Adenosine signaling inhibits erythropoiesis and promotes myeloid differentiation

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A. Supplemental methods

Blood samples

Fresh peripheral blood samples were obtained from healthy donors (age range: 18-70, as per Etablissement Français du Sang/EFS criteria) in accordance with the Declaration of Helsinki protocols and approved by the Ethical Committee of the National Institute of Blood Transfusion (INTS) (Paris, France).

Antibodies

Monoclonal antibodies used to characterize GPA^{neg} cells and to monitor human erythropoiesis are listed in the supplemental Table S1.

Ex vivo erythropoiesis: Purification and culture of CD34$^+$ cells

Peripheral blood mononuclear cells (PBMCs) were obtained from the peripheral blood samples of healthy donors (n=20) using a Ficoll density gradient separation (Pancoll 1.077 g/ml, PAN BIOTECH). CD34$^+$ cells were then purified using anti-CD34 conjugated microbeads and manual cell separation columns as per the manufacturers' instructions (StemCell Technologies Inc; MACS Miltenyi Biotec). The cell culture procedure was comprised of 2 phases as previously described by (Fibach et al., 1989; Reihani et al., 2016)[1,2]. Briefly, isolated peripheral blood CD34$^+$ cells were incubated for 7 days in Iscove's Modified Dulbecco's Medium IMDM Glutamax (Gibco) supplemented with 15% BIT 9500 (BSA, human insulin, human holo-transferin) (Stem Cell Technologies), 100 U/ml Penicillin Streptomycin (Gibco) and 2 mM L-Glutamine (Gibco), 100 ng/mL human recombinant (hr) interleukin-6 (IL-6) (Miltenyi Biotec), 10 ng/mL hr interleukin-3 (IL-3) (Miltenyi Biotec), and 50 ng/mL stem cell factor (SCF) (Miltenyi Biotec). On day 7, the cells were harvested and cultured for 12 days with the second phase medium (10 ng/mL hr IL-3, 100 ng/mL hr SCF, and 2 U/mL hr Erythropoietin (EPO) at

a density of 10^5 cells/ml. For cultures treated with adenosine, cells were incubated with different concentrations of adenosine (5, 10, 20, 25, 50, 200 µM, Sigma-Aldrich) or dimethyl sulfoxide (DMSO) < 0.1 % (v/v) starting from day 0 of the differentiation phase. In experiments combining adenosine treatment to nucleoside transporter's inhibitor, S-(4-Nitrobenzyl)-6-thioinosine (NBTI) (Sigma-Aldrich) was added. In experiments testing other nucleosides, 50 µM of guanosine, uridine or cytidine (Sigma-Aldrich) were added to the culture media. Adenosine receptor (AR) activation during cell culture was induced, as indicated, by addition of AR agonists BAY 60-6583 (Sigma-Aldrich) and 2-Cl-IB-MECA (Tocris Biosciences) at final concentration of 25 µM.

May-Grünwald Giemsa staining

1×10^5 cells were used to prepare cytospin preparations on coated slides, using the Thermo Scientific Shandon 2 Cytospin. The slides were stained with May-Grünwald (Sigma MG500) pure solution for 5 minutes, with May-Grünwald diluted twice for 5 minutes, and subsequently stained with Giemsa solution (Sigma GS500) for 15 minutes, then rinsed twice in demineralized water for 4 minutes. The cells were imaged using a Nikon Eclipse Ti-S inverted microscope. The same protocol was used to stain blood smears. For blood smear preparation, a drop of blood was placed on one end of a glass slide and dispersed over the slide's length.

Flow cytometry analysis

The characterization of $CD34^+$ progenitors before and during erythropoiesis were performed monitoring the expression level of several surface markers using the corresponding monoclonal antibodies listed in Table S1. Briefly, 0.1×10^6 cells were suspended in 20µL PBS buffer supplemented with 2% bovine serum albumin (BSA) and stained with fluorochrome-conjugated antibodies for 30 minutes on ice. Cells were then washed three times with PBS 2% BSA before analysis. Each cocktail of antibodies contained a viability marker: 7-

Aminoactinomycin D (7AAD) (BD Bisiciences) or Sytox Blue (Invitrogen). The Syto 16 green dye (Invitrogen) was used to identify the enucleated reticulocytes at the end of differentiation. For cell cycle analysis, GPA$^+$ cells sorted at day 4 were incubated for 90 minutes at 37°C with CytoPhase^{TM} Violet (Biolegend). At least 10 000 cells were acquired using BD FACS Canto II flow cytometer coupled with the FACS Diva software (version 8.0.1), and analyzed by FlowJo software (V10).

