Adenosine signaling inhibits erythropoiesis and promotes myeloid differentiation

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Abstract

Intracellular uptake of adenosine is essential for optimal erythroid commitment and differentiation of hematopoietic progenitor cells. The role of adenosine signaling is well documented in the regulation of blood flow, cell proliferation, apoptosis, and stem cell regeneration. However, the role of adenosine signaling in hematopoiesis remains unclear. In this study, we show that adenosine signaling inhibits the proliferation of erythroid precursors by activating the p53 pathway and hampers the terminal erythroid maturation. Furthermore, we demonstrate that the activation of specific adenosine receptors promotes myelopoiesis. Overall, our findings indicate that extracellular adenosine could be a new player in the regulation of hematopoiesis.

Introduction

Hematopoiesis is the process by which all blood cells are produced from hematopoietic stem cells (HSC) in the bone marrow. Erythropoiesis and granulopoiesis are two main branches of hematopoiesis leading, respectively, to the production of red blood cells (RBC) and neutrophils. While both erythroid and myeloid differentiation derive from the same common myeloid progenitor (CMP), the interplay between the two processes is complex and tightly orchestrated by different intrinsic and extrinsic factors regulating the commitment of progenitor cells towards one cell lineage or the other.[1] Terminal erythropoiesis and granulopoiesis occur in erythroblastic islands, which are specialized microenvironments in the bone marrow consisting of a central macrophage surrounded by erythroid and neutrophil precursors.[2] These structures constitute a unique cellular microenvironment, and play a crucial role in supporting cell proliferation and differentiation by providing essential nutrients, removing cellular debris, and secreting cytokines and growth factors.[3] A growing body of evidence showed that balanced microenvironmental cues taking place in these niches, as well as metabolite transport and signaling, are additional key regulators of HSC commitment and differentiation.[1,4-6] Under stress or pathological conditions such as hypoxia, infection, inflammation, and anemia, the balance of these factors can be perturbed leading to the overproduction of one cell lineage at the expense of the other.

Glucose, lipid, amino acid and purinergic metabolisms, as well as nucleotide biosynthesis, have all been shown to be important for erythropoiesis.[1,4-6] Furthermore, we have recently shown that the intracellular uptake of adenosine, a pleiotropic and ubiquitous purine nucleoside, is essential for optimal erythroid commitment and differentiation of progenitor cells.[7] However, adenosine is also a major signaling molecule that activates cellular signaling pathways through a family of four different G protein-coupled adenosine receptors (AR): A_1, A_{2A}, A_{2B}, and A_3.[8] Adenosine signaling pathways play critical roles in tissue homeostasis by regulating cellular activation, differentiation, and cell cycling.[9] Extracellular levels of adenosine are very low at steady-state condition, ranging from 10 to 200 nM, due to the tightly regulated balance between its production from ATP/AMP by ectonucleotidases (CD39, CD73), its rapid cellular uptake by nucleoside transporters, and its degradation by adenosine deaminase (ADA).[10,11] However, the extracellular

levels of adenosine can be rapidly elevated up to 100 μM by release of intracellular adenosine from damaged cells or in response to hypoxia and inflammation, as well as tissue injury.[10,12] In sickle cell disease (SCD), plasma adenosine levels are increased, and adenosine signaling is enhanced and associated with an increase in RBC sickling through A_{2B} activation[13] and a reduction in inflammation via the activation of iNKT cells.[14] The erythrocyte equilibrative nucleoside transporter 1 (ENT1) plays a key role in the regulation of plasma adenosine concentrations and thereby controls adenosine signaling in RBC.[15] Under hypoxia, increased extracellular adenosine levels activate the adenosine A_{2B} membrane receptor in RBC, which triggers a downregulation of erythrocyte ENT1 through its proteasomal degradation. These reduced ENT1 levels prevent adenosine cellular uptake and allow its rapid accumulation in the plasma leading to a further activation of adenosine receptors.[15] Our previous work demonstrates that ENT1-mediated adenosine uptake is important for erythropoiesis.[7] However, the role of extracellular adenosine signaling in erythropoiesis remains to be elucidated.

Here, we show that adenosine signaling via its plasma membrane receptors, A_{2B} and A_3, influences hematopoiesis both by inhibiting erythropoiesis through the activation of p53 and cell cycle arrest, and by promoting myeloid cell differentiation.

Methods

Detailed protocols for all methods are provided in the *Online Supplementary Methods*.

