletion antibody (50 μg; InVivoMAb antimouse Ly6G, clone 1A8, Biozol Diagnostica) or isotype control (50 μg; InVivoMAb antirat Kappa Immunoglobulin Light Chain, clone MAR 18.5, Biozol Diagnostica) intraperitoneally (i.p.) 24 and 2 hours preischemia, as described before [47]. Afterward, AMI was induced as described in Section 2.1 (Figure 5F).

2.6 | Antibody-induced CD40L deficiency

To induce CD40L deficiency, C57BL/6J mice were injected i.p. with 10 μg/g of Ultra-LEAF Purified antimouse CD154 Antibody (clone MR1, 25 mg/11 mL, BioLegend) or correspondingly the isotype control (10 μg/g, Ultra-LEAF Purified Armenian Hamster IgG Isotype Ctrl Antibody, Clone HTK888, 25 mg/11.57 mL, BioLegend) once 24 hours before and once directly after the surgery (Figure 6A).

2.7 | Genetically induced platelet CD40L deficiency

Mice genetically lacking CD40L in platelets and control littermates were used for the induction of AMI. Homozygous $Cd40l^{fl/fl}$ mice were backcrossed to platelet factor 4 (Pf4)-Cre transgenic mice to generate $Cd40l^{fl/fl}$/Pf4-Cre^{tg}–positive (+) and their Cre^{wt} littermates as previously described [48]. These mice lack CD40L specifically in megakaryocytes and platelets (Figure 6D).

All experiments as stated by the European Convention of the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes were approved by the State Agency for Nature, Environment and Consumer Protection North-Rhine Westphalia, Germany (no. 2023.A124). Animals were treated according to the institutional guidelines (Council of Europe Treaty Series no. 123). All procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. We used the ARRIVE checklist when writing our report [49].

2.8 | Patients

In a translational approach, we analyzed patients from the all-comer prospective SYSTEMI trial (ClinicalTrials.gov ID: NCT03539133) [50]. Informed written consent was given prior to the inclusion of subjects in the study. Patients with STEMI, and successful PCI, were followed up for 365 days. All patients with suspected STEMI received a prehospital injection of loading dose ASA as acute treatment by the paramedic teams during transportation to our hospital. Details on characteristics such as comedication and comorbidities were collected, including whether patients took permanent oral ASA medication 3 months prior to STEMI (Supplementary Table S1). We compared 1 group with documented prior ASA medication vs 1 group without preexisting ASA medication. MACE, as a composite of death from any cause, reinfarction, and rehospitalization due to HF, were evaluated as a combined endpoint and each event as a single endpoint. Blood was taken before PCI from the arterial sheath for the

phase [D, E]) after AMI. Statistical test: *t*-test for graphs B–E. (B) IS/AAR was lower (33.65% ± 6.23% vs 46.26% ± 5.81%, $P = .0021$) in mice 24 hours after AMI, treated with ASA intraischemia ($n = 6$), than in the control group (injected with NaCl [$n = 8$]). AAR/LV did not differ in mice 24 hours after AMI, treated with ASA, compared with that in control (43.52% ± 6.30% vs 46.12% ± 8.45%, $P = .5401$). Exemplary TTC stainings are shown on the right (scale bar, 1 mm). Vital tissue is marked by Evans Blue, AAR is stained red by TTC, and nonviable infarcted tissue remains white. (C) Functional parameters were measured in echocardiography 24 hours post-AMI. HR was similar in the ASA and the control group (450.60 ± 27.84 vs 465.40 ± 46.85 beats/min, $P = .5066$). SV tended to be higher in the ASA-treated mice but did not reach statistical significance (SV: 25.07 ± 2.55 vs 20.13 ± 6.26 μL, $P = .0953$). CO and EF were both significantly higher with ASA than with control treatment (CO: 11.31 ± 1.44 vs 7.37 ± 2.58 mL/min, $P = .0058$; EF: 40.60% ± 3.69% vs 32.24% ± 7.19%, $P = .0238$). (D) Scar size formation in the LV 21 days after AMI was not different in mice with ASA medication ($n = 7$) and in NaCl-treated mice ($n = 8$; 24.80% ± 7.16% vs 28.17% ± 7.40%, $P = .3885$). Exemplary Sirius Red stainings are shown on the right scar tissue; ie, collagen fibers are dyed red (scale bar, 1 mm). (E) After 21 days, functional parameters such as SV and EF were higher with ASA than with control treatment (SV: 30.66 ± 3.57 vs 24.16 ± 2.33 μL, $P = .0017$; EF: 39.04% ± 5.73% vs 32.33% ± 3.82%, $P = .0242$). CO and HR were not different between the 2 groups in the long-term phase after AMI (CO: 14.14 ± 2.51 vs 13.24 ± 1.37 mL/min, $P = .4157$; HR: 503.9 ± 49.86 vs 549.5 ± 43.42 beats/min, $P = .0933$). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. AAR, area at risk; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; CO, cardiac output; EF, ejection fraction; HR, heart rate; IS, infarct size; LAD, left anterior descending; LV, left ventricle; SV, stroke volume; TTC, 2,3,5-triphenyl tetrazolium chloride.

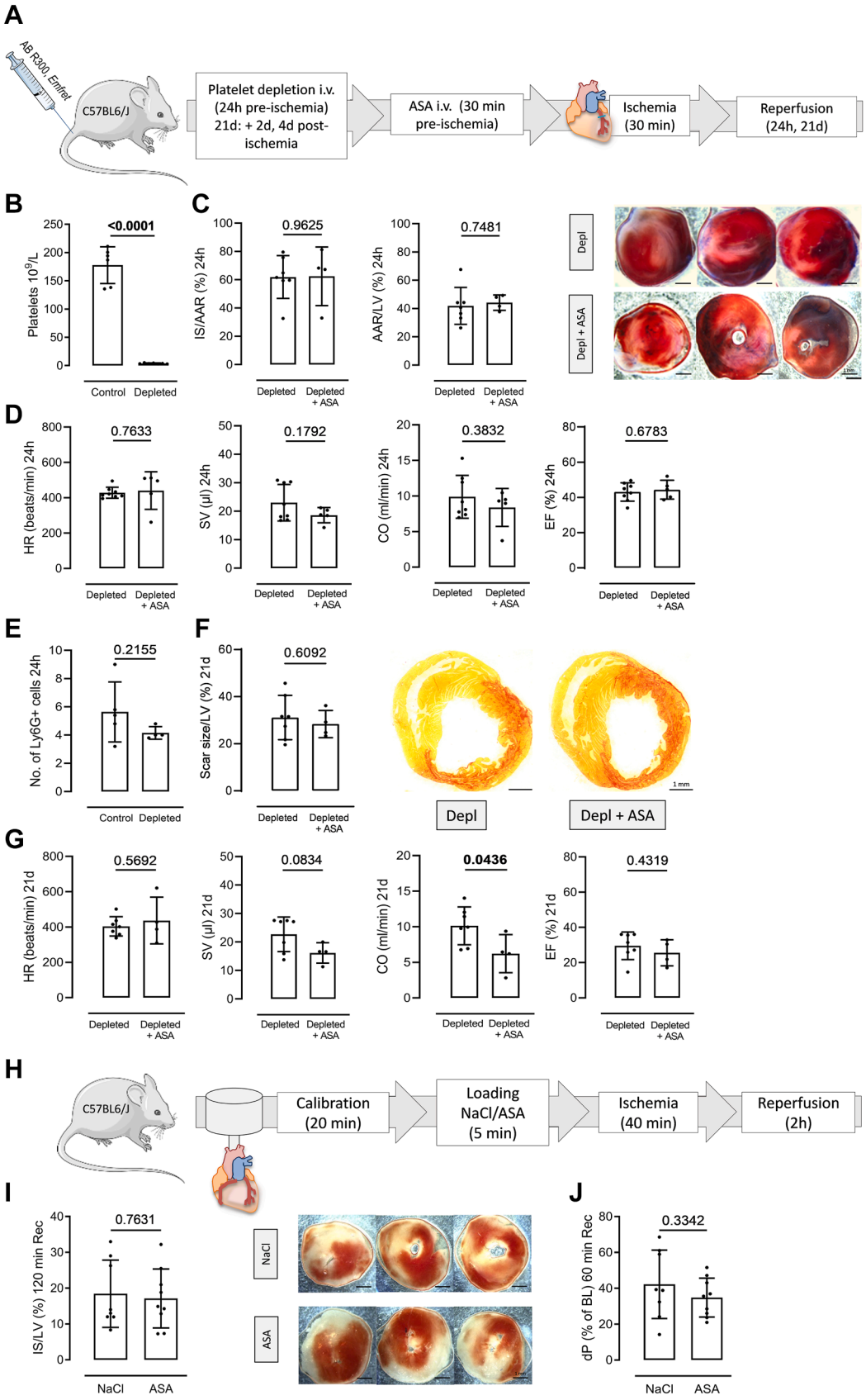

FIGURE 3 ASA-mediated improvement of cardiac outcome post-AMI is platelet-dependent. (A) C57BL6 mice were injected i.v. with a platelet-depletion antibody (AB R300, Emfret) 24 hours prior to myocardial ischemia. In the 21-day group, this injection was repeated on days 2 and 4 after ischemia. ASA was administered i.v. 30 minutes before AMI induction. Here, surgery was performed via a closed chest access due to a higher bleeding risk. Ischemia was maintained by LAD ligation for 30 minutes. Afterward, reperfusion was initiated for either 24 hours (C–E) or

determination of laboratory parameters. This study conformed to the principles of the Declaration of Helsinki and to the ethics committee of the Medical Faculty of the Heinrich Heine University Hospital Düsseldorf (vote no. 2019-557/5961R).

