Evidence of Platelet Activation at Medically Used Hypothermia and Mechanistic Data Indicating ADP as a Key Mediator and Therapeutic Target

Andreas Straub, Stefanie Krajewski, Jan David Hohmann, Erik Westein, Fu Jia, Nicole Bassler, Carly Selan, Julia Kurz, Hans Peter Wendel, Shala Dezfooli, Yuping Yuan, Harshal Nandurkar, Shaun Jackson, Michael J. Hickey, Karlheinz Peter

Objective—Hypothermia is used in various clinical settings to inhibit ischemia-related organ damage. However, prothrombotic effects have been described as potential side effects. This study aimed to elucidate the mechanism of hypothermia-induced platelet activation and subsequent prothrombotic events and to develop preventative pharmacological strategies applicable during clinically used hypothermia.

Methods and Results—Platelet function was investigated ex vivo and in vivo at clinically used hypothermia (28°C/18°C). Hypothermic mice demonstrated increased expression of platelet activation marker P-selectin, platelet-leukocyte aggregate formation, and thrombocytopenia. Intravital microscopy of FeCl₃-injured murine mesenteric arteries revealed increased platelet thrombus formation with hypothermia. Ex vivo flow chamber experiments indicated increased platelet-fibrinogen adhesion under hypothermia. We show that hypothermia results in reduced ADP hydrolysis via reduction of CD39 (E-NTPDase1) activity, resulting in increased levels of ADP and subsequent augmented primary and secondary platelet activation. In vivo administration of ADP receptor P₂Y₁₂ antagonists and recombinant soluble CD39 prevented hypothermia-induced thrombus formation and thrombocytopenia, respectively.

Conclusion—The platelet agonist ADP plays a key role in hypothermia-induced platelet activation. Inhibition of receptor binding or hydrolysis of ADP has the potential to protect platelets against hypothermia-induced activation. Our findings provide a rational basis for further evaluation of novel antithrombotic strategies in clinically applied hypothermia. (Arterioscler Thromb Vasc Biol. 2011;31:1607-1616.)

Key Words: platelet receptor blockers • platelets • ADP • CD39 • hypothermia

Cooling as a means of reducing oxygen requirements of organs was initially reported in 1950 by Bigelow et al. This concept was first implemented in cardiac surgery in 1952, when John Lewis and his team used total-body hypothermia for the closure of an atrial septal defect. Since then, hypothermia has been routinely used in cardiac surgery as an adjunct to extracorporeal circulation (ECC) with the aim of protecting organs against ischemia-related damage. Temperatures applied are usually between 28°C and 32°C. Furthermore, during deep hypothermic circulatory arrest (which is used either in congenital cardiac surgery, for operations on the thoracic aorta in adults, or in neurosurgical operations for the treatment of cerebral aneurysms), temperatures lower than 20°C are used. Mild hypothermia is also used successfully in emergency and intensive care medicine to improve survival and neurological outcomes of patients after cardiac arrest.

Potential prothrombotic events of medically used hypothermia are of major concern. In fact, increased activation, aggregation, and sequestration of platelets and (micro)vascular thrombus formation have all been seen in both in vitro and in vivo settings at hypothermia. An increased platelet response to activation at temperatures less than 37°C could be considered part of human physiology. It has been proposed that platelets act as thermosensors, being less responsive to thrombogenic stimuli at the core body temperature of the central circulation, where coronary or cerebral thrombus formation could be lethal. However, at the lower temperatures of external body surfaces, the sites most susceptible to bleeding throughout evolutionary history, platelets would be primed for activation. Under clinical conditions, hypothermia-induced platelet aggregation is associated with an added risk of cognitive dysfunction in patients undergoing...
cardiac surgery, probably caused by microvascular occlusion in the brain.20 Hypothermia used during ECC has also been identified as an independent predictor of late thrombocytopenia following cardiac operations.21 In addition, fatal intravascular thrombus formation in hypothermic patients has been reported.22 Therefore, hypothermia-associated thromboembolic complications potentially leading to substantial disability and mortality are a significant problem. The mechanism of hypothermia-induced thrombosis is not understood, and therefore no therapeutic approach to preventing or treating this potentially deleterious effect has been established.

Our study systematically evaluates mechanistic changes in platelet function under hypothermia using ex vivo and in vivo platelet function assays. (1) Platelet activation as measured by P-selectin expression on the platelet surface, binding of fibrinogen to the platelet glycoprotein (GP) IIb/IIIa receptor, and expression of the platelet GPIb receptor.23,24 (2) Binding of platelets to von Willebrand factor (vWF), which mediates tethering of platelets to extracellular matrix on vascular injury,25 and adhesion of platelets to fibrinogen, which typically mediates binding of platelets to each other on activation and aggregate formation.26,27 (3) Platelet aggregation and thrombus formation as evaluated by intravital microscopy26, platelet-leukocyte aggregate formation as determined with flow cytometry,27 and hypothermia-associated changes in platelet and leukocyte counts. Furthermore, temperature-dependent changes of enzymatic activity of the ADP-metabolizing enzyme ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39)28,29 and temperature-dependent degradation of the platelet agonist ADP were investigated. Using these assays, we provide evidence for platelet activation at hypothermia, describe a novel pathomechanism, and, most importantly, report therapeutic strategies with the potential of preventing prothrombotic complications during medically applied hypothermia.

Materials and Methods
A full description of all methods can be found in the Supplemental Data, available online at http://atvb.ahajournals.org.

Mice
All in vivo experiments were performed with anesthetized wild-type C57BL/6 mice. The rectal temperature was monitored using a digital thermometer and maintained at either 37°C or 28°C using a heat pad.

Blood Sampling in Human Subjects
Blood was collected by venipuncture from nonmedicated healthy subjects into tubes with preloaded heparin (final concentration, 3 IU/mL), citric acid (final concentration, 10.6 mmol/L), or EDTA.

Analysis of Platelet Activation, Platelet-Leukocyte Aggregate Formation, and Blood Count at Hypothermia
Blood was sampled from mice with body temperatures of 37°C or 28°C, and platelet activation, platelet-leukocyte aggregates, and blood counts were evaluated.