SDS-PAGE and Western Blotting

Whole-cell lysates of cultured erythroblasts were solubilized in Laemmli buffer (10 mM Tris-HCl pH=6.8,1 mM EDTA, 5% final SDS) containing 1X cOmplete™ protease cocktail inhibitor (Roche; 11697498001), heated at 110°C for 5 min and sonicated. Protein quantification was performed by Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, 23225), and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked, and immunoblotting was realized using the corresponding primary antibodies overnight at 4 °C. Antibodies for western blotting were directed against TP53 (Sigma #5816), CDKN1A/p21 (Cell Signaling #2947), Caspase3 (Cell Signaling #9662), and cleaved-Caspase3 (Cell Signaling #9661), Proteins of interest were revealed using HRP-conjugated secondary antibodies (1:2000, Cell Signaling Technology) and the Enhanced Chemiluminescence (ECL) Prime kit (GE Healthcare, RPN2236). Images were acquired by ChemiDoc™ Imaging Systems (Bio-Rad) and bands intensity were quantified with Image Lab software (Bio-Rad). HRP-conjugated rabbit anti-actin (1/5000, Cell Signalling #13E5) was used as loading control.

Global proteomic analysis

Sample preparation for proteomic analyses

Cell pellets were solubilized in lysis buffer (4% SDS, 100mM Tris-HCl, pH 8.5) and boiled 5 minutes at 95°C. Thirty milligrams of each protein extracts were digested using trypsin

(Promega) and Strap Micro Spin Column was used according to the manufacturer's protocol (Protifi, Farmingdale, NY, USA). Peptides were than speed-vacuum dried.

Liquid Chromatography-coupled Mass spectrometry analysis (nLC-MS/MS)

nLC-MS/MS analyses were performed on a Dionex U3000 RSLC nano-LC- system coupled to a TIMS-TOF Pro mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). After drying, peptides were solubilized in 30 μL of 0.1% TFA containing 2% acetonitrile (ACN). Two μL were loaded, concentrated and washed for 3min on a C_{18} reverse phase column (5μm particle size, 100 Å pore size, 300 μm inner diameter, 0.5 cm length, from Thermo Fisher Scientific). Peptides were separated on an Aurora C18 reverse phase resin (1.6 μm particle size, 100Å pore size, 75μm inner diameter, 25cm length mounted to the Captive nanoSpray Ionisation module, from IonOpticks, Middle Camberwell Australia) with a 4h run time with a gradient ranging from 98% of solvent A containing 0.1% formic acid in milliQ-grade H2O to 35% of solvent B containing 80% acetonitrile, 0.085% formic acid in mQH2O.

The mass spectrometer acquired data throughout the elution process and operated in DIA PASEF mode with a 1.17 second/cycle, with Timed Ion Mobility Spectrometry (TIMS) enabled and a data-independent scheme with full MS scans in Parallel Accumulation and Serial Fragmentation (PASEF). Ion accumulation and ramp time in the dual TIMS analyzer were set to 100 ms each and the ion mobility range was set from $1/K0 = 0.63$ Vs cm^{-2} to 1.43 Vs cm^{-2}. Precursor ions for MS/MS analysis were isolated in positive polarity with PASEF in the 100-1.700 m/z range by synchronizing quadrupole switching events with the precursor elution profile from the TIMS device.

Protein identifications and quantifications

The mass spectrometry data were analyzed using DIA-NN version 1.8.1 (Demichev V. et al., Nature Methods, 2019). The database used for *in silico* generation of spectral library was a concatenation of Human sequences from the Uniprot and-Swissprot databases (release 2022-05) and a list of contaminant sequences. M-Terminus exclusion and carbamidomethylation of cysteins was set as permanent modification and one trypsin misscleavage was allowed. Precursor false discovery rate (FDR) was kept below 1%. The "match between runs" (MBR) option was allowed.

Statistics

Statistical analyses were performed using GraphPad Prism 7. Statistical tests corresponding to each experiment are indicated in the figure legends. Comparisons between values were performed using unpaired t-test and all comparisons with a p value less than 0.05 ($p < 0.05$) were considered statistically significant. $p < 0.0001$, $p < 0.001$, $p < 0.01$, and $p < 0.05$ are indicated with four, three, two, or one star (s), respectively. The results are expressed as the means ± the standard error of the mean (SEM), unless otherwise mentioned.