2.9 | Statistical analysis

Statistical analyses were conducted with GraphPad Prism and SPSS (IBM). Normality of distribution was tested with the Shapiro–Wilk test. Normally distributed continuous variables were analyzed using a *t*-test or one-way analysis of variance, where applicable. Fisher's exact test was used for categorical variables. Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. The characteristics differentiating between the ASA-premedicated and the ASA-naive patients with STEMI were analyzed by a propensity score method—an inverse probability of treatment–weighted (IPTW) Cox regression—as formerly described [21]. This resulted in covariate-adjusted hazard ratios (HRs) with 95% CIs. Stabilized weights in IPTW are used to prevent inflation of sample size and, therefore, an increase in type I error rate. However, due to the weighting process, number of patients in groups might change to a minor extent. The statistical computation is not affected.

3 | RESULTS

3.1 | Preischemic and intraischemic ASA treatment improves murine cardiac outcome post-AMI

Mice with ASA injection preischemia showed decreased IS in the early phase 24 hours after AMI compared with NaCl/control mice. Area at risk (AAR) of the LV did not differ (Figure 1B). Regarding functional parameters, heart rate (HR) was lower in the ASA than in the control group. Stroke volume (SV) and cardiac output (CO) tended to be higher in ASA-treated mice but did not reach significance. EF was significantly better with pre-ASA treatment (Figure 1C).

After 21 days, LV scar size formation was lower in mice that underwent preischemic ASA treatment than in control (Figure 1D). Systolic functions such as SV, CO, and EF were markedly higher in the ASA group. HR was not different between the groups (Figure 1E).

ASA treatment intraischemia led to decreased IS and higher CO and EF 24 hours post-AMI. After 21 days, SV and EF were also higher than those of the control group. However, scar size was not reduced with intraischemic ASA treatment (Figure 2).

3.2 | ASA-mediated improvement of cardiac outcome post-AMI is platelet-dependent

To investigate the platelet dependency of ASA-mediated effects, we depleted platelets in mice with an antibody (Figure 3A, B). In the short-term phase after AMI, ASA did not show any beneficial effect in platelet-depleted mice. Neither IS (Figure 3C) nor LV function was altered in comparison to untreated platelet-depleted mice (Figure 3D). The myocardial tissue of platelet-depleted animals showed a numerically lower expression of Ly6G-positive cells (ie, neutrophils), although this observation was not statistically significant (Figure 3E). Regarding the long-term results, LV scar tissue formation was not different between the groups (Figure 3F). The functional parameters HR, SV, and EF were similar (Figure 3G).

Supporting this result, in Langendorff experiments, IS/LV after 120 minutes of recovery was not different between ASA- or NaCl-loaded hearts (Figure 3H, I). Hemodynamics were not different, which were measured as LV pressure gradient in percentage from

21 days (F–G). Statistical test: *t*-test for graphs B–G, I, and J. (B) Success of platelet depletion was proven by distinct reduction of platelet count (3.74 ± 0.89 vs 177.80 ± 32.59 × 10^9/L, $P < .0001$). (C) Twenty-four hours after AMI, ASA did not add any beneficial effects in platelet-depleted mice (group: depleted + ASA [$n = 4$]) compared with depleted mice with control (NaCl) treatment (group: depleted [$n = 7$]). AAR/LV did not differ between ASA and NaCl treatment (44.17% ± 5.42% vs 41.86% ± 13.06%, $P = .7481$). IS/AAR was not lower in the depleted + ASA group (62.45% ± 20.71% vs 61.93% ± 15.15%, $P = .9625$). Exemplary TTC stainings are shown on the right (scale bar, 1 mm). (D) Echocardiographic parameters did not differ in both groups 24 hours post-AMI. HR was 440.60 ± 106.30 vs 428.50 ± 31.17 beats/min ($P = .7633$). SV and CO were not higher in the depleted + ASA group (SV: 18.64 ± 2.68 vs 23.01 ± 6.39 μL, $P = .1792$; CO: 8.39 ± 2.66 vs 9.89 ± 3.04 mL/min, $P = .3832$). EF was the same with 44.40% ± 5.31% vs 43.12% ± 5.23% ($P = .6783$). (E) Myocardial tissue of platelet-depleted animals showed a slightly lower expression of Ly6G-positive cells (ie, neutrophils), although this observation was not statistically significant (5.64 ± 2.12 vs 4.15 ± 0.44 cells per field, $P = .2155$). (F) LV scar tissue formation was not different between the ASA-treated depleted mice ($n = 4$) and the depleted mice with control treatment ($n = 7$; 28.36% ± 5.79% vs 31.15% ± 9.45%, $P = .6092$). Exemplary Sirius Red stainings are shown on the right (scale bar, 1 mm). (G) After 21 days, the functional parameters HR, SV, and EF were similar with ASA and control treatment (HR: 436.80 ± 132.20 vs 404.0 ± 54.65 beats/min, $P = .5692$; SV: 16.15 ± 3.59 vs 22.70 ± 6.08 μL, $P = .0834$; EF: 25.59% ± 7.39% vs 29.55% ± 7.79%, $P = .4319$). CO measured 6.22 ± 2.68 vs 10.13 ± 2.65 mL/min ($P = .0436$). (H) Langendorff experiments on isolated perfused wild-type mouse hearts were performed to differentiate between an endothelium- or platelet-dependent effect of ASA. Blood is washed out in this system and replaced by buffer solution. Therefore, platelets are nonexistent in this experimental setup. Differences in results should thus be attributed to the endothelium. Calibration was executed for 20 minutes, loading with NaCl as control ($n = 8$) or ASA ($n = 9$) for 5 minutes, myocardial ischemia for 40 minutes, and reperfusion/recovery time for 120 minutes. (I) IS/LV after 120 minutes of Rec was not different between ASA- or NaCl-loaded hearts (17.14% ± 8.21% vs 18.45% ± 9.40%, $P = .7631$). Exemplary TTC stainings are shown on the right, and the infarcted avital area remains white (scale bar, 1 mm). (J) Hemodynamics were not different between the 2 groups, measured as a LV pressure gradient (dP) in percentage from BL at 60 minutes of Rec (34.82% ± 10.83% vs 42.22% ± 19.10%, $P = .3342$). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. AAR, area at risk; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; BL, baseline; CO, cardiac output; EF, ejection fraction; HR, heart rate; IS, infarct size; i.v., intravenously; LAD, left anterior descending; LV, left ventricle; Rec, recovery; SV, stroke volume; TTC, 2,3,5-triphenyl tetrazolium chloride.

baseline at 60 minutes of recovery (Figure 3J). The Langendorff heart is explanted and perfused with KHB—therefore separated from its circulatory system—to be analyzed isolated and devoid of circulating cells. Therefore, ASA could not exert its cardioprotective effects in absence of (a) circulating cells (as seen in the Langendorff hearts) and more precisely (b) platelets (as seen in platelet-depleted mice). Circulating blood cells must be thus relevant for the mechanism by which ASA improves cardiac outcome. As platelets and neutrophils interact in thromboinflammatory processes, we next looked at circulatory markers of thromboinflammation.

3.3 | Circulatory markers of thromboinflammation reveal only minor differences with preischemic or intraischemic ASA

Our measurement of circulatory markers of thromboinflammation showed a trend toward reduced platelet activation, measured by P-selectin expression, in the ASA groups 24 hours after AMI, which was significant in the ASA-preischemia group (Figure 4A). This proves the antiplatelet effect of ASA. Furthermore, a reduction in CD40L and CD40 expression with ASA 24 hours post-AMI was detectable, which was significant for CD40L in the ASA intraischemia group (Figure 4B). Neutrophils, measured as Ly6G expression, were numerically reduced in the pre-ASA groups 24 hours and 3 days post-AMI, but not in the 24-hour intra-ASA group (Figure 4A–C [second column from the left]). These data are indicative of reduced thromboinflammation. Other circulatory markers on day 3 post-AMI did not reveal relevant changes. No significant difference in platelet-neutrophil aggregates (PNAs), expressed as Ly6G- and CD41-positive complexes, could be observed (Figure 4A–C [middle column]).

3.4 | ASA-mediated improvement of outcome post-AMI is neutrophil-dependent and reduces myocardial and circulating NET formation

As further marker of thromboinflammation, myocardial and circulating NET formation was analyzed. As described above, mice were injected with ASA 30 minutes before induction or 15 minutes after induction of AMI (Figure 5A). Twenty-four hours after AMI, NET counts were slightly lower in the ASA-preischemia group than in the control group in the myocardium and, more pronounced, in the plasma. In the ASA intraischemia group, 24 hours post-AMI, there was also a trend toward lower NET counts in the myocardium and in the plasma. Three days after AMI with preischemic ASA treatment, no relevant differences could be found in the myocardium or in the plasma. Five days post-AMI, NETs were significantly reduced in the ASA-pretreated group, in contrast to the control group (Figure 5B–E).