Binding of Oregon Green–labeled fibrinogen to human platelets at 37°C or 18°C was analyzed by flow cytometry. Citrated human whole blood was incubated at 37°C, 28°C, or 18°C for 30 minutes. Expression of platelet surface receptors GPⅠbα was analyzed by flow cytometry as previously described.10,30

Ex Vivo Flow Studies
Flow studies were performed as previously described31 using microcapillary tubes coated with vWF or fibrinogen. Heparinized human blood was treated as either control or with platelet antagonists and perfused through the tubes at 37°C, 28°C, or 18°C on either vWF for 2 minutes (shear rate: 1800 seconds⁻¹) or on fibrinogen for 5 minutes (shear rate: 150 seconds⁻¹). Platelet-surface interactions were visualized in real time using differential interference contrast microscopy.

In Vivo Thrombosis Model
Intravital microscopy of mesenteric arteries from mice with body temperatures of either 37°C or 28°C was performed as previously described.26 All mice received heparin (0.25 IU/g of body weight) to simulate conditions of cardiac surgery, in which all patients undergoing routine ECC procedures are anticoagulated with heparin. Furthermore, antiplatelet agents (2-MeSAMP [3.2 μg/g of body weight] bolus injection only or in combination with bolus injection of MRS2179 [4.2 μg/g of body weight] or cangrelor solution [40 ng/μL] administered as initial bolus of 50 μL followed by continuous infusion of 4 μg/g of body weight per minute) or saline (control) were administered. After vessel injury with ferric chloride, thrombus formation of rhodamine-labeled platelets was monitored for 40 minutes or until full occlusion. The following parameters were examined: thrombus surface in relation to vessel surface 5 minutes after injury and embolization rate of thrombi over a period of 1 minute.

Production, Characterization, and Administration of Soluble CD39
Soluble (sol) CD39 was cloned into a mammalian expression vector and transfected into Hek293F cells. Transfected cells were cultured for 7 to 9 days before being purified via a polyhistidine tag by fast protein liquid chromatography. Enzyme activity was measured at 3 different temperatures (18°C, 28°C, and 37°C) using a bioluminometric assay as previously described.32 For in vivo studies with solCD39, mice with body temperatures of either 37°C or 28°C received either PBS as a control substance (vehicle) or solCD39 via the left jugular vein, and blood cell count was measured after 30 minutes.

ADP Degradation at Hypothermia and Effect on Platelet Activation
To investigate temperature-dependent differences of ADP degradation, ADP (2 μmol/L) was added to cell-free plasma samples at 37°C or 18°C. After 10 minutes, ADP concentrations were conserved by EDTA addition. Platelet-rich plasma was resuspended in these samples, and platelet P-selectin expression was analyzed by flow cytometry. ADP levels in cell-free plasma samples were determined using a previously described bioluminometric assay.32

Nucleotide Release From Blood Cells and Platelet Activation in Hypothermia
EDTA-anticoagulated whole blood was incubated at 3 different temperatures. After 60 minutes, P-selectin expression on single platelets was measured, and nucleotide plasma levels above nucleotide background concentration (directly after blood taking) were measured as previously described.32 In short, ADP was converted to ATP by the pyruvate kinase reaction, and total ATP concentration was determined in a bioluminescence assay on a microplate luminometer (Microlumat.Plus, Berthold).

Statistics
Data are depicted as means and SEM unless otherwise indicated. Differences between 2 data sets were evaluated using unpaired 2-tailed t tests for unmatched data and using paired t tests for matched data. If not otherwise indicated, differences among 3 or more data sets were evaluated by repeated-measures ANOVA with the indicated post hoc tests for matched data and using 1-way
ANOVA with the indicated post hoc tests for unmatched data. A probability value of $<0.05$ was defined to indicate a statistically significant difference.

**Results**

**Prothrombotic Effects of Hypothermia on Platelet Function In Vivo**

**Hypothermia Induces Platelet Activation, Platelet-Leukocyte Aggregate Formation, Thrombocytopenia, and Leukocytopenia In Vivo**

To evaluate the in vivo effect of hypothermia on platelet function, expression of the activation marker P-selectin on nonstimulated platelets was determined in mice with body temperatures of 28°C and 37°C using flow cytometry (see Figure 1A and 1B for representative dot plot and histograms). At 28°C, platelet P-selectin expression was significantly increased compared with 37°C ($P<0.05$, Figure 1C). Furthermore, in vivo platelet-leukocyte aggregate formation was significantly increased at hypothermia ($P<0.05$, Figure 1D). Analysis of whole blood counts revealed that at hypothermia, platelet count ($P<0.05$, Figure 1E) and white blood cell count ($P<0.05$, Figure 1F) were significantly lower compared with 37°C. No effect of hypothermia on red blood cell counts was observed (Figure 1G).

**Hypothermia Increases In Vivo Platelet Thrombus Formation and Thrombus Stability**

Intravital microscopy allows direct visualization and quantification of the dynamic process of platelet accumulation and thrombus formation at injured or diseased vessels.33 Five minutes after application of ferric chloride to murine mesenteric arteries, the surface area of platelet thrombi in mice that were cooled to 28°C was increased (2.2-fold, $P<0.001$) compared with mice with a body temperature of 37°C (Figure 2A). Disaggregation analysis of thrombi revealed that the thrombus dissolving rate was 5-fold lower at 28°C compared with 37°C ($P<0.0001$) indicating stronger binding of platelets and therefore decreased thrombus dissolvement at hypothermia. (Figure 2B). Supplemental Video I shows representative examples of thrombus formation and disaggregation at 37°C and 28°C.

**Effect of Hypothermia on GPIbα Expression and Platelet-vWF Adhesion Under Flow**

The interaction of the platelet receptor GPIbα with vWF plays a key role in promoting initial platelet tethering to the vessel wall.34 To evaluate a potential role of hypothermia on platelet-vWF interaction, we analyzed the platelet surface expression of GPIbα and platelet binding on a vWF matrix under ex vivo flow conditions. GPIbα expression was unchanged at 28°C and showed a small reduction at 18°C in comparison to 37°C (Supplemental Figure I), which is consistent with decreased

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**Figure 1.** Hypothermia induces platelet activation, platelet-leukocyte aggregate formation, thrombocytopenia, and leukocytopenia in mice. A and B, Murine platelets are depicted in a forward scatter/side scatter dot plot (A), and binding of a fluorescein isothiocyanate-labeled antibody against the activation marker P-selectin on the platelet surface from mice with body temperatures of 37°C (B: curve with gray shading) and 28°C (B: overlaid curve without shading) was analyzed in histograms. C, Geometric mean fluorescence of anti-P-selectin–fluorescein isothiocyanate (FITC) antibody binding on platelets from mice with body temperatures of 37°C and 28°C ($n=15$ per group; $^*P<0.05$). D, Platelet-leukocyte aggregate formation in mice with body temperatures of 37°C and 28°C ($n=7$ per group; $^*P<0.05$). E to G, Whole blood from mice ($n=7$ per group) with body temperatures of 37°C and 28°C was analyzed for differences in platelet count (E; $^*P<0.05$), white blood cell count (F; $^*P<0.05$), and red blood cell count (G). Data are given as means and SEM; $^*$probability value derived from 2-tailed paired $t$ test.
expression of GPIbα via receptor shedding on platelet activation at hypothermia. The evaluation of platelet tethering on a vWF matrix under flow conditions did not reveal a significant difference between 18°C and 37°C (Supplemental Video II).

