SUPPLEMENTAL MATERIAL

Supplemental Methods:

Preparation of PRP and washed platelets

To prepare Platelet Rich Plasma (PRP), blood was collected by cardiac puncture from mice anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL) as described \(^1\). Blood (about 800 µl) was withdrawn into a syringe containing 80 µl of 3.15% sodium citrate (1:9 ratio). The sample was centrifuged at 200 x g for 8 min in an Eppendorf 5702 centrifuge (Eppendorf, Westbury, New York). PRP was collected from the supernatant and platelet number was counted on HEMAVET HV950FS (Drew Scientific, Inc, Oxford, CT). Platelet Poor Plasma (PPP) was prepared from the rest of the blood by centrifugation at 1500 x g for 15 min. To prepare washed platelets, 0.5 µM prostacyclin (PGI\(_2\), Sigma Aldrich, St. Louis, MO) was added to PRP and centrifuged at 1300 x g for 5 min. After 2 washes, the platelet pellet was resuspended in Tyrode’s buffer (10 mM HEPES [pH 7.4], 5.56 mM glucose, 137 mM NaCl, 12 mM NaHCO\(_3\), 2.7 mM KCl, 0.36 mM NaH\(_2\)PO\(_4\), 1 mM MgCl\(_2\)) with 0.02 U/ml apyrase (Sigma Aldrich, St. Louis, MO) and 0.5 µM PGI\(_2\) at a density of 3 x 10\(^8\) platelets/mL, as described in \(^2\). Platelets were kept at 37°C throughout all experiments. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline level. A 200-µL aliquot of platelet suspension was stirred at 1100 rpm and activated by the addition of 20 µL agonist. PRP was diluted with Platelet Poor Plasma (PPP) at a platelets density of 1.8 x 10\(^8\) /mL and washed platelets were adjusted with Tyrode’s buffer at a platelets density of 3 x 10\(^8\) /mL for aggregation measurement. Platelet
aggregation in response to 5 µM ADP (Sigma Aldrich, St. Louis, MO) or 10 µg/ml collagen (Chrono-log, Harvertown, PA) was measured in the absence or presence of A2-type receptor agonist 10 µM NECA (Sigma Aldrich, St. Louis, MO), while selectively blocking the A2bAR with 10 µM MRS 1754 (Sigma Aldrich, St. Louis, MO). A2aAR agonist CGS 21680 (10 µM, Sigma Aldrich, St. Louis, MO) was used as control. Ligand- and vehicle-treated platelets were compared. Experiments were repeated in presence of adenosine deaminase (1U/ml, Roche Applied Science, Indianapolis, IN).

**Purification of MKs**

Bone marrow cells were isolated as described before. MKs were purified by the MACS® magnetic bead purification system (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, fresh bone marrow cells were labeled with a MK specific antibody, anti-CD41-FITC (BD Pharmingen, Franklin Lakes, NJ) using a 1:150 dilution in running buffer (0.5% BSA, 2 mM EDTA, and PBS, pH 7.2), and were incubated at 4°C for 25 minutes. Following two washes in running buffer, bone marrow cells were resuspended in 270 µL of buffer and 30 µL of anti-FITC labeled microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). After 15 minute incubation at 4°C, cells were washed, spun down, and resuspended in 500 µL of running buffer, before loading onto an equilibrated large cell separation column (Miltenyi Biotech, Bergisch Gladbach, Germany) fitted with a 25 gauge needle for flow resistance. The column was washed three times with running buffer to remove unlabeled cells. The column was then removed from the magnetic stand and bound CD41-FITC labeled MKs were eluted with 1 mL of running buffer. The MKs percentage after purification is in the range of 70-80% by number and over 95% by mass (considering MK size) in both freshly isolated and
cultured cells. At day 3 in culture, MKs were treated for 24 hours with adenylyl cyclase activator Forskolin (2 µM, Sigma Aldrich, St. Louis, MO), phosphodiesterase (PDE) inhibitor IBMX (10 µM, Sigma Aldrich, St. Louis, MO), NECA (10 µM), CGS 21680 (10 µM) and MRS 1754 (10 µM), as indicated. MRS 1754 was pre-incubated for 10 min before NECA. At day 4, the MK-rich fraction was harvested as above. Cell pellets were subjected to RNA isolation as described below.

**RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)**

Cells were immediately spun down and pellets were homogenized using the QiaShredder® system (Qiagen, Valencia, CA). Lysates were stored at -80°C until RNA isolation, as described below. MKs RNA was prepared with the Rneasy® Mini Kit (Qiagen, Valencia, CA) and complementary DNA (cDNA) was made using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. mRNA expression was quantified by TaqMan® Gene Expression system (Applied Biosystems, Foster City, CA) and was run using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Data are normalized with housekeeping gene 18s rRNA expression.