References

1. Fibach, E., Manor, D., Oppenheim, A. & Rachmilewitz, E. A. Proliferation and maturation of human erythroid progenitors in liquid culture. *Blood* (1989). doi:10.1182/blood.v73.1.100.bloodjournal731100

2. Reihani, N. *et al.* Unexpected macrophage-independent dyserythropoiesis in Gaucher disease. (2016). doi:10.3324/haematol.2016.147546
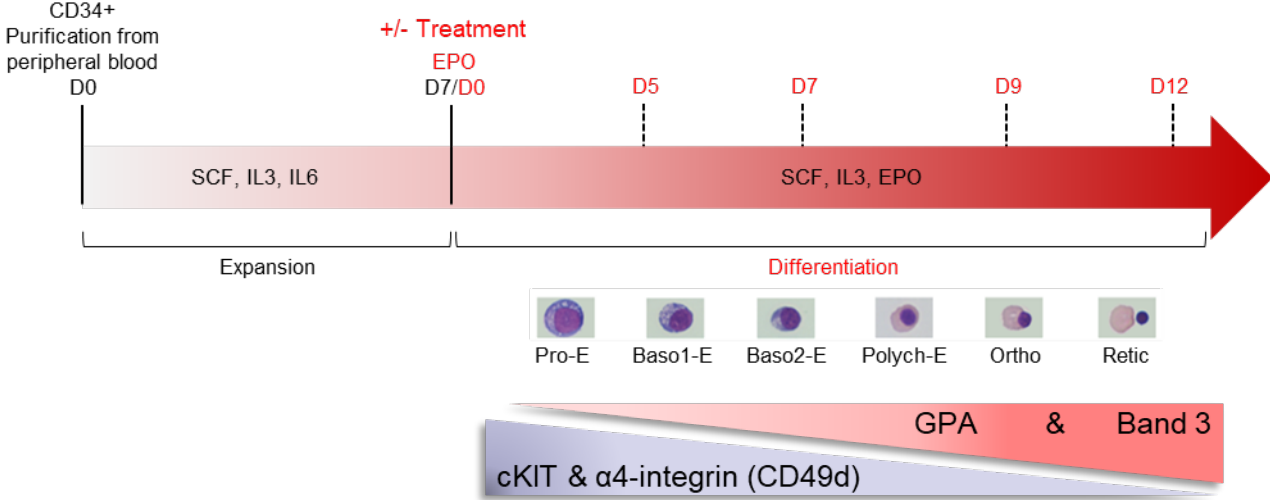
B. Supplemental figures

Figure S1. Schematic representation of human erythropoiesis and experimental design. Adult CD34$^+$ cells isolated from peripheral blood of healthy donors were expanded for 7 days then differentiated using a liquid culture system (containing rEPO) that recapitulates human erythropoiesis up to the enucleated reticulocyte. Representative MGG-stained cells are shown for corresponding differentiation stages. Expected expression patterns of cell surface markers during terminal erythroid differentiation are presented, GPA and Band3 expression is increased (red) while cKIT and α4-integrin expression is decreased (purple).
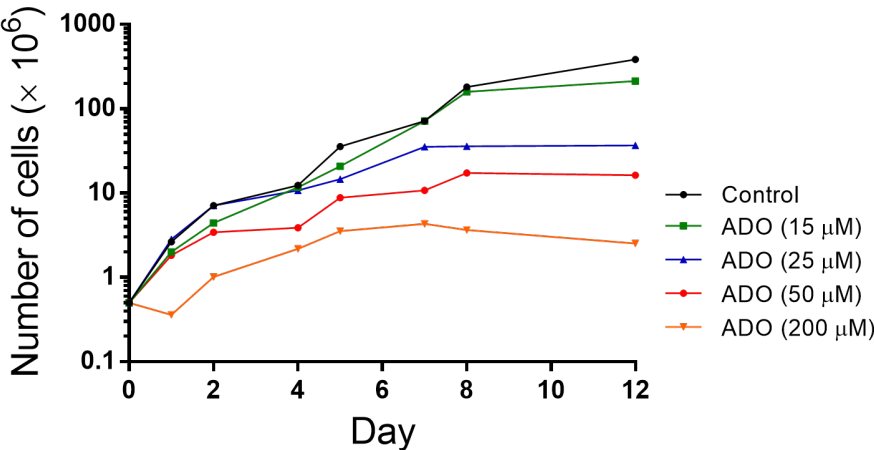