**Hypothermia Increases Fibrinogen Binding to Platelets and Platelet Adhesion on Fibrinogen Under Flow Conditions**

Binding of fibrinogen to platelets is an important mechanism mediating platelet aggregate formation and thrombus growth. Fibrinogen binding to platelets was 1.6-fold higher ($P < 0.05$) at hypothermia compared with normothermia (Figure 3A).

Furthermore, on a fibrinogen matrix under ex vivo flow conditions, the mean number of adherent platelets was increased 2.2-fold ($P < 0.001$) at both 28°C and 18°C (Figure 3B and 3C) relative to experiments performed at 37°C.

**Potential Mechanisms for Prothrombotic Effects of Hypothermia**

**Hypothermia Inhibits Activity of Soluble CD39, Thereby Increasing ADP Levels and Subsequently Causing Platelet Activation**

It has previously been postulated that the platelet agonist ADP may be involved in hypothermia-induced platelet acti-
ADP Degradation in Plasma Is Decreased At Hypothermia

It has previously been reported that ADP is metabolized in whole blood and plasma. This is certainly of central importance for the regulation of platelet function. To investigate whether the described effect of hypothermia on ADP metabolism is also present in plasma, we examined ADP degradation in plasma at 37°C compared with 18°C. In these experiments, ADP (2 μmol/L) was added to plasma samples, which were then exposed to a temperature of 37°C or 18°C for 10 minutes. Measurement of ADP concentrations at the end of this period demonstrated that samples that had been exposed to a temperature of 18°C contained a significantly higher ADP concentration (mean, 1.75 μmol/L) compared with 37°C control. ADP receptor blockers prevented platelet activation and ADP release caused by hypothermia.

Figure 4. Hypothermia decreases CD39 activity and ADP degradation, thereby causing platelet activation. A. Recombinant soluble CD39 was incubated at 3 different temperatures with 50 μmol/L ADP for 15 minutes. Using a bioluminometric assay, the remaining ADP was measured, and the specific activity of solCD39 was quantified. Data are given as means and SEM (n=4), and groups were compared using 1-way ANOVA with Dunnett’s multiple comparison test (*P<0.05, **P<0.001 compared with enzyme activity at 37°C). ADP degradation in plasma was reduced at hypothermia. B. ADP (2 μmol/L) was added to plasma, which was then exposed to a temperature of 37°C or 18°C. After 10 minutes, remaining ADP levels were determined using a bioluminometric assay. C. Human platelets were resuspended in the respective plasma samples, and platelet surface expression of P-selectin was evaluated using flow cytometry. Data are given as means and SEM; groups were compared using repeated-measures ANOVA with Bonferroni’s multiple comparison test (**P<0.01, ***P<0.001).

Antibody to untreated platelets (D) and after P2Y12 and P2Y1 blockade (F) was measured in flow cytometry to evaluate platelet activation and aggregation. However, this has not been shown in vivo and the underlying mechanism of this phenomenon has not yet been elucidated. The E-NTPDase CD39, which hydrolyzes extracellular nucleotides ATP and ADP to AMP, is expressed by the endothelium and thereby presents a physiologically important platelet-inhibitory surface. For our experiments, we generated CD39 in soluble form (solCD39), for which enzymatic properties have been described previously in detail. We tested the specific activity of solCD39 at different temperatures in vitro using a bioluminometric assay. We found a significant decrease of solCD39 activity at 28°C and 18°C compared with 37°C (Figure 4A).

ADP Degradation in Plasma Is Decreased At Hypothermia

Adenosine diphosphate (ADP) is hydrolyzed in human cell-free plasma because of ADPase activity. Altogether, the in vitro conversion of ADP in plasma samples in our experiments can therefore be related to the action of CD39 on microparticles, as well as to soluble plasma phosphatases. Nevertheless, in the in vivo...
setting of blood circulation, CD39 is proposed to be the major ADP hydrolyzing enzyme on the basis of its broad expression on endothelial cells and leukocytes.

**Hypothermia Does Not Induce Nucleotide Leakage**

Activated platelets secrete ADP from intracellular storage granules. ADP activates circulating platelets and amplifies platelet aggregation and stable aggregate formation.

To investigate whether nucleotides (ATP and ADP) spontaneously leak from whole blood cells at hypothermia, whole blood was incubated for 60 minutes at 3 different temperatures (37°C, 28°C, 18°C). Potential differences of nucleotide hydrolysis rates at different temperatures were negated by EDTA addition. Samples were treated as controls or with a combination of P2Y12 and P2Y1 receptor blockers to avoid active ADP release resulting from platelet activation. To analyze a potential direct effect of hypothermia on platelet degranulation, expression of the platelet activation marker P-selectin and ADP and ATP plasma levels were investigated.

A decrease in temperature resulted in an increase of P-selectin expression and nucleotide plasma concentrations in control samples (Figure 4D and 4E). In the presence of ADP receptor blockers, P-selectin expression and nucleotide levels were not significantly different among the 3 temperatures (Figure 4F and 4G). To closely explore potential hypothermia-induced nucleotide leak by platelets alone, the same assay (including P2Y receptor blockade) was repeated with washed platelets alone. Under these conditions, platelets also did not secrete ADP to a greater extent at hypothermia (data not shown).