In a different setup, neutrophil depletion was achieved, as previously performed [47] (Figure 5F, G). Twenty-four hours after AMI, ASA did not add any beneficial effects in neutrophil-depleted mice vs depleted control mice. IS/AAR was not lower in the $Ly6G^{-}$ + ASA group (Figure 5H). Echocardiographic parameters did not differ in both groups 24 hours post-AMI (Figure 5I).

ASA was not able to exert its cardioprotective effects in the absence of neutrophils. This suggested that neutrophils must play an important role in the mechanism by which ASA improves cardiac outcome.

3.5 | ASA-mediated improvement of cardiac outcome post-AMI depends on the CD40–CD40L crosstalk

C57BL/6J mice received CD40L antibody i.p. 24 hours prior to AMI. Control (isotype-treated) and CD40L-AB–treated mice received NaCl or ASA (Figure 6A). We could reproduce the finding from Figure 1B and prove again that IS was lower with preischemic injection of ASA than with the control substance. When comparing CD40L-AB + ASA with CD40L-AB + NaCl, the protective effect of ASA was blunted, as both groups did not differ. IS was distinctly lower in the control + ASA group than in the CD40L-AB + ASA group (Figure 6B). No relevant functional differences were found for HR and SV. In the isotype cohort, CO and EF were higher in ASA-treated mice. In the CD40L-AB cohort, ASA did not improve functional parameters. CO and EF were significantly lower in the CD40L-AB + ASA group than in the control + ASA group (Figure 6C). No differences were found between NaCl + isotype control vs NaCl + CD40L-AB.

For the genetic approach, $Cd40l^{fl/fl}$/Pf4-Cre^{tg}-positive (Pf4-Cre^{+}) and their Cre^{wt} littermates (Pf4-Cre^{-}) received ASA or NaCl intravenously 30 minutes prior to the 30-minute myocardial ischemia and 24-hour reperfusion phase (Figure 6D). IS was lower in the ASA than in the control Pf4-Cre^{-} group. In the Pf4-Cre^{+} group, ASA did not have an impact. IS was reduced in the Pf4-Cre^{-} + ASA compared with that in the Pf4-Cre^{+} + ASA group (Figure 6E).

Regarding cardiac function, EF was significantly higher in the ASA- than in the NaCl-treated Pf4-Cre^{-} cohort. In the Pf4-Cre^{+} cohort, ASA did not improve systolic function. EF was significantly lower in the Pf4-Cre^{+} + ASA than in the Pf4-Cre^{-} + ASA group. The other parameters showed no relevant differences (Figure 6F). No differences were found between NaCl + Pf4-Cre^{-} and NaCl + Pf4-Cre^{+}.

Overall, both pharmacologic and platelet-specific genetic inhibition of the CD40L receptor resulted in ineffectiveness of ASA treatment. This suggested a defining role of platelet CD40L in the pathway of ASA-mediated cardioprotection.

3.6 | MACE after STEMI are lower in patients with prior ASA medication

In our SYSTEMI analysis [50], 651 patients with successful PCI received follow-up for 365 days (Figure 7A). Four hundred sixty patients did not take, and 191 patients took ASA as regular medication prior to STEMI. Several baseline characteristics differed between the ASA-pretreated and the ASA-naive group. Patients with

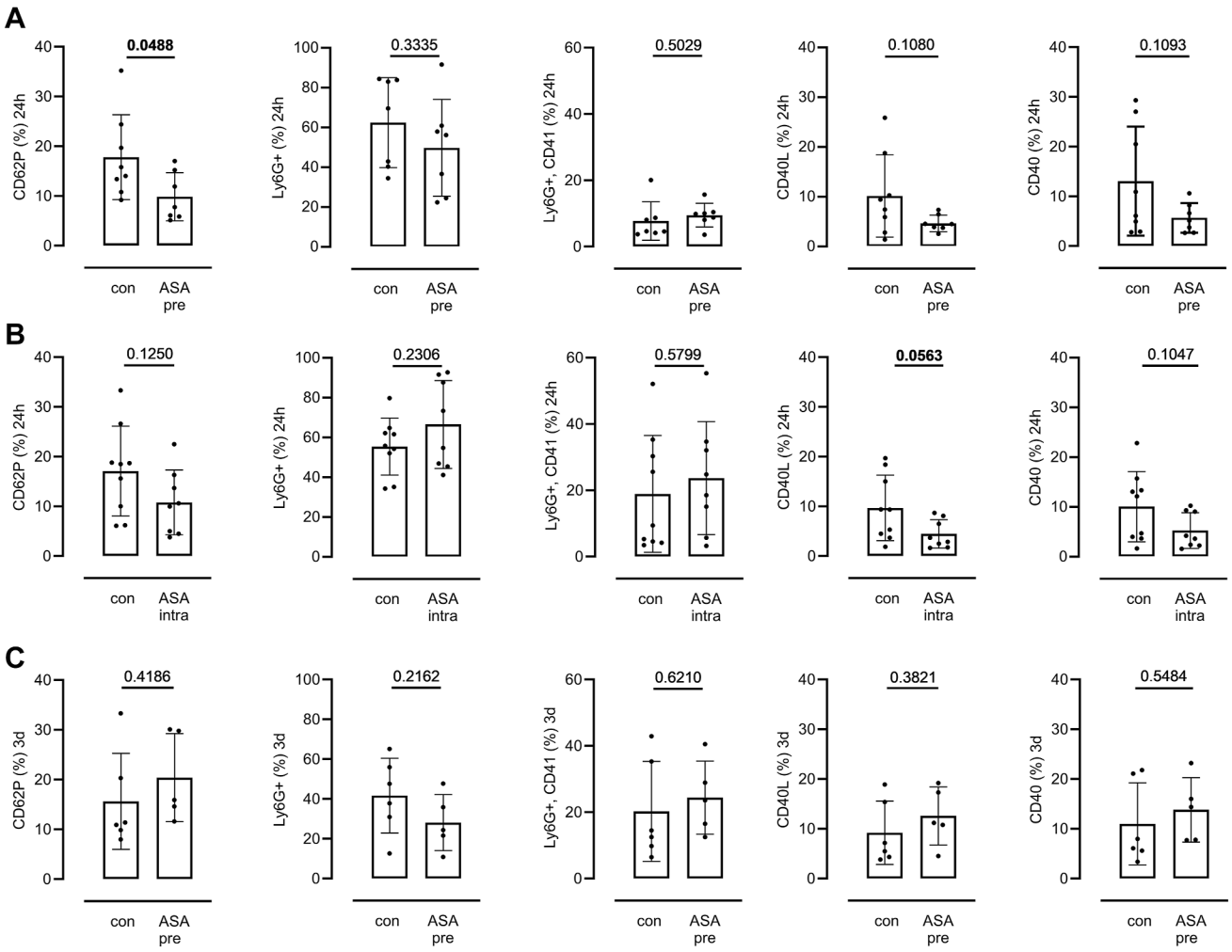

FIGURE 4 Circulatory markers of thromboinflammation reveal only minor differences with pre- or intraischemic ASA. C57BL6 mice were injected with ASA (n = 5-7) or NaCl (n = 6-8) 30 minutes before induction of AMI or 15 minutes after induction of ischemia. Transient ischemia was maintained for 30 minutes by LAD ligation and subsequent reperfusion for 1 or 3 days. Flow cytometric measurements were conducted afterward. (A) Twenty-four hours post-AMI, activated platelets (P-selectin/CD62P) were significantly reduced with preischemic ASA application (17.81% ± 8.54% vs 9.85% ± 4.84%, P = .0488). Neutrophils (Ly6G+) were numerically lower but not statistically significant (62.46% ± 22.62% vs 49.81% ± 24.30%, P = .3335). Platelet–neutrophil aggregates (Ly6G+, CD41) showed no relevant difference (7.71% ± 5.80% vs 9.49% ± 3.58%, P = .5029). CD40L and CD40 levels were numerically reduced, which did not reach significance (CD40L: 10.16% ± 8.27% vs 4.65% ± 1.65%, P = .1080; CD40: 13.05% ± 10.96% vs 5.67% ± 2.99%, P = .1093). (B) 24 hours post-AMI, CD62P levels were slightly reduced with intraischemic ASA application (17.09% ± 9.04% vs 10.80% ± 6.53%, P = .1250). Neutrophils (Ly6G+) were slightly higher but not statistically significant (55.46% ± 14.31% vs 66.60% ± 22.09%, P = .2306). Platelet–neutrophil aggregates (Ly6G+, CD41) showed no relevant difference (18.91% ± 17.61% vs 23.68% ± 17.05%, P = .5799). CD40L was distinctly reduced with ASA intraischemia (9.69% ± 6.59% vs 4.47% ± 2.87%, P = .0563), and CD40 levels were slightly lower (10.07% ± 7.05% vs 5.29% ± 3.59%, P = .1047). (C) Three days post-AMI, circulatory markers showed no relevant differences with ASA preischemia (CD62P: 15.62% ± 9.66% vs 20.40% ± 8.86%, P = .4186; Ly6G: 41.68% ± 18.78% vs 28.10% ± 14.11%, P = .2162; Ly6G+, CD41: 20.25% ± 15.04% vs 24.40% ± 11.00%, P = .6210; CD40L: 9.19% ± 6.37% vs 12.60% ± 5.83%, P = .3821; and CD40: 10.99% ± 8.24% vs 13.82% ± 6.46%, P = .5484). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. AMI, acute myocardial infarction; ASA, acetylsalicylic acid; LAD, left anterior descending.