**cAMP measurement in platelets and MKs**

Cells were treated for 10 min with Forskolin (2 µM), NECA (10 µM) and CGS 21680 (10µM). MRS 1754 (10 µM) was pretreated for 10 min before NECA. Adenosine deaminase (1U/mL) was added in all experiments to eliminate the effect of endogenous adenosine. All experiments were pursued in presence of 0.5 mM PDE inhibitor Papaverine hydrochloride (Sigma Aldrich, St. Louis, MO). Cells were lysed in 0.1 N HCl
with 0.5% Triton X-100. Levels of cAMP were normalized to protein amount measured with the Bradford assay (Protein Assay Kit, Bio-Rad Laboratories, Inc., Hercules, CA).

**Mouse femoral artery injury model**

WT and A2bAR KO male mice (10-12 week-old) were anaesthetized, an incision was made in the groin, a clamp was used to occlude the femoral artery below the inguinal ligament, a cut was made distal to the epigastric artery, and a 0.25 mm angioplasty guide wire was introduced. The clamp was then removed and the guide wire advanced 3 cm, ten times. After removal of the guide wire, the artery was ligated and the incision was closed, all as described in 1. Sham surgery included all of the procedures described except that no guide wire was introduced.

**β-galactosidase assay in MKs and platelets**

MKs and platelets were prepared as described in Methods. Cell pellets were re-suspended in fixative buffer for 20 min (0.5% glutaraldehyde, 0.02% NP-40 in 1X PBS without MgCl₂), washed twice with PBS, and then incubated in 1 ml X-gal staining solution (4 mM K₄Fe(CN)₆·3H₂O (Sigma Aldrich, St. Louis, MO), 4 mM K₃Fe(CN)₆ (Sigma Aldrich, St. Louis, MO), 1mM MgCl₂, 1mg/ml X-gal (American Bioanalytical, Natick, MA) in PBS) for 16-20 hrs at 37°C. Cells were mounted onto slides and blue precipitates (indicative of β-galactosidase activity) were visualized via Olympus IX70 microscope combined with a Hamamatsu charge-coupled device camera (C4742-95). DAPI (Vector Laboratory, Inc., Burlingame, CA) staining was used to observe nuclei on MK slides. β-galactosidase expression in platelets was also analyzed at the ultrastructural level in the above described mouse femoral artery injury model. Mouse
tissue stained for β-galactosidase was processed for electron microscopy by using a modification of a previously published protocol. At day 1 after injury, the mouse was perfused with 4.3% glutaraldehyde (pH 7.4, EM grade, Polysciences, Inc., Warrington, PA) for 20 min and the injured limb was dissected and subjected to bluo-gal (Sigma Aldrich, St. Louis, MO) staining solution (4 mM K₄Fe(CN)₆·3H₂O, 4 mM K₃Fe(CN)₆, 1mM MgCl₂, 1mg/ml bluo-gal in PBS) for 16-20 hrs at 37°C. Following washes with PBS, the samples were kept in 4.3% glutaraldehyde overnight at 4°C. The samples were rinsed three times with sodium barbital-sodium acetate buffer containing potassium chloride (0.07 M) for 15 minutes each. This was followed by dehydration in a graded series of ethanol starting with 50% ethanol, embedded in a 1:1 mixture of Araldite 502 and dodecenyl succinic anhydride at 60°C. After polymerization of the Araldite mixture, sections were cut on an LKB Ultratome V. Ribbons of sections showing gray, silver or slightly gold interference colors were pick up on uncoated 200 mesh Athene Thin Bar copper grids.

**Platelet preparation for electron microscopy**

Two WT (C57BL/6 background) and two A2bAR KO mice are used for this experiment. To prepare Platelet Rich Plasma (PRP), blood was collected by cardiac puncture from mice anesthetized with isoflurane (Baxter Healthcare Corporation, Deerfield, IL). Blood (about 800 µl) was withdrawn into a syringe containing 80 µl of 3.15% sodium citrate (1:9 ratio). The sample was centrifuged at 200 x g for 8 min in an Eppendorf 5702 centrifuge (Eppendorf, Westbury, New York). PRP was collected from the supernatant and platelet number was counted on HEMAVET HV950FS (Drew Scientific, Inc, Oxford,
Platelet Poor Plasma (PPP) was prepared from the rest of the blood by centrifugation at 1500 x g for 15 min. PRP was diluted with Platelet Poor Plasma (PPP) at a platelets density of 1.8 x 10^8 /mL. 200 μl PRP sample was treated with or without 5 μM ADP (Sigma Aldrich, St. Louis, MO) for 2 min. The samples were fixed by adding 1.0 ml 4.3% glutaraldehyde (Polysciences; Warrington, PA) in 0.03 M sodium barbital-sodium acetate buffer (pH 7.4) containing 0.07M potassium chloride for 10 min at 37 ºC. The samples were centrifuged at 1300 x g for 5 min, and the platelet pellets were resuspended in the glutaraldehyde solution at 4 ºC for 24 h. The samples were then rinsed with sodium barbital-sodium acetate buffer solution three times for 15 min each, and post-fixed in a solution of 1% osmium tetroxide (Ted Pella; Redding, CA) in 0.03 M sodium barbital-sodium acetate buffer (pH 7.4) in 0.07M potassium chloride for 1-2 hrs at room temperature. This was followed by dehydration in a graded series of ethanol starting with 70% ethanol, embedded in a 1:1 mixture of Araldite 502 and dodecenyl succinic anhydride at 60°C. After polymerization of the Araldite mixture, sections were cut on an LKB Ultratome V. Ribbons of sections showing gray, silver, or slightly gold interference colors were picked up on uncoated 200 mesh Athene Thin Bar copper grids. Electron micrographs were obtained from sections stained with uranyl acetate followed by lead citrate and examined in a JEOL electron microscope.