**Therapeutic Implications for the Prevention of Hypothermia-Induced Prothrombotic Effects**

**Blockade of P2Y12 and P2Y1 Decreases Hypothermia-Induced Platelet-Fibrinogen Binding Under Ex Vivo Flow Conditions**

The pathomechanistic role of ADP that has been described provides the unique opportunity to develop novel therapeutic approaches with the aim of preventing hypothermia-induced platelet activation. Therefore, we systematically investigated whether inhibition of platelet ADP receptors could potentially be used therapeutically. First, we used the established flow chamber model. Heparinized whole blood samples, which had been treated with the P2Y12 antagonist 2-MeSAMP in combination with the P2Y1 antagonist MRS2179 (P2Y block), were perfused over fibrinogen at 37°C, 28°C, or 18°C at a shear rate of 150 seconds⁻¹. In this model, at 37°C, no inhibitory effect of ADP receptor blockade was observed. However, at both 28°C and 18°C, the combination of P2Y12 and P2Y1 inhibition significantly decreased platelet adhesion (28°C, Figure 5A, P<0.001; 18°C, Figure 5B, P<0.001), returning the levels of platelet adhesion to values close to those observed at 37°C. This finding indicates that in our ex vivo flow model, ADP-induced platelet activation is of much greater importance at hypothermia than at normothermia. This further emphasizes the essential role of ADP as key platelet agonist in hypothermia.

**In Vivo Blockade of ADP Receptors Decreases Hypothermia-Induced Platelet Thrombus Formation in Mice**

ADP receptor blockade has proven to be clinically highly beneficial in patients with coronary artery disease, and major drug developments are providing a plethora of various ADP receptor blockers, including the short-acting intravenously applicable cangrelor. To provide proof of concept for a potential therapeutic approach in preventing hypothermia-induced platelet activation, we evaluated ADP receptor blockade in intravital microscopy. Again, the P2Y12 antagonist 2-MeSAMP alone and in combination with the P2Y1 antagonist MRS2179 were used as experimentally available ADP receptor blockers. In addition, the P2Y12 antagonist cangrelor, which has been developed as a promising therapeutic agent, was administered intravenously to mice.
before mesenteric artery injury. At a body temperature of 28°C, 2-MeSAMP and cangrelor significantly decreased (P<0.01) thrombus surface in mesenteric arteries after ferric chloride injury (Figure 6A). The disaggregation rate of platelet thrombi at 28°C was also significantly (P<0.01) increased after treatment with ADP receptor blockers including cangrelor (Figure 6B). The finding that cangrelor displayed a favorable potency in comparison with the experimental P2_Y1/P2_Y12 blockade supports the therapeutic potential of this short-acting ADP receptor blocker.

**SolCD39 Inhibits Hypothermia-Induced Platelet Aggregation and Thrombocytopenia**

We aimed to evaluate the potential effect of solCD39 administration on hypothermia-induced platelet dysfunction. First, we tested whether solCD39 would inhibit platelet aggregation at hypothermic temperatures in vitro. Our results indicate that solCD39 indeed inhibits ADP-induced platelet aggregation at hypothermia, but clearly less efficiently than at 37°C (Supplemental Figure II). This finding confirms our statement that hypothermia has an inhibitory effect on CD39 function. However, a significant antiaggregatory effect of solCD39 is still present at 28°C and 18°C. To investigate the effect of solCD39 in vivo, we administered it to mice at body temperatures of 37°C and 28°C. Our results indicate that treatment with solCD39 abolishes hypothermia-induced platelet loss (see Figure 6C). These findings support the suitability of solCD39 as a potential agent for the prevention of platelet activation and thrombosis at hypothermia.

**Discussion**

This systematic ex vivo and in vivo study on platelet function reports on platelet activation and prothrombotic effects and provides mechanistic data indicating ADP as a key mediator and important therapeutic target in medically used hypothermia.

Primary platelet activation by potent agonists, such as thrombin, ADP, or collagen, causes the release of the platelet agonist ADP from platelet dense granules. As a secondary activating effect, ADP recruits additional circulating platelets and amplifies activation of platelets within the platelet clot (ADP augmentation pathway). We postulate a potential pathomechanism indicating primary and secondary effects of ADP in hypothermia-induced platelet activation. At hypothermia, ADP hydrolysis and especially the activity of the main ADP-metabolizing enzyme, CD39 (NTPDase1), are significantly decreased, resulting in a lower rate of hydrolysis and thus higher levels of the platelet agonist ADP. This can promote primary platelet activation as reflected by platelet adhesion on fibrinogen under flow and by increased platelet P-selectin expression. Once platelets have become activated, more ADP is actively released into plasma via the ADP augmentation pathway. The increase in plasma ADP concentration, which will again be amplified by decreased ADP temperatures for 30 minutes after injection. Whole blood was sampled for platelet count analysis. Data are given as means and SEM; groups were compared using one-way ANOVA with Bonferroni’s multiple comparison test (P<0.05).
hydrolysis at hypothermia, will in turn cause further activation of platelets, resulting in the systemic activation of platelets that is observed at hypothermia. This hypothesis is supported by our finding that platelet ADP receptor P2Y12 blockade decreases hypothermia-induced platelet adhesion and aggregation under ex vivo and in vivo conditions. Additional support for the important role of ADP in platelet function at hypothermia is provided by our finding that administration of solCD39 inhibits hypothermia induced decreases of platelet and leukocyte counts. Our findings are also supported by an in vitro study by Xavier et al demonstrated that hypothermia-induced platelet aggregation is inhibited by ADP-receptor blockade.

With the identification of ADP as the major mediator of hypothermia-associated platelet activation, other parameters of platelet physiology may be influenced by hypothermia as well. Although it is possible that changes in membrane fluidity, receptor clustering, or microhemodynamics may occur under hypothermia, our findings that ADP hydrolysis or P2Y12 blockade can prevent hypothermia-induced platelet activation does not suggest a sufficient role for these mechanisms. Also notably, the inhibition of the thromboxane pathway by aspirin, which inhibits thromboxane A2 synthesis, is reported not to inhibit hypothermia-induced platelet aggregation.

Shear stress during ECC as used in cardiac surgery results in substantial ADP release from platelets and erythrocytes in amounts that are sufficient to induce platelet aggregation. ADP-mediated platelet activation may therefore be of particular importance during ECC, which is often used together with hypothermia. In addition, the artificial ECC surface is a strong inducer of platelet activation. Elevated ADP levels at hypothermia may significantly enhance the prothrombotic side effects of ECC. Therefore, the approach of specifically inhibiting ADP-induced platelet activation via inhibition of P2Y12 at hypothermia may be of particular benefit during ECC.