ASA therapy were older (67 ± 12 vs 63 ± 12 years, P < .001) and had more comorbidities than ASA-naive patients: 61.3% vs 50.9% (P = .015) were smokers, 48.7% vs 32.8% had diabetes mellitus, 74.3% vs 55.9% had hypertension, and 45.5% vs 28.5% had hyperlipidemia (all P < .001). Accordingly, in the ASA-pretreated group, more people were also treated with comedication such as dual antiplatelet therapy, beta-blockers, angiotensin-converting enzyme inhibitors, diuretics, and statins (all P < .001). Gender inequalities were not found (female: 25.7% vs 24.6%, P = .770). As expected, more patients in the ASA pregroup had previous AMI, stroke, or PCI (Supplementary Table S1).

By matching the groups using IPTW, 465 patients were allocated to the ASA-naive group and 184 patients were allocated to the ASA pregroup, and covariates, including comedication, were assimilated

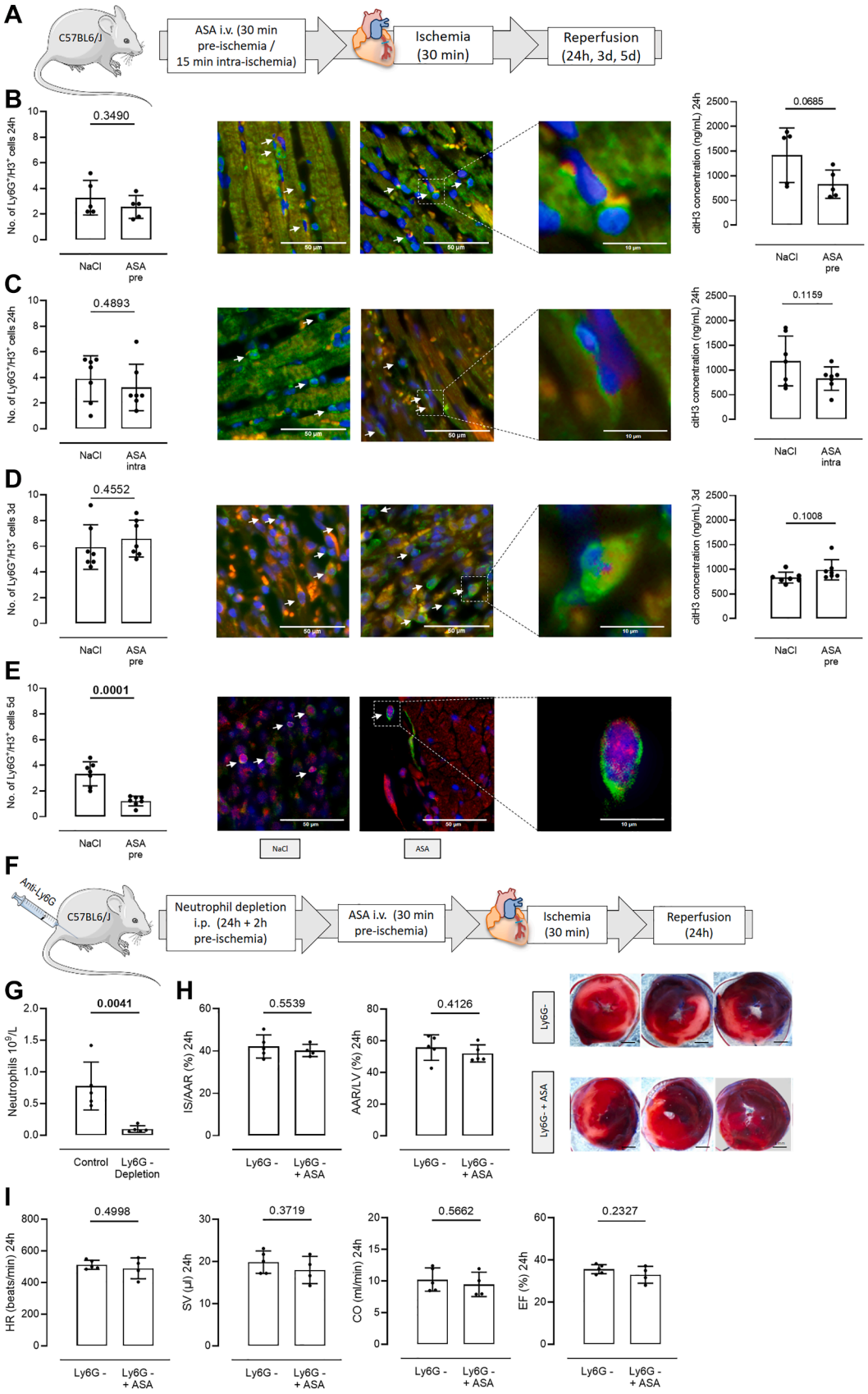

FIGURE 5 ASA-mediated improvement of cardiac outcome post-AMI is neutrophil-dependent and reduces the formation of NETs. (A) Similar to the experimental setup before, C57BL6 mice were injected with ASA (n = 5-7) or NaCl (n = 5-7) 30 minutes before induction of AMI or 15 minutes after induction of ischemia. Transient ischemia was maintained for 30 minutes by LAD ligation and subsequent reperfusion for

(Supplementary Table S2). After IPTW Cox regression, MACE occurred less (10.2% vs 22.6%; HR, 0.42; 95% CI, 0.24-0.74; $P = .002$) in patients with ASA therapy (Figure 7B). Regarding the single components of MACE, mortality rates were notably lower (5.2% vs 16.7%; HR, 0.30; 95% CI, 0.16-0.55; $P < .001$) in the ASA premedication group than in the ASA-naive group. Reinfarction and rehospitalization due to HF did not differ between the groups (reinfarction: 1.7% vs 3.2%; HR, 0.45; 95% CI, 0.09-2.40; $P = .352$; rehospitalization: 3.4% vs 3.6%; HR, 0.87; 95% CI, 0.24-3.21; $P = .835$; Figure 7C–E and Supplementary Table S3).

4 | DISCUSSION

The main findings of this study are as follows: (1) ASA reduces murine IS and myocardial NET formation and improves echocardiographic function after AMI, (2) ASA application before—but not after—the initiation of ischemia reduces scar tissue formation, (3) these effects are platelet- and neutrophil-dependent and mediated via CD40–CD40L, and (4) MACE are lower in patients with STEMI and previous ASA medication.

In summary, we present a new beneficial cardioprotective mechanism of ASA beyond mere platelet inhibition and reduction of thrombus formation. The earlier the ASA treatment begins, the more protective it seems to be: beginning an ASA treatment only after AMI is beneficial for short-term results and even for long-term cardiac function, but it does not reduce scar size formation. The human STEMI cohort supports the divergent long-term effects, as MACE after 12 months were significantly higher with the ASA-naive group, receiving ASA only after the STEMI. Moreover, we could show that preischemic ASA alters cardiac remodeling and improves outcome post-AMI by inhibiting thromboinflammation. This mechanism is platelet- and neutrophil-dependent via the CD40–CD40L axis (Figure 8 [visual abstract]).

Our measurement of circulatory markers of thromboinflammation showed a reduction in P-selectin–mediated platelet activation with ASA medication 24 hours after AMI, which was significant in the ASA-preischemia group (Figure 4A). This proves the antiplatelet effect of ASA. Furthermore, a reduction in CD40L and CD40 expression with ASA after 24 hours was detectable, which was significant for CD40L in the ASA intraischemia group (Figure 4B). These data are indicative of reduced thromboinflammation. Data on circulatory markers on day 3 post-AMI and on PNAs, expressed as Ly6G- and CD41-positive complexes, were rather inconclusive and could not substantiate our hypothesis. Comparison of myocardial and circulating NET formation showed a similar trend: NET counts tended to be reduced both with preischemic and intraischemic ASA 24 hours after AMI. This observation was not significant. On day 3, again no relevant changes could be detected. On day 5, however, NET formation was distinctly reduced with preischemic ASA (Figure 5B–E). This points to the peak of inflammatory response 5 days post-AMI.