Ethics statement

Blood bags from healthy donors were obtained from the Établissement Français du Sang (EFS). The study was performed in accordance with the protocols of the Declaration of Helsinki and was approved by the Ethical Committee of the National Institute of Blood Transfusion (INTS), Paris, France. All the participants gave written informed consent.

Ex vivo erythropoiesis

Ex vivo erythropoiesis of $CD34^+$ cells isolated from the peripheral blood of healthy donors was performed following the protocol described in *Online Supplementary Figure S1*, in the presence of increasing extracellular adenosine concentrations of up to 200 μM, which corresponds to adenosine levels in stress conditions.

Flow cytometry

Viability, cell cycle and expression of surface markers were monitored every two or three days using fluorochrome-conjugated antibodies (*Online Supplementary Methods*).

Western blots

Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with the indicated antibodies, followed by HRP anti-rabbit or anti-mouse immunoglobulin.

Proteomic

At day 4 of the differentiation phase, adenosine-treated and untreated cells were washed and conserved as dry pellets at -80°C until protein extraction. Proteomic analysis was performed using liquid chromatography-coupled mass spectrometry analysis (nLC-MS/MS), and DIA-NN version 1.8.1 for identification.

Results

High dose of exogeneous adenosine inhibits erythroid proliferation and activates the p53 apoptotic pathway

In order to investigate the role of adenosine signaling in regulating erythroid commitment and terminal erythroid maturation, we performed *ex vivo* erythropoiesis of $CD34^+$ cells purified from peripheral blood of healthy donors, and supplemented with different concentrations of adenosine. Our results showed that adenosine decreases erythroid proliferation in a dose-dependent manner (*Online Supplementary Figure S2*). When treated with 50 μM of adenosine, erythroid precursors exhibited over a 16-fold lower cell growth than untreated controls by day 12 of terminal differentiation ($P<0.0001$) (Figure 1A). To explore the potential mechanisms by which adenosine inhibited erythroblast proliferation, we assessed whether adenosine induced apoptosis in erythroid cells. Annexin V and Sytox blue staining revealed that 14% and 31% of cells treated with adenosine are in early and late stage of apoptosis, respectively, at day 5 of differentiation (Figure 1B). The apoptotic effect of adenosine is significantly enhanced in immature erythroblasts and extenuated during terminal erythroid maturation (Figure 1C). In line with the increased apoptosis, adenosine treatment was found to induce the activation of the p53 pathway and its downstream target p21, as well as the increase in the cleaved form of caspase 3 (Figure 1D). Moreover, cell cycle assays revealed that adenosine treatment led to cell accumulation in the G1 phase and a delayed progression towards the S and G2/M phases ($P<0.05$) (Figure 1E). To further understand how adenosine signaling activates the p53 pathway in immature erythroblasts, we performed a whole proteomic analysis of day 4 erythroblasts cultured in the presence of adenosine (n=2). Interestingly, adenosine treatment led to the overexpression of several proteins involved in the p53 apoptotic signaling pathway (APAF1, PRKCD, HTRA2,

TADA3, and DAPK1) and in repair of reactive oxygen species (ROS)-related DNA damage (TP53, DDB2, and TP53I3) (*Online Supplementary Figure S3*). These data suggest that adenosine signaling may increase DNA damage which leads to the activation of the p53 pathway. The impact of adenosine on DNA has been previously reported in HL-60 cells.[16] On the other hand, numerous myeloid lineage-specific proteins were overexpressed in adenosine-treated cells as compared to untreated control cells, which suggests that adenosine modulates erythroid and myeloid differentiation (*Online Supplementary Figure S3*).