The platelet ADP receptor P2Y12 is an important target for antiplatelet drugs, particularly in the setting of stent implantation in coronary arteries. Using a whole set of ex vivo and in vivo experiments, we have demonstrated that hypothermia-induced prothrombotic effects are reversed by pharmacological P2Y12 inhibition. The direct-acting and reversible P2Y12 antagonist cangrelor, which can be administered intravenously and which has a half-life of less than 5 minutes, has been developed for clinical application. Cangrelor’s short half-life offers a major advantage in comparison to other antiplatelet agents. With the idea of exploiting this advantage, cangrelor is currently under clinical trial (BRIDGE trial; http://clinicaltrials.gov ID: NCT00767507) to enable safe bridging from oral P2Y12 blockade to coronary bypass graft surgery. Based on our finding that cangrelor inhibits hypothermia-induced platelet activation, this intravenously applicable P2Y12 blocker with a short half-life appears to be an ideal candidate for platelet protection during medically induced hypothermia. Particularly in cardiac surgical procedures using hypothermia, the concept of administering a short-acting P2Y12 antagonist only during the phase of hypothermia is very attractive, providing protective antithrombotic effects during hypothermia (as well as during ECC and during surgical procedures with increased risk of thrombosis) but ensuring fully functional platelets after rewarming at the end of the operation when a fully functional hemostasis is needed to prevent postoperative bleeding complications.

In animal models, administration of solCD39 has been shown to minimize platelet deposition and leukocyte recruitment at sites of vascular endothelial injury and to reduce cerebral infarct size. Our experiments demonstrate that solCD39 can prevent hypothermia-induced reduction of platelet and leukocyte counts. Therefore, administration of solCD39 may represent an alternate pharmacological approach to inhibit hypothermia-associated platelet dysfunction. Hypothermia-induced thrombocytopenia and leukocytopenia can be explained by the fact that hypothermia induces a prothrombotic state characterized by in vivo platelet/platelet and platelet/leukocyte aggregation and cell sequestration, as well as consecutive microinfarctions in liver and pancreas.

Besides several literature reports, which indicate deleterious prothrombotic effects of hypothermia, it has also been reported that under clinical conditions, hypothermia can induce coagulopathy and bleeding tendency. It has also been demonstrated that the main effect of hypothermia on platelets is the induction of prothrombotic events, namely typical signs of platelet activation (ie, GPIIb/IIIa activation and P-selectin expression) and augmented formation of platelet thrombi with increased stability at sites of endothelial injury.

Although our in vitro data have been obtained using human platelets, our in vivo data were obtained in mice. Therefore, there is a general caveat that the latter data may not be fully transferable to the human situation. Nevertheless, our findings warrant further testing of our hypothesis and potential therapeutic approaches in patients during perioperatively applied hypothermia.

In conclusion, our ex vivo and in vivo findings indicate that medically applied hypothermia induces platelet activation and thrombus formation/stabilization. The platelet agonist ADP plays a central role in hypothermia-induced platelet activation. Decreased NTPDase1 (CD39) activity and thus decreased ADP hydrolysis contribute to platelet activation at hypothermia. Inhibition of receptor binding or hydrolysis of ADP has the potential to protect platelets against hypothermia-induced activation. Our findings provide a rational basis for further evaluation of this novel antithrombotic approach in clinically applied hypothermia.

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Disclosures

None.

References


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Supplement Material

Evidence of platelet activation in medically used hypothermia and mechanistic data indicating ADP as key mediator and therapeutic target

Straub: Platelet activation/protection at hypothermia

Andreas Straub\textsuperscript{1,2}, Stefanie Krajewski\textsuperscript{1,2}, Jan David Hohmann\textsuperscript{1}, Erik Westein\textsuperscript{4}, Fu Jia\textsuperscript{1}, Nicole Bassler\textsuperscript{1}, Carly Selan\textsuperscript{6}, Julia Kurz\textsuperscript{2}, Hans Peter Wendel\textsuperscript{3}, Shala Dezfooli\textsuperscript{5}, Yuping Yuan\textsuperscript{4}, Harshal Nandurkar\textsuperscript{5}, Shaun Jackson\textsuperscript{4}, Michael J Hickey\textsuperscript{6}, Karlheinz Peter\textsuperscript{1}

\textsuperscript{1} Atherothrombosis & Vascular Biology, Baker IDI Heart & Diabetes Institute, Melbourne, Australia
Departments of \textsuperscript{2}Anesthesiology and \textsuperscript{3}Paediatric Heart Surgery,
University of Tübingen, Germany

\textsuperscript{4} Australian Centre for Blood Diseases, Monash University, Melbourne, Australia

\textsuperscript{5} St. Vincent’s Hospital, Department of Haematology, Melbourne, Australia.

\textsuperscript{6} Centre for Inflammatory Diseases, Monash Medical Centre, Melbourne, Australia
Supplemental figure legends

Supplemental figure I

*Effect of hypothermia on GP Ibα expression and platelet-vWF adhesion under flow*

Whole blood, anticoagulated with citrate, was incubated at 3 different temperatures (18°C, 28°C and 37°C) for 30 minutes. Binding of an anti-CD42bα-PE mAb to platelets was measured using flow cytometry. Data are given as means ± SEM; n = 3; groups were compared using RM-ANOVA with Bonferroni’s Multiple Comparison Test (*p<0.05).

Supplemental figure II

*Inhibition of ADP-induced platelet aggregation with solCD39*

Citrated platelet-rich plasma was incubated with PBS (vehicle control) or solCD39 (fc: 1.6 µg/ml) at 18°C, 28°C or 37°C for 30 minutes and platelet aggregation was induced by ADP (10 µM). Data are given as means ± SEM; groups were compared using RM-ANOVA with Bonferroni's Multiple Comparison Test (**p<0.01; *p<0.05).
Supplemental figure I

![Graph showing CD42b expression with error bars.]

**Supplemental figure II**

![Graph showing aggregation with error bars.]

Legend: 
- **37°C**
- **28°C**
- **18°C**
Legends for videos

**Video 1**, which accompanies figure 2 as online data supplement, shows representative examples of *in vivo* platelet thrombus formation and disaggregation. At 37°C, platelet thrombi rapidly disaggregate and re-aggregate at the site of vessel injury. At 28°C, platelet attachment is much more stable.

**Video 2**, which accompanies supplemental figure 1 shows representative examples of rolling of single platelets over a vWF surface at 37°C and 18°C at a shear rate of 1800 sec-1 *in vitro*. 
Expanded Materials and Methods

Blood sampling in healthy human subjects
Blood was collected from non-medicated healthy subjects, who gave signed informed consent, by venipuncture with a 21-gauge butterfly needle from an antecubital vein. The first 2 ml of blood were discarded before additional blood samples were drawn for analysis. Blood was sampled into tubes with preloaded heparin [final concentration (fc): 3 I.U./ml], or with citrate (Sarstedt, Nümbrecht, Germany, fc of citrate: 10.6 mM), or with EDTA (Sarstedt, Nümbrecht, Germany, fc of EDTA: 1.2 - 2 mg EDTA/ml blood). All subjects were free of platelet-affecting drugs for 14 days. Study and blood sampling procedures were approved by the Research and Ethics Unit of the Alfred Hospital, Melbourne, Australia.