ASA in the primary prevention of CAD has been under debate [9]. The underlying studies found an unfavorable safety profile, driven by major bleedings [10] or all-cause mortality [11]. However, patient selection might have affected these results. The ASCEND (A Study of Cardiovascular Events in Diabetes) trial population on ASA in patients with diabetes was solely British. ASA-associated major bleeding excess was mainly gastrointestinal. However, only 1 quarter of the patients were treated with proton pump inhibitors. Simultaneous prescription of ASA and proton pump inhibitors could reduce bleedings and strengthen the benefit of MACE reduction [10]. The ASPREE (Aspirin in Reducing Events in the Elderly) trial investigated ASA for primary prevention in the elderly. No cardiovascular benefit could be demonstrated at the cost of higher bleeding risk and enhanced cancer-related mortality. The latter was unexpected and

1, 3, or 5 days. (B–E) NET formation was assessed by immunofluorescence staining and presented as the number of Ly6G-positive and citrullinated histone (H3)–positive cells on an arbitrary 100 × 100 μm field. Exemplary images are shown on the right, and white arrows indicate $Ly6G^+/H3^+$ cells (scale bar, 50 μm). Magnifications of relevant cells can be found in the zoomed-in images (scale bar, 10 μm). Statistical test: *t*-test for graphs B–E and G–I. (B) Twenty-four hours after AMI, NET counts were slightly lower in the ASA-preischemia group than in the control group in the myocardium (3.28 ± 1.35 vs 2.56 ± 0.89 cells, $P = .3490$) and, more pronounced, in the plasma (1416 ± 554 vs 830 ± 286 ng/mL, $P = .0685$). (C) In the ASA intraischemia group 24 hours post-AMI, NET counts were slightly lower in the myocardium (3.91 ± 1.79 vs 3.23 ± 1.81 cells, $P = .4893$) and in the plasma (1183 ± 506 vs 826 ± 237 ng/mL, $P = .1159$). (D) Three days after AMI with preischemic ASA treatment, no relevant differences could be found in the myocardium (5.94 ± 1.74 vs 6.60 ± 1.43 cells, $P = .4552$) or in the plasma (833 ± 111 vs 991 ± 207 ng/mL, $P = .1008$). (E) NETs were significantly reduced in the ASA-preischemia group in contrast to the control group 5 days after AMI (1.22 ± 0.38 vs 3.34 ± 0.94 cells, $P = .0001$). (F) In a different setup, C57BL6 mice were injected intraperitoneally with a Ly6G-antibody (mAB clone 1A8, Biozol) 24 hours and 2 hours preischemia to achieve neutrophil depletion. As before, mice received either ASA (group: $Ly6G^- + ASA$ [$n = 4$]) or NaCl (group: $Ly6G^-$ [$n = 5$]) i.v. 30 minutes before AMI. Myocardial ischemia was performed for 30 minutes, followed by 24 hours of reperfusion. (G) Successful neutrophil depletion was proven by distinct reduction of neutrophil count (0.78 ± 0.38 vs 0.09 ± 0.05 × 10^9/L, $P = .0041$). (H) Twenty-four hours after AMI, ASA did not add any beneficial effects in neutrophil-depleted mice compared with depleted control mice. AAR/LV did not differ between ASA and control mice (52.01% ± 5.41% vs 55.77% ± 8.07%, $P = .4126$). IS/AAR was not lower in the $Ly6G^- + ASA$ group (40.25% ± 2.91% vs 42.15% ± 5.45%, $P = .5539$). Exemplary TTC stainings are shown on the right (scale bar, 1 mm). (I) Echocardiographic parameters did not differ in both groups 24 hours post-AMI. HR was 489.80 ± 66.02 vs 512.80 ± 28.42 beats/min ($P = .4998$). SV and CO were not higher in the $Ly6G^- + ASA$ group (SV: 18.0 ± 3.23 vs 19.86 ± 2.65 μL, $P = .3719$; CO: 9.48 ± 1.93 vs 10.24 ± 1.85 mL/min, $P = .5662$). EF was the same with 32.96% ± 3.99% vs 35.66% ± 2.16% ($P = .2327$). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. AAR, area at risk; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; CO, cardiac output; EF, ejection fraction; HR, heart rate; IS, infarct size; LAD, left anterior descending; LV, left ventricle; NET, neutrophil extracellular trap; SV, stroke volume; TTC, 2,3,5-triphenyl tetrazolium chloride.

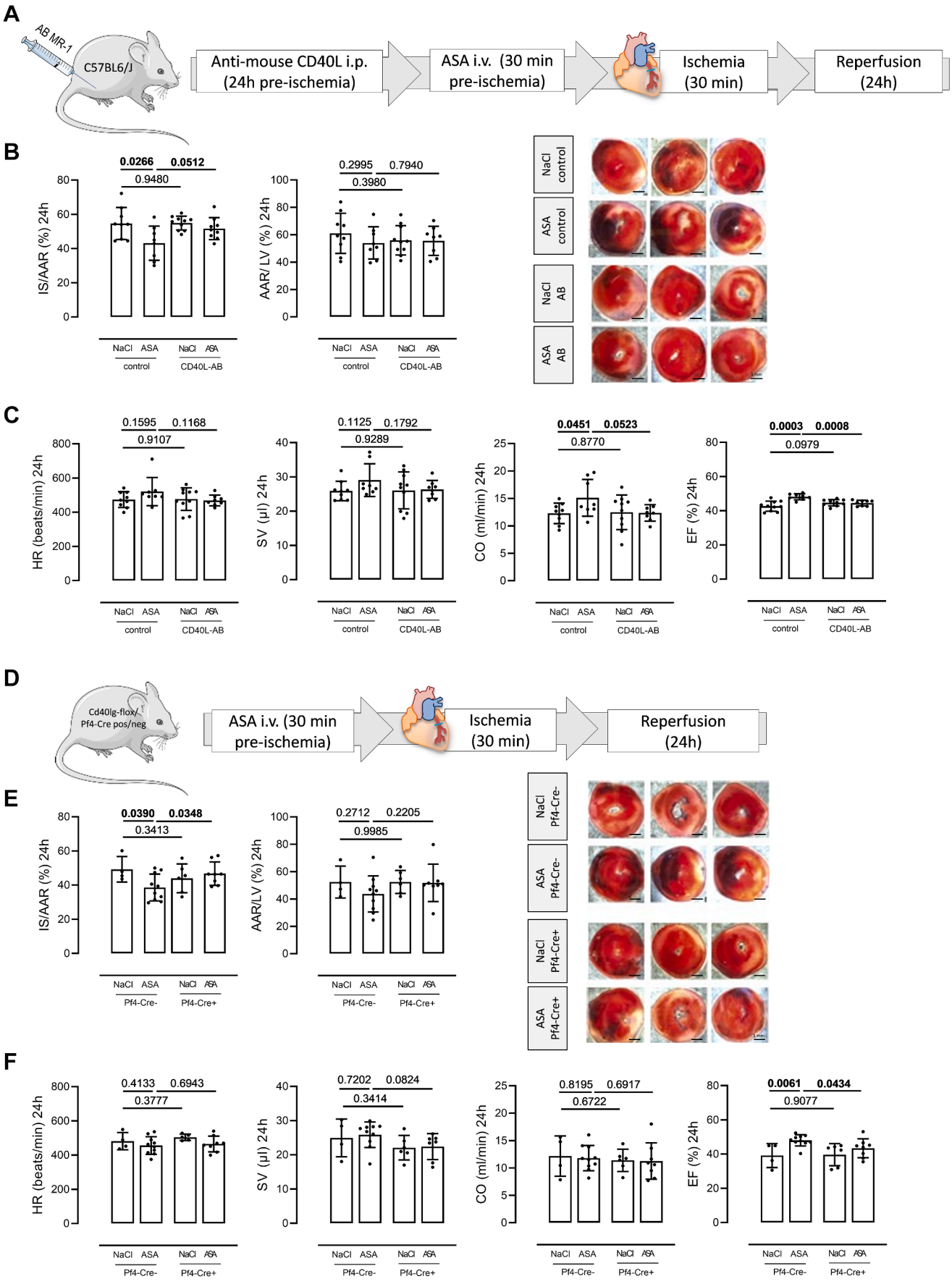

FIGURE 6 ASA-mediated improvement of cardiac outcome post-AMI is dependent on the CD40–CD40L axis. Here, we differentiated between pharmacologic inhibition (A–C) and genetic deletion (D–F) of the CD40L receptor. Statistical test: *t*-test. (A) C57BL6 mice received CD40L antibody (CD154 Antibody, Clone MR1, BioLegend) intraperitoneally 24 hours prior to myocardial infarction. The following sequence

should be interpreted with caution in the context of the apparently healthy elderly cohort [11]. ARRIVE with no relevant differences in MACE with ASA was designed for a moderate cardiovascular risk group, although the study population reflected a low-risk group, as event rates were lower than expected. The authors attributed this to contemporary strategies for risk management [12]. These trials only looked as far as the first event without investigating if ASA might have cardioprotective effects in post-AMI remodeling. Our findings suggest that these studies might not have chosen the appropriate outcomes and patient selection could be improved.