**Western blot analysis**

Platelets were washed three times with cold 1 X PBS, followed by addition of ice-cold Radioimmunoprecipitation Assay (RIPA) buffer (1 X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)), freshly supplemented with 1 X
protease inhibitor cocktail (Cat. No. 11697498001, Roche Applied Science, Indianapolis, IN). The lysed cells were collected and centrifuged at maximum speed for 10 min, at 4 °C. The supernatants were applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western Blotting as previously described.

Supplemental figure legends:

Supplemental Fig 1: cAMP level in megakaryocytes (MKs) derived from WT and A2bAR KO mice. One set of experiments was performed with WT MKs (A) and one with WT as compared to A2AR KO MKS (B). MKs were isolated, cultured with TPO and treated with ligands as detailed in the legend to Fig 6. cAMP was measured in MKs derived from WT mice after treatment with vehicle or different ligands for 10 min. To prevent cAMP degradation, PDE inhibitor Paraverine hydrochloride was added before treatment. MRS1754 was pretreated for 10 min before NECA. Data are presented as average ± SD (n = 3). P < 0.05 is considered statistically significant.

Supplemental Fig 2: A larger magnification of the image in Fig 3A depicting an electron microscopic examination of an artery and platelets following femoral artery injury in A2bAR KO mice. The green arrow indicates platelets. The red arrow indicates A2bAR promoter driven β-gal expression (black signal) in platelets.

Supplemental Fig 3: Expression of the A2bAR gene in MKs at base line and under inflammation. A2bAR mRNA expression is measured by qRT-PCR. mRNA was prepared from freshly isolated MKs derived from control and LPS (5 μg/g i.p, 16 h) treatment mice. Data is normalized with 18s mRNA expression.
Supplemental Fig 4: β-galactosidase staining of cultured MKs derived from A2bAR KO mice. A 200X magnification picture is shown. Blue staining in A2bAR KO MKs indicates the expression of β-galactosidase gene driven by A2bAR gene promoter. The yellow arrow indicates the big cell and the red arrow indicates the small cell. The blue staining is shown in both big and small cells.

Supplemental Fig 5. Platelet counts before and after TPO injection in WT and A2bAR KO mice. TPO (0.05 μg/g) or PBS was injected to the mice through tail vein injection. After 4 days, blood was collected through cardiac puncture and platelets were counted using a HEMAVET HV950FS.

Supplemental references:


Supplemental Fig 1A

The figure shows the cAMP level in MKs (pmol/mg protein) under different conditions:

- **Control**
- **NECA**
- **MRS 1754 + NECA**
- **MRS 1754**

The graph indicates that:

- The cAMP level in MKs treated with NECA is significantly higher compared to the control group.
- The cAMP level in MKs treated with MRS 1754 + NECA is significantly higher compared to the control group.
- The cAMP level in MKs treated with MRS 1754 is significantly lower compared to the control group.

Statistical significance is indicated by:

- **P < 0.05**

The data suggests that NECA and MRS 1754 + NECA increase cAMP levels, while MRS 1754 decreases them.
Supplemental Fig 1B

![Graph showing cAMP levels in MKs with different treatments.](image)

- Control
- Forskolin
- CGS 21680
- NECA
- MRS 1754 + NECA
- MRS 1754

*P < 0.05*
Supplemental Fig 3

A2bAR mRNA expression in MKs (fold)

- Control
- LPS 16h

Bar chart showing A2bAR mRNA expression in MKs. The expression is significantly higher in the LPS 16h group compared to the control group.
Supplemental Fig 5

The graph shows platelet counts in two conditions: Control and TPO. The x-axis represents different conditions (Control and TPO) and the y-axis represents platelet counts (k/ul). The bars indicate the average platelet counts with error bars showing the variability. The bars for TPO indicate higher platelet counts compared to the Control condition.