Preparation of platelet-rich, platelet-poor and cell-free plasma
Citrated human blood was centrifuged (180 g, 10 minutes), the supernatant [platelet rich plasma (PRP)] collected and kept at 37°C. The remainder was centrifuged (2,500 g, 10 minutes) to obtain platelet-poor plasma (PPP). Cell-free plasma (CFP) was prepared by passing PPP through a 0.2 µm filter (Acrodisc syringe filter, Pall Corporation, Ann Arbor, MI, USA) according to previously described methods.¹

Mice
All in vivo experiments were performed with wild-type C57BL/6 mice (4-5 weeks old). Animal experiments were approved by the animal ethics committees of the Monash University, the Baker IDI Heart and Diabetes Institute (Melbourne, Australia) and the University of Tübingen, Germany. For experiments involving intravital microscopy, determination of whole blood counts and platelet P-selectin expression mice were
anesthetized via intraperitoneal injection with a ketamine/xylazine (150:10 mg/kg) mixture. For experiments involving platelet-leukocyte interaction, mice were anesthetized via intraperitoneal injection with a fentanyl/midazolam/medetomidine (0.05:5:0.5 mg/kg) mixture. Mice were not actively chilled. Rather more, body temperature drops spontaneously in anesthetized mice while kept at room temperature. The rectal body temperature was measured using a thermometer, which was connected to a warming pad in order to maintain stable body temperatures of 28°C or 37°C.

**Analysis of P-selectin expression, platelet-leukocyte interaction and whole blood count in mice**

Anesthetized mice were kept at either 37°C or 28°C using a warming pad and rectal thermometer. After 30 minutes, blood was sampled via cardiac puncture in citrate for flow cytometry or via an abdominal incision from the inferior vena cava in EDTA for blood cell count analysis.

For analysis of P-selectin expression and platelet-leukocyte interaction in murine blood citrated whole blood samples (14 µl) were incubated with an anti-CD42bα-PE antibody (2 µl, EMFRET Analytics, Eibelstadt, Germany) in PBS (2 µl) and with an anti-CD62P-FITC antibody (2 µl, EMFRET Analytics) or with an anti-CD45-FITC antibody (BD Biosciences). Samples were incubated at 37°C and were fixed using CellFix® (BD Biosciences) after 30 minutes if not otherwise indicated.

Blood cells were counted using an automated blood cell counter (Sysmex KX-21N, Sysmex Corporation, Kobe, Japan).
Analysis of GP Ibα expression and fibrinogen binding of human platelets in flow cytometry

Incubation steps were performed according to the principles of previously described methods. 

For analysis of GP Ibα expression on human platelets whole blood was diluted (D=1:50) in modified Tyrode’s buffer and 25 µl were incubated with 5 µl of an anti-CD42bα-PE antibody. (Beckman Coulter, Marseille, France). For analysis of fibrinogen binding (GP IIb/IIIa activation) 45 µl purified human platelets in Tyrode’s buffer were incubated with 5 µl of Oregon-green labeled fibrinogen. Samples were incubated at 37°C, 28°C, or 18°C as indicated and were fixed using CellFix® (BD Biosciences) after 30 minutes if not otherwise indicated.

Ex vivo sample preparation with platelet antagonists

Human blood samples were either pre-treated for 5 minutes with the P₂Y₁₂ antagonist 2-MeSAMP (Sigma-Aldrich, Castle Hill, NSW, Australia) at 100 µM, and/or with the P₂Y₁ antagonist MRS2179 (Tocris, Ellisville, MO, USA) at 100 µM, or with the GP IIb/IIIa blocker tirofiban (Aggrastat®, MSD Sharp & Dohme GmbH, Haar, Germany) at 500 ng/ml, or with an equivalent volume of PBS buffer as control. The concentration for 2-MeSAMP and MRS2179, was employed according to a previously published study reporting that ADP-induced platelet aggregation was markedly decreased by administration of 100 µM 2-MeSAMP alone and almost abolished by additional administration of 100 µM MRS2179. The employed concentration of tirofiban allows to achieve stable inhibition of platelet aggregation.
Ex vivo flow studies

Flow studies were performed according to previously described methods. Microcapillary tubes (Vitrotubes, VitroCom, NJ, USA) with a diameter of 0.1 x 1 mm were coated with vWF and tubes with a diameter of 0.2 x 2 mm were coated with fibrinogen. Tubes were treated with solutions of vWF (50 µg/ml) or fibrinogen (50 µg/ml) for 1 hour at room temperature (RT) and were then blocked with 2% BSA for 1 hour at RT. The tubes were mounted on a microscope stage within a plexiglas chamber that was kept at 37°C, 28°C, or 18°C using a microscope heater or air conditioner. Human whole blood anticoagulated with heparin (3 I.U./ml) was kept at 37°C until the beginning of the flow experiment. It was then treated as either control or with platelet antagonists as indicated and perfused through the microcapillary tubes on vWF at 1800 sec⁻¹ for 2 minutes or on fibrinogen at 150 sec⁻¹ for 5 minutes at the respective temperatures in the flow chamber.