In secondary prevention, recent trials tend to drop or replace ASA in antiplatelet regimes, focusing on P2Y12 inhibitors [51,52]. The OPT-BIRISK trial evaluated extended antiplatelet therapy with clopidogrel alone vs clopidogrel plus ASA in patients with high-risk ACS after PCI. The difference in favor of clopidogrel monotherapy for the primary endpoint was mainly driven by Bleeding Academic Research Consortium 2, not 3 or 5 (ie, noncritical and nonfatal) bleedings. MACE were lower in the clopidogrel monotherapy group, although the single components of MACE were not significantly different. Furthermore, the study population was presorted, and all

of ischemia and reperfusion was conducted as described before. Control (isotype-treated) mice received NaCl (n = 9) or ASA (n = 8), and CD40L-AB–treated mice received NaCl (n = 10) or ASA (n = 9) as well. Controls (control + NaCl vs CD40L-AB + NaCl) showed no difference. Statistical test: one-way ANOVA for graphs B, C, E, and F. (B) AAR/LV was similar in all groups 24 hours after AMI (control + ASA vs control + NaCl: 54.05 ± 11.78 vs 61.03% ± 14.61%, P = .2995; CD40L-AB + ASA vs CD40L-AB + NaCl: 55.54% ± 10.65% vs 55.96% ± 10.74%, P = .9351; control + ASA vs CD40L-AB + ASA: 54.05% ± 11.78% vs 55.54% ± 10.65%, P = .7940; and control + NaCl vs CD40L-AB + NaCl: 61.03% ± 14.61% vs 55.96% ± 10.74%, P = .3980). IS/AAR was lower in the ASA than in the NaCl control group (43.13% ± 9.96% vs 54.65% ± 9.37%, P = .0266). CD40L-AB + ASA vs CD40L-AB + NaCl did not differ: 51.64% ± 6.43% vs 54.87% ± 4.03% (P = .2025). IS was lower in the control + ASA than in the CD40L-AB + ASA group: 43.13% ± 9.96% vs 51.64% ± 6.43% (P = .0512). Control + NaCl vs CD40L-AB + NaCl: 54.65% ± 9.37% vs 54.87% ± 4.03%, P = .9480. Exemplary TTC stainings for all 4 groups are shown on the right (scale bar, 1 mm). (C) HR was numerically highest in the control + ASA group but did not differ significantly between the groups (control + ASA vs control + NaCl: 520.80 ± 82.16 vs 473.60 ± 46.46 beats/min, P = .1595; CD40L-AB + ASA vs CD40L-AB + NaCl: 468.90 ± 31.10 vs 476.60 ± 65.86 beats/min, P = .7638; control + ASA vs CD40L-AB + ASA: 520.80 ± 82.16 vs 468.90 ± 31.10 beats/min, P = .1168; control + NaCl vs CD40L-AB + NaCl: 473.60 ± 46.46 vs 476.60 ± 65.86 beats/min, P = .9107). SV was numerically highest in the control + ASA group but failed to reach statistical significance (control + ASA vs control + NaCl: 29.05 ± 4.76 vs 25.89 ± 2.85 μL, P = .1125; CD40L-AB + ASA vs CD40L-AB + NaCl: 26.34 ± 2.62 vs 26.07 ± 5.39 μL, P = .9012; control + ASA vs CD40L-AB + ASA: 29.05 ± 4.76 vs 26.34 ± 2.62 μL, P = .1792; control + NaCl vs CD40L-AB + NaCl: 25.89 ± 2.85 vs 26.07 ± 5.39 μL, P = .9289). In the isotype control cohort, CO and EF were higher in ASA-treated mice in contrast to NaCl-treated mice. In the CD40L-AB cohort, ASA did not improve functional parameters. CO and EF were lower in the CD40L-AB + ASA group than in the control + ASA group (CO in mL/min: control + ASA vs control + NaCl: 15.11 ± 3.35 vs 12.29 ± 1.87, P = .0451; CD40L-AB + ASA vs CD40L-AB + NaCl: 12.36 ± 1.50 vs 12.48 ± 3.16, P = .9284; control + ASA vs CD40L-AB + ASA: 15.11 ± 3.35 vs 12.36 ± 1.50, P = .0523; control + NaCl vs CD40L-AB + NaCl: 12.29 ± 1.87 vs 12.48 ± 3.16, P = .8770; EF in percentage: control + ASA vs control + NaCl: 48.16 ± 1.77 vs 42.73 ± 3.09, P = .0005; CD40L-AB + ASA vs CD40L-AB + NaCl: 44.52 ± 1.64 vs 45.45 ± 2.56, P = .3869; control + ASA vs CD40L-AB + ASA: 48.16 ± 1.77 vs 44.52 ± 1.64, P = .0008; and control + NaCl vs CD40L-AB + NaCl: 42.73 ± 3.09 vs 44.67 ± 1.87, P = .0979). (D) Cd40lg-flox/Pf4-Cre–positive and negative mice received ASA or NaCl i.v. 30 minutes prior to 30 minutes of myocardial ischemia and 24 hours of reperfusion (Pf4-Cre$^-$ + NaCl [n = 4], Pf4-Cre$^-$ + ASA [n = 10], Pf4-Cre$^+$ + NaCl [n = 6], and Pf4-Cre$^+$ + ASA [n = 8]). Controls (Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl) showed no difference. (E) AAR/LV was similar in all groups 24 hours after AMI (Pf4-Cre$^-$ + ASA vs Pf4-Cre$^-$ + NaCl: 43.69% ± 13.21% vs 52.45% ± 11.64%, P = .2712; Pf4-Cre$^+$ + ASA vs Pf4-Cre$^+$ + NaCl: 51.81% ± 13.68% vs 52.47% ± 8.43%, P = .9197; Pf4-Cre$^-$ + ASA vs Pf4-Cre$^+$ + ASA: 43.69% ± 13.21% vs 51.81% ± 13.68%, P = .2205; and Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl: 52.45% ± 11.64% vs 52.47% ± 8.43%, P = .9985). IS/AAR was lower in the ASA than in the NaCl Pf4-Cre$^-$ group: 38.63% ± 7.87% vs 49.28% ± 7.47%, P = .0390. Pf4-Cre$^+$ + ASA vs Pf4-Cre$^+$ + NaCl did not differ: 46.76% ± 6.84% vs 44.00% ± 8.42%, P = .5103. IS was lower in the Pf4-Cre$^-$ + ASA group than in the Pf4-Cre$^+$ + ASA group (38.63% ± 7.87% vs 46.76% ± 6.84%, P = .0348; Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl: 49.28% ± 7.47% vs 44.00% ± 8.42%, P = .3413). Exemplary TTC stainings for all 4 groups are shown on the right (scale bar, 1 mm). (F) HR (beats/min) did not differ between the groups: Pf4-Cre$^-$ + ASA vs Pf4-Cre$^-$ + NaCl: 455.90 ± 51.61 vs 481.60 ± 50.45, P = .4133; Pf4-Cre$^+$ + ASA vs Pf4-Cre$^+$ + NaCl: 465.20 ± 46.17 vs 504.20 ± 18.35, P = .1028; Pf4-Cre$^-$ + ASA vs Pf4-Cre$^+$ + ASA: 455.90 ± 51.61 vs 465.20 ± 46.17, P = .6943; and Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl: 481.60 ± 50.45 vs 504.20 ± 18.35 beats/min, P = .3777). CO (mL/min) also did not differ between the groups (Pf4-Cre$^-$ + ASA vs Pf4-Cre$^-$ + NaCl: 11.80 ± 2.31 vs 12.18 ± 3.67, P = .8195; Pf4-Cre$^+$ + ASA vs Pf4-Cre$^+$ + NaCl: 11.26 ± 3.33 vs 11.39 ± 2.04, P = .9357; Pf4-Cre$^-$ + ASA vs Pf4-Cre$^+$ + ASA: 11.80 ± 2.31 vs 11.26 ± 3.33, P = .6917; and Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl: 12.18 ± 3.67 vs 11.39 ± 2.04 mL/min, P = .6722). SV (in microliters) was highest in the Pf4-Cre$^-$ + ASA group but failed to reach statistical significance (Pf4-Cre$^-$ + ASA vs Pf4-Cre$^-$ + NaCl: 25.87 ± 3.74 vs 24.95 ± 5.51, P = .7202; Pf4-Cre$^+$ + ASA vs Pf4-Cre$^+$ + NaCl: 22.42 ± 3.80 vs 22.08 ± 3.58, P = .8693; Pf4-Cre$^-$ + ASA vs Pf4-Cre$^+$ + ASA: 25.87 ± 3.74 vs 22.42 ± 3.80, P = .0824; and Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl: 24.95 ± 5.51 vs 22.08 ± 3.58 μL, P = .3414). EF was higher in the ASA- in contrast to the NaCl-treated Pf4-Cre$^-$ cohort. In the Pf4-Cre$^+$ cohort, ASA did not improve systolic function. EF in percentage was significantly lower in the Pf4-Cre$^+$ + ASA than in the Pf4-Cre$^-$ + ASA group (Pf4-Cre$^-$ + ASA vs Pf4-Cre$^-$ + NaCl: 48.01 ± 3.29 vs 39.17 ± 6.99, P = .0061; Pf4-Cre$^+$ + ASA vs Pf4-Cre$^+$ + NaCl: 43.41 ± 5.55 vs 39.68 ± 6.44, P = .2677; Pf4-Cre$^-$ + ASA vs Pf4-Cre$^+$ + ASA: 48.01 ± 3.29 vs 43.41 ± 5.55, P = .0434; and Pf4-Cre$^-$ + NaCl vs Pf4-Cre$^+$ + NaCl: 39.17% ± 6.99% vs 39.68% ± 6.44%, P = .9077). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. AAR, area at risk; AMI, acute myocardial infarction; ANOVA, analysis of variance; ASA, acetylsalicylic acid; CO, cardiac output; EF, ejection fraction; HR, heart rate; IS, infarct size; i.v., intravenously; LV, left ventricle; SV, stroke volume; TTC, 2,3,5-triphenyl tetrazolium chloride.