Figure S2. Growth curves of erythroid precursors from cells treated with increased concentrations of adenosine shown in absolute number of cells during the differentiation phase.
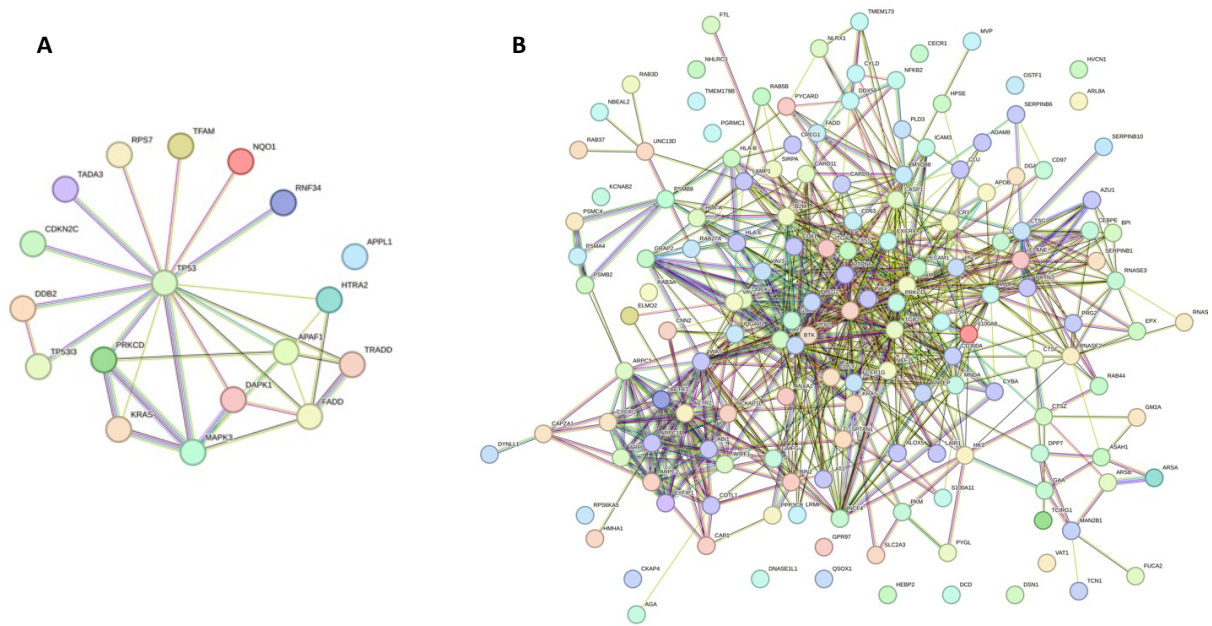
Figure S3: Protein–protein interaction network analyzed by String software. Network analysis form overexpressed proteins from ex vivo erythropoiesis in the presence of adenosine at day 4 (n=2). Two clusters of proteins were detected: **A.** Proteins involved in the P53 pathway. **B.** Proteins involved in myeloid lineage.

C. Supplemental tables

Table S1. Antibodies used for flow cytometry

Antibody	Fluorescence	Clone	Dilution	Brand	Reference
			Human		
CD235a (GPA)	BV 421	GA-R2 (HIR2)	1/20	BD Pharmingen	562938
CD36	APC	CB38 (NL07)	1/20	BD Pharmingen	550956
CD49d (α4β1 integrin)	APC	MZ18-24A9	1/20	Miltenyi Biotec	130-093-281
CD233 (Band3)	PE	BRIC6	1/100	IBGRL	9439PE
CD11a	PE	TS2/4	1/20	Biolegend	350605
SYTOX blue	Pacific Blue	N/A	1/1000	Invitrogen	S34857
Syto 16 green	FITC	N/A	1/1000	Invitrogen	S7578
CD33	BV421	WM53	1/20	BD Pharmingen	565949
CD117 (cKIT)	APC	104D2	1/20	eBioscience	17-1178-42
CD13	PE	WM15	1/20	BD Pharmingen	560998
CD18	FITC	CBR LFA-1/2	1/20	Biolegend	366305
Annexin V	PE	N/A	1/100	BD Pharmingen	51-65875X

N/A. not available