All flow experiments were performed using a Leica DMIRBE inverted microscope (Leica Microsystems, North Ryde, Australia). Platelet adhesion was visualized in real time using differential interference contrast (DIC) microscopy and images were DVD-recorded for off-line analysis. Flow experiments on the different matrixes were evaluated according to the following criteria: On a vWF surface platelets dynamically bind, roll and often detach again without forming aggregates. Therefore, the velocities of platelets, which were visualized in 63-fold magnification while travelling over the vWF-surface were determined using DiaTrack software version 3.0 (Semasopht, Chavannes pres Renens, Switzerland). On a fibrinogen surface the number of attached and aggregated platelets increase over the time-course of the experiment. Platelets were visualized in 63-fold magnification and the number of adherent platelets per field of view was recorded after 5 minutes of flow.
**In vivo administration of platelet antagonists to mice**

Adjusting for blood volume according to body weight (BW), the dosages for intravenous (i.v.) administration of 2-MeSAMP (3.2 µg/g BW) and MRS 2179 (4.2 µg/g BW) were calculated in order to achieve *in vivo* levels of 100 µM for both agents. For both compounds the *in vivo* concentration of 100 µM is equivalent to concentrations used in our *ex vivo* experiments with human blood. Further, *ex vivo* experiments with heparinized mouse blood revealed that the addition of 100 µM 2-MeSAMP distinctly decreases P-selectin expression and aggregation of ADP-stimulated platelets and that administration of both 100 µM 2-MeSAMP and 100 µM MRS2179 results in a slight additive effect regarding platelet inhibition compared to administration of 100 µM 2-MeSAMP only (data not shown). Cangrelor (The Medicines Company, Parsippany, NJ) in a solution with a concentration of 40 ng/µl was administered as initial 50 µl bolus followed by continuous infusion of 4 ng/g BW per minute. The initial cangrelor bolus injection was performed for technical reasons in order to prefill the jugular vein catheter with cangrelor solution. The dosage of the continuous cangrelor infusion is equivalent to the clinically relevant cangrelor dosage of 4 µg/kg per minute.⁷

**In vivo thrombosis model**

Intravital experiments were performed according to a previously described method.⁸ Anesthetized mice were placed on a heat pad to maintain the rectal temperature at either 37°C or 28°C as monitored using a digital thermometer. Rectal temperature was chosen for continuous temperature monitoring because pilot experiments indicated that a temperature probe placed at the exposed mesentery might interfere with the mesenteric preparation, measurements and visualizing. Furthermore, pilot experiments showed that the rectal temperature as well as the temperature
measured at the exposed mesentery are very similar. A catheter was inserted into the jugular vein. The mesentery was exteriorized through a midline abdominal incision. The mesenteric preparation was constantly kept moist by application of small amounts of saline solution. Using a micrometer gauge mesenteric arteries, with an average size of 100 µm were identified and visualized with 20-fold magnification using a Zeiss Axioplan 2 Imaging microscope (Zeiss, Oberkochen, Germany), captured with a DageMTI VE 1000 SIT camera (Dage MTI, Michigan City, IN, USA), and recorded on videotape. Before the induction of vessel injury all mice received heparin [0.25 IU/g BW] in order to simulate coagulatory conditions in cardiac surgery, where all patients undergoing routine ECC procedures are heparinized. Furthermore, antiplatelet agents [2-MeSAMP - 3.2 µg/g BW only or in combination with MRS2179 - 4.2 µg/g BW, or cangrelor solution (40 ng/µl) as initial 50 µl bolus followed by continuous body-weight adjusted infusion (4 ng/g BW per minute)], or saline (control) were injected and 50 µl rhodamine dye (0.05 %) was administered in order to label platelets. Five minutes after initial drug administration a filter paperstrip of 1 x 4 mm was immersed in a 462 mM (7.5 %) FeCl$_3$ solution for 3 seconds and then applied to the respective artery. After 4 minutes the filter paper was removed and the area of vessel injury flushed with saline. Vessels were monitored for 40 minutes after injury or until full occlusion (blood flow stopped). Parameters assessed were: [1] Thrombus surface in relation to vessel surface five minutes after injury, expressed as a percentage. [2] “Embolization rate” of thrombi with a diameter greater than 20 µm and representing the number of emboli with a diameter greater than 10 µm counted over a period of one minute.

The examiner was not blinded during the actual experiments and whilst choosing the vessel areas from where video recordings were taken. However, afterwards, analyses of thrombus surface and embolization rate were performed blinded. ImageJ
software (Version 10.2) was used for determination of thrombus surface area and
counting of emboli was performed manually.

Limitations of the intravital microscopy model
According to technical limitations blood flow velocity and shear rates in examined
blood vessels were not assessed.

ADP-degradation at hypothermia
To investigate temperature-dependent differences in ADP degradation, CFP samples
were incubated in a thermomixer (Eppendorf, North Ryde, Australia) at 37°C or 18°C.
ADP in a concentration of 2 µM was added to CFP at the respective temperature.
After 10 minutes EDTA (4 mM) was added to remove calcium in order to block the
action of ADP-metabolizing plasma enzymes, thereby fixing the ADP concentration.

Resuspension of PRP in CFP and sample preparation for flow cytometry
Before flow cytometric analysis, samples were processed as follows: One pair of
EDTA-CFP samples that had been exposed in the thermomixer to 18°C and another
pair that had been exposed to 37°C were incubated at 37°C. To one sample of each
pair, 2-MeSAMP (fc: 100 µM) was added in order to determine ADP-independent
baseline platelet activation. PRP was added in a dilution of 1:50 to all samples. Five
minutes after PRP addition, 35 µl of the respective CFP-PRP sample was incubated
with 5 µl of anti-CD41-FITC Ab (SZ 22, Beckman-Coulter GmbH, Krefeld, Germany)
and 5 µl of anti-CD62P-PE Ab (BD Biosciences, Heidelberg, Germany). After 20
minutes samples were fixed with 500 µl CellFix® (BD Biosciences) to inhibit further
platelet activation. Aliquots of the EDTA-fixed CFP samples were stored at -20°C
until luminometric measurement of ADP concentrations.
**Bioluminometric determination of ADP levels**

These procedures were performed according to a previously described method. In CFP samples ADP was converted to ATP by the pyruvate kinase reaction: To 100 µl EDTA-CFP 33 µl solution containing 40 U/ml pyruvate kinase, 4 µM phosphoenolpyruvate (PEP; Sigma-Aldrich), 10 mM KCl, and 40 mM MgSO₄ in 40 mM tricine buffer (pH 7.75) were added. After 5 minutes incubation, each sample was divided into two aliquots of 50 µl each. ATP (representing ATP and ADP) was determined in a bioluminescence assay on a microplate luminometer (Berthold MicroLumatPlus, Bundoora, Australia) by adding 50 µl luciferase reagent (ATP bioluminescence assay kit CLS II; Roche, Mannheim, Germany) to each sample. Corresponding blank CFP samples (without addition of pyruvate kinase and PEP) were also analyzed in doublets to determine basal ATP content. ADP levels were determined by subtracting basal ATP content from the ATP content in samples that had been processed with the pyruvate kinase reaction. Standard samples containing different concentrations of ADP in EDTA-CFP were also measured in order to establish a standard curve for the back-calculation of ADP levels.