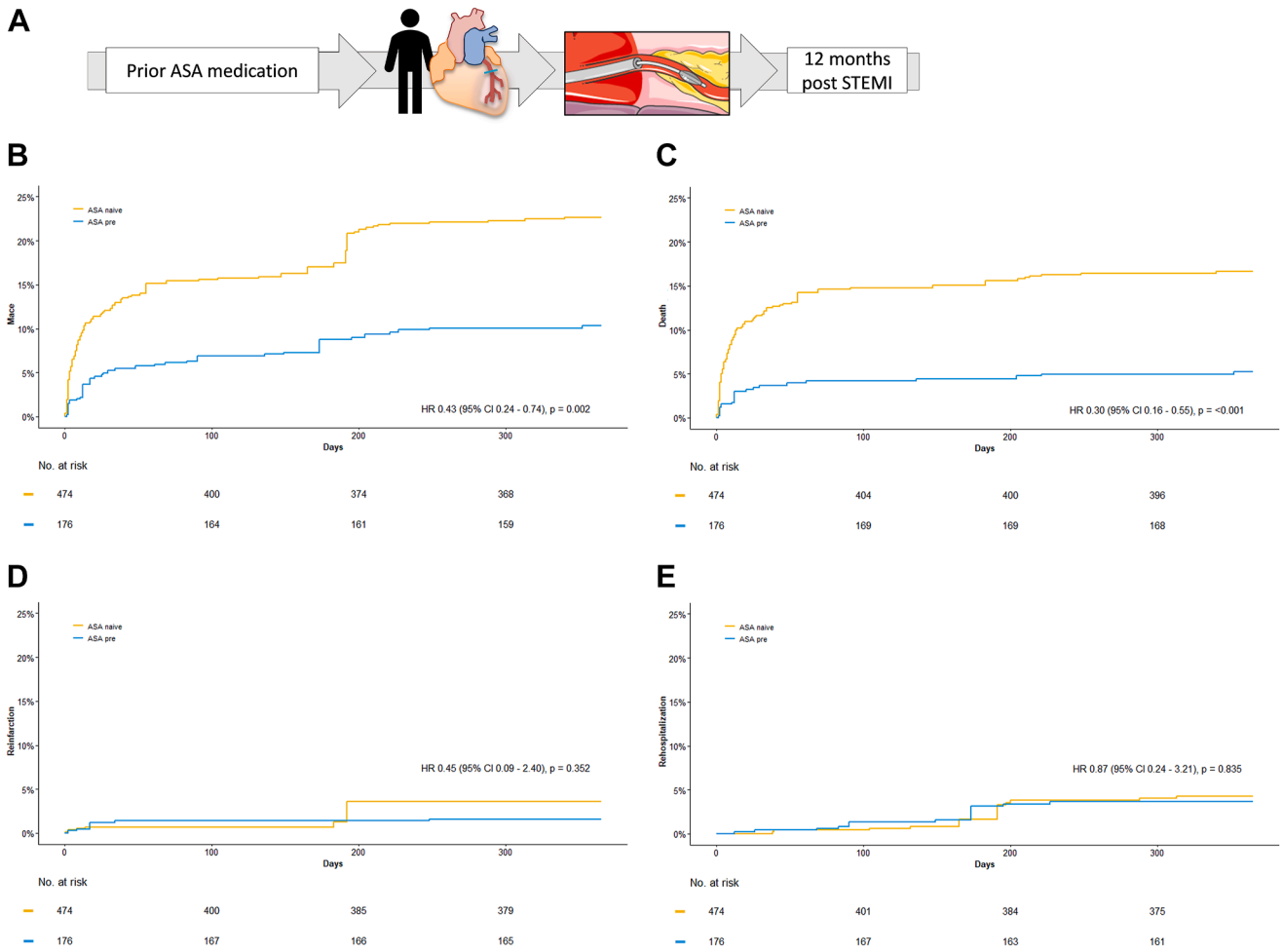

FIGURE 7 Adverse event rates after STEMI are lower in patients with prior ASA medication. (A) Six hundred fifty-one patients with STEMI and successful percutaneous coronary intervention were included in this human substudy and followed up for 12 months/365 days. Events such as death from any cause, reinfarction and rehospitalization due to heart failure, and the composite endpoint MACE were documented. Baseline characteristics such as premedication (no ASA medication [ASA naive] vs preexisting ASA therapy [ASA pre] prior to STEMI), comedication, and comorbidities were collected. (B) Survival analysis of 184 ASA pre patients (blue curve) vs 465 ASA naive patients (yellow curve) 365 days after STEMI. After IPT-weighted Cox regression, MACE rates were lower in the ASA pre group (10.2% vs 22.6%; HR, 0.42; 95% CI, 0.24-0.74; P = .002). (C) Survival analysis of single endpoints in ASA pre patients vs ASA naive patients. After IPT-weighted Cox regression, death from any cause occurred less in the ASA pre group (5.2% vs 16.7%; HR, 0.30; 95% CI, 0.16-0.55; $P < .001$). (D) Reinfarctions did not differ between ASA pre and ASA naive patients in the survival analysis 365 days after STEMI (1.7% vs 3.2%; HR, 0.45; 95% CI, 0.09-2.40; P = .352). (E) The rate of rehospitalization was not different between the 2 groups in IPT-weighted Cox regression analysis (3.4% vs 3.6%; HR, 0.87; 95% CI, 0.24-3.21; P = .835). Continuous parameters were presented as bar graphs with error bars and given as mean ± SD. ASA, acetylsalicylic acid; HR, hazard ratio; IPTW, inverse probability of treatment; MACE, major adverse cardiac event; STEMI, ST-elevation myocardial infarction.

patients came from the study's country of origin. Patients had to be event-free for the first 9 to 12 months of dual antiplatelet therapy, before being eligible for the trial [16]. As 1 event-free year is equal to the stable phase toward chronic coronary syndrome (CCS), results are hardly transferable to patients with ACS. The HOST-EXAM Extended study investigated the long-term outcomes of antiplatelet therapy with ASA vs clopidogrel monotherapy after PCI. Clopidogrel, vs ASA, was associated with lower rates of the primary endpoint and the secondary thrombotic and secondary bleeding endpoint. Patients with an adverse event between 6 and 18 months after PCI were not found eligible, and therefore, results only apply for event-free dual antiplatelet therapy patients [13].

In CCS, studies disagree whether ASA or clopidogrel monotherapy should be applied as the standard regime after PCI. The STOPDAPT-2 (Short and Optimal Duration of Dual Antiplatelet Therapy-2) trial favored 1 month of dual antiplatelet therapy (DAPT) followed by mono-clopidogrel over 12 months of DAPT. CAPRIE stated that clopidogrel was more effective than, but as safe as, long-term ASA therapy. However, an opposing finding from the CORONOR (Suivi d'une cohorte de patients COROnariens stables en région NORd-Pas-de-Calais) registry was that a large proportion of patients with CCS were treated with mono-clopidogrel, but ischemic or bleeding event rate was similar in ASA-treated patients [14,15,17]. In our study, patients with previous continuous ASA therapy showed