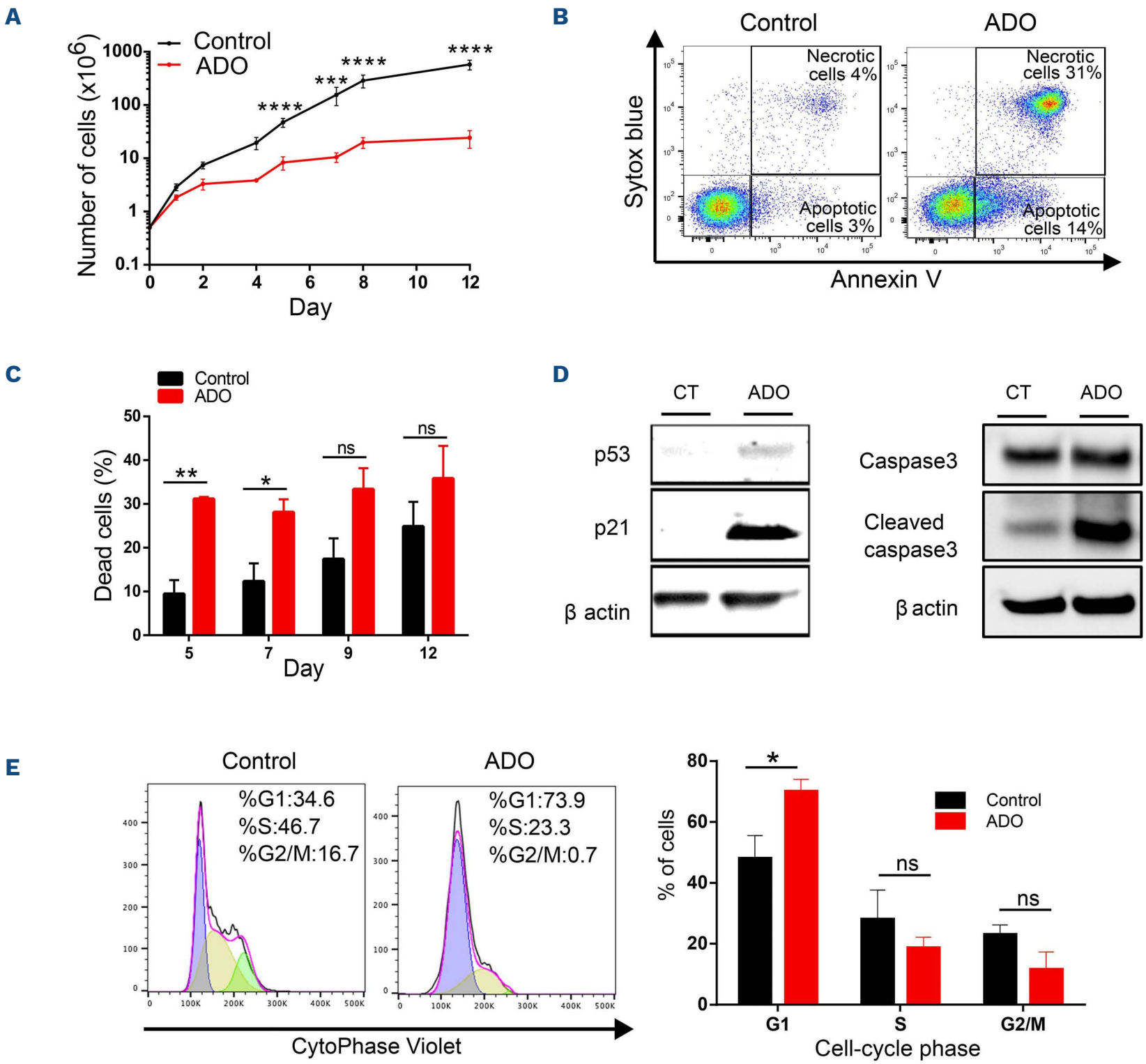

Figure 1. High dose of adenosine inhibits erythroid proliferation and induces cell death through activation of p53 pathway. (A) CD34⁺ progenitors from peripheral blood of healthy donors were stimulated with erythropoietin in the absence or presence of adenosine (50 μM) at day 0 of the differentiation phase. Growth curves of erythroid precursors from cells treated with adenosine (red line) and control (black line) cells are shown in absolute number of cells during the differentiation phase. Mann-Whitney test: ***P=0.0002, ****P<0.0001; N=9 independent experiments. (B) Percentages of apoptotic, necrotic, and living cells as monitored by Sytox blue and Annexin V co-staining of cells treated or not with adenosine at day 5 of differentiation. (C) Dead cell rate was determined by Sytox blue staining in flow cytometry and percentages of cells were plotted in bar histograms of mean cell % ± Standard Error of Mean. Unpaired t test: *P<0.05, **P<0.005, ns: not significant; N=3 independent experiments. (D) Representative western blot of p53, p21, caspase3, cleaved caspase3 expression and corresponding expression of β-actin in control (CT) and adenosine-treated (ADO) cells at day 5 of differentiation phase. (E) Cell cycle of GPA⁺-sorted cells was determined by CytoPhase Violet staining at day 4 of differentiation phase. G1, S, and G2/M phase are represented in blue, yellow, and green, respectively. Percentages of cells in each cell cycle phases are plotted in bar histograms. Two-way Anova test: *P=0.04; N=3 independent experiments.