**Nucleotide release from blood cells at hypothermia**

EDTA-anticoagulated whole blood from human subjects was either pre-treated for 5 minutes with a combination of 2-MeSAMP and MRS2179 (100 µM each) or with an equivalent volume of PBS buffer as control and then incubated for 60 minutes at 18°C, 28°C and 37°C. EDTA anticoagulation was employed in order to remove calcium and thereby to block the action of ADP-metabolizing enzymes in whole blood. Directly after blood was taken as well as after 60 minutes of incubation 10 µl of whole blood was diluted (D=1:50) in modified Tyrode’s buffer and 50 µl were
incubated with 10 µl of an anti-CD62P-PE antibody. (BD Biosciences, Heidelberg, Germany) for 20 minutes and analyzed using flow cytometry.

Remaining samples were centrifuged (2,500 g, 20 minutes) to obtain platelet-poor plasma (PPP). ADP was converted to ATP as described above. Total ATP concentrations were measured directly after blood sampling as well as after incubation for 60 minutes at 18°C, 28°C and 37°C. Basal nucleotide (ATP and ADP) concentration, directly after blood had been sampled, was subtracted from nucleotide concentration after 60 minutes of incubation to determine the nucleotide concentration released after 60 minutes.

**Analysis of human and murine platelet activation in flow cytometry**

Platelet activation was evaluated in flow cytometry within 6 hrs after fixation on a FACSCalibur-cytometer (Becton Dickinson, Heidelberg, Germany) as previously described.² ⁴ In brief, for analysis of P-selectin expression human platelets were detected by triggering on a preset threshold of the platelet marker SZ 22-FITC fluorescence and were depicted by their typical forward scatter (FSC) / sideward scatter (SSC) criteria in a dot plot. Fluorescence of anti-CD62P-PE Ab was detected and served as measure for platelet activation.

For analysis of GP Ibα expression and fibrinogen binding platelets were detected according to their typical FSC and SSC criteria. Fluorescence of anti-CD42-PE Ab and Oregon-green fibrinogen was detected and served as a measure for platelet GP Ibα expression and fibrinogen binding / GP IIb/IIIa activation respectively. A total of 10,000 events were counted in each measurement. Antibody fluorescence was analyzed in histograms and is given as mean fluorescence or as the percentage platelets with positive Ab binding. P-selectin expression that was induced by ADP in EDTA-CFP samples, was calculated as follows: the amount of ADP-independent
baseline P-selectin expression that was present in 2-MeSAMP-incubated samples was subtracted from the respective samples that contained EDTA-CFP without 2-MeSAMP addition.

For flow cytometric analysis of murine platelets the same principles as described above for the analysis of P-selectin expression were applied. However, the detection threshold of the flow cytometer was set for the anti-CD42bα-PE Ab fluorescence, and P-selectin expression was analyzed according to the fluorescence of the anti-CD62P-FITC Ab. Leukocyte content of platelet-leukocyte aggregates, which were detected according to anti-CD42bα-PE Ab fluorescence and their typical FSC/SSC criteria was analyzed according to anti-CD45-FITC fluorescence. In all experiments suitable isotype controls were used to adjust for non-specific antibody binding.

**Soluble CD39 production and functional testing**

CD39 was PCR modified from pCDNA3-CD39\(^{10}\) in order to add restriction sites that would allow for cloning into the pSectag2a vector (Invitrogen, USA). For increased solubility the CD39 was additionally truncated to remove its 2 transmembrane domains. CD39 was hence modified with the following primers to create solCD39:

Sense strand primer:
5’-tcagtaGCGGCCGCCAACCAGAAACAAAGCATT-3’ and antisense strand primer: 5’-atcgcaCTCGAGTGGTGGAGAGAGGAGAGAG-3’.

After amplification by PCR, the constructs were then digested with NcoI and XhoI restriction enzyme (NEB, USA), and cloned into pSectag2A cut with the same restriction enzyme. Ligation of the plasmids was performed with T4 ligase (NEB, USA) at 16°C overnight. The resulting plasmid constructs were then transformed into Turbo Competent *E. coli* cells (NEB, USA). Transformed bacteria were grown in LB
media containing 100 µg/ml ampicillin at 37°C and the plasmids were purified using Plasmid Mini Kit (Qiagen, Australia). Obtained colonies were confirmed by DNA sequencing.

Larger DNA preparations were performed by Plasmid Maxi Kit (Qiagen, Australia) and then transfected using Lipofectamine 2000 (Invitrogen, USA) as specified by Invitrogen.

After 7-9 days, supernatant medium was collected and dialyzed against PBS overnight. This dialyzed solution was then purified using a Biorad FPLC with Nickel Beads (Qiagen) using the His-tag of the solCD39, with washing at 10 and 20 mM imidazol, and eluted at 250 mM imidazol. The eluted protein was then dialyzed to remove the imidazol against PBS before being aliquotted and stored at -80°C.

Recombinant soluble CD39 (final concentration: 0.1 µg/ml) was incubated with ADP (final concentration: 50 µM) at 18°C, 28°C and 37°C. After 15 minutes remaining ADP was converted to ATP by the pyruvate kinase reaction and generated ATP was determined in a bioluminescence assay as described above.

**Inhibition of ADP-induced platelet aggregation with solCD39**

PRP from human whole blood was prepared as described above. PRP was incubated with PBS (vehicle control) or solCD39 (1.6 µg/ml) at 18°C, 28°C or 37°C for 30 minutes. Platelet aggregation was induced with ADP (10 µM) and measured using aggregometry (AggRAM™ System, Helena Laboratories, Beaumont, TX, USA).

**In vivo administration of solCD39 to mice**

Wild-type C57BL/6 mice were anesthetized and body temperature was allowed to drop to 28°C or maintained at 37°C. When stable body temperatures were reached
100 µl PBS (vehicle control) or recombinant solCD39 (15 µg/10g body weight in 100 µl PBS) was administered in the left jugular vein. Thirty minutes post-injection, blood was sampled via an abdominal incision from the inferior vena cava in EDTA and blood cell count was measured.

**Statistics**

Data are depicted as means with standard errors of the mean (SEM) if not otherwise indicated. Differences between two data sets were evaluated using unpaired two-tailed t-tests for unmatched data and using paired t-tests for matched data. If not otherwise indicated, differences between three or more data sets were evaluated by repeated measures ANOVA with Dunnett's multiple comparison test for matched data and using one-way ANOVA with indicated post-hoc tests for unmatched data. A p-value of <0.05 was defined to indicate a statistically significant difference.
References