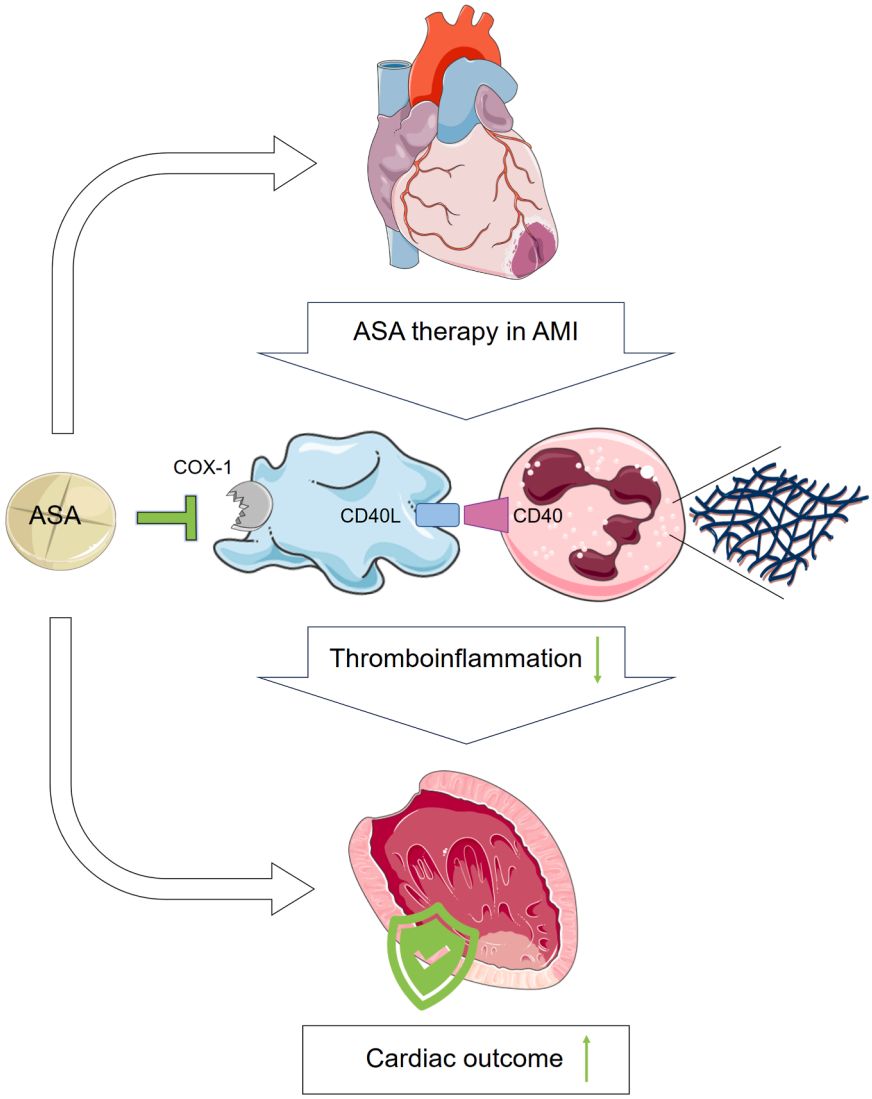

FIGURE 8 Visual abstract. ASA for primary prevention of AMI has been under debate. Also in secondary prevention, ASA's role has been questioned lately. We showed that ASA's effects reach beyond the known antithrombotic properties. Both existing ASA therapy and ASA treatment after AMI reduced the infarct size and myocardial NET formation and improved cardiac function. Only existing ASA therapy also reduced the scar size as a long-term parameter. The underlying mechanism is platelet- and neutrophil-dependent and mediated via the CD40–CD40L axis. Thus, existing ASA therapy shows pleiotropic effects in the reduction of thromboinflammation and improvement of outcome after AMI, independent of its effects on the occurrence of ischemia itself. This figure was made with the help of Servier Medical Art, licensed under CC BY 4.0. AMI, acute myocardial infarction; ASA, acetylsalicylic acid; NET, neutrophil extracellular trap.

better outcome with less MACE after 1 year. Our data suggest not to avoid ASA in standard therapy of CAD, preventing patients from their stable disease turning into ACS. Moreover, although platelet function testing is not generally recommended [25,26], clopidogrel nonresponders should be borne in mind, for whom new P2Y12-inhibitor–based strategies could be harmful.

MACE in our study were mainly driven by lower mortality rates in patients with pre-existing ASA therapy. The other endpoints such as reinfarction and rehospitalization were not different (Figure 7). Death occurred most likely due to malignant arrhythmias, although we did not distinguish between cardiovascular and noncardiovascular death. This human pilot analysis was not the focus of this study. But in murine data, we showed better cardiac function with ASA medication (Figures 1 and 2), which seemed to be associated with death. However, there was no difference in the "known" function of ASA to reduce rethrombosis in humans. Here, it seems to suffice, if ASA is applied after, respectively during, AMI.

Recent research on platelets has upgraded them from "just" being the backbone of primary hemostasis to playing an essential role in inflammation. This interplay is called thromboinflammation. Platelets are linked to inflammation by the complement system and connecting elements of the plasmatic coagulation cascade, such as factor XII. They are involved in endothelial tissue repair, apoptosis, and angiogenesis [53]. Moreover, platelets secrete a plethora of immune-modulatory mediators from granules, microparticles, or exosomes—some of them are thought to be beneficial in cardiac recovery [54,55]. The effect of platelet depletion on AMI itself is relevant, as IS was diminished in thrombocytopenic mice in previous work [44]. However, in our study, we focused on the additional role of ASA on top of platelet depletion.

Neutrophils are another key player in inflammation. They release NETs upon stimulation not only due to bacteria but also during sterile inflammation [31]. Moreover, platelets induce NET release by interacting with neutrophils [56,57]. ASA is known to inhibit platelets, and

thus, it could inhibit platelet–neutrophil interaction and NET release, as shown in our results (Figure 5). NETs have been investigated as important components in AMI [34,58]. Neutrophils extrude NETs at the culprit lesion site. This is discussed to serve as a scaffold for platelets and red blood cells to form a coronary thrombus [59]. The authors found a positive correlation between myocardial IS, duration until the resolution of ST elevation, and NET levels [60]. Moreover, an association of high double-stranded DNA levels, a NET component, with increased adverse cardiac remodeling post-AMI was found [61]. NETs have been discussed as new circulating marker for the diagnosis of ACS [33]. We could prove that NETs were enhanced in STEMI, associated with the clinical outcome, and identify patients with enhanced MACE risk [32].

The effect of ASA has been questioned as simple blocking of secondary platelet activation and subsequent cell–cell contact with other cell types, such as neutrophils. Nonetheless, ASA treatment resulted in reduced platelet–leukocyte aggregates and blocking of CD40L release [55]. Platelets, next to T lymphocytes, are the main source of CD40L and soluble CD40L (sCD40L) [37,62]. Platelet CD40L interacts with leukocyte CD40 and forms platelet–leukocyte aggregates (Figure 4). Enhanced levels of these aggregates were found in patients with atherosclerosis, resulting in increased cytokine production [63]. Correspondingly, CD40–CD40L interactions initiate endothelial inflammatory responses (ie, expression of adhesion receptors, chemokine production, and matrix metalloproteinases) [64,65]. Inhibition of the CD40–CD40L axis reduced the atherosclerosis burden and encouraged the formation of stable plaques [48,62] Additionally, sCD40L has a predictive value for postinfarction outcome in patients with STEMI [66,67]. Similarly, patients with ACS along with enhanced levels of sCD40L had higher MACE risk [68,69]. Moreover, enhanced sCD40L levels have been associated with heart failure following AMI [70].

Evidently, other ligands are involved in platelet–leukocyte crosstalk and PNA formation. Of note, CD40L can also be expressed on neutrophils [42] and activate platelets together with reactive oxygen species, which then results in secretion of soluble platelet CD40L [36]. However, our experiments on genetic deletion of platelet CD40L provide evidence that the platelet CD40L receptor is important for the ASA-mediated mechanism of reduction of thromboinflammation.

Another ligand, P-selectin, is presented on the platelet's surface upon activation and binds to P-selectin glycoprotein ligand-1 on neutrophils [71]. In our data, P-selectin (CD62P) expression was reduced with ASA premedication (Figure 4A), as a sign of ASA's platelet inhibition. Reduced platelet activation leads to decreased neutrophil attraction. This could also be observed in platelet-depleted mice with reduced neutrophil expression in the myocardial tissue, in comparison to nondepleted mice (Figure 3E).

The formation of PNAs is further strengthened by a conformational change of β2 integrin Mac1 on neutrophils upon activation. This enhances the attachment of neutrophils to platelets by ICAM-2 [72], GPIbα [73], and fibrinogen, which is bound to platelet αIIbβ3 integrin [74]. Recent data suggest that under shear flow conditions, a newly discovered protein on neutrophils, SLC44A2, binds to the GPαIIbβ3 integrin [75]. Moreover, molecules such as CXCL4, CXCL1, and CXCL2 are also involved in PNA formation [71].

Our study has several limitations. Regarding animal experiments, we did not investigate the subcellular and molecular mechanisms by which ASA affects the CD40–CD40L axis. Furthermore, we only investigated the CD40–CD40L connection, knowing that other receptors exist between platelets and immune cells. We did not investigate the role of sCD40L in mice or humans and the role of platelet CD40L in humans. In further studies, we plan to examine these gaps in the pathway in more detail. Regarding the human STEMI data—beside the reduction of MACE—no improvement of cardiac function or reduction of IS was observed with ASA. This might have been due to the limited sample size in this pilot analysis. By matching the patients with IPTW, we could not generate truly equal groups, as they were allocated by a propensity score method.

As a conclusion, ASA therapy shows pleiotropic effects in the reduction of CD40–CD40L–mediated thromboinflammation and outcome improvement after AMI, independent of its effects on the occurrence of ischemia itself. The sooner begun, the better the ASA effect, especially regarding long-term effects. This should be considered while choosing timing of initiation and the optimal antithrombotic regime post-AMI in patients with CAD.

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AUTHOR CONTRIBUTIONS

A.P., N.G., C.H., and M. Benkhoff designed the study, analyzed and interpreted data, and wrote the manuscript. P.M., D.D., C.J., T.H., B.L., T.P., O.B., N.G., and M.K. supervised the study and critically revised the manuscript. M.C., J.H., and K.A. collected and analyzed the human data. C.H., L.D., M. Benkhoff, M. Barcik, H.H., S.B., and A.A. performed animal experiments and analyzed and interpreted data. M.O.-B., S.W., A.L., and N.G. provided expertise and experimental support in CD40L experiments.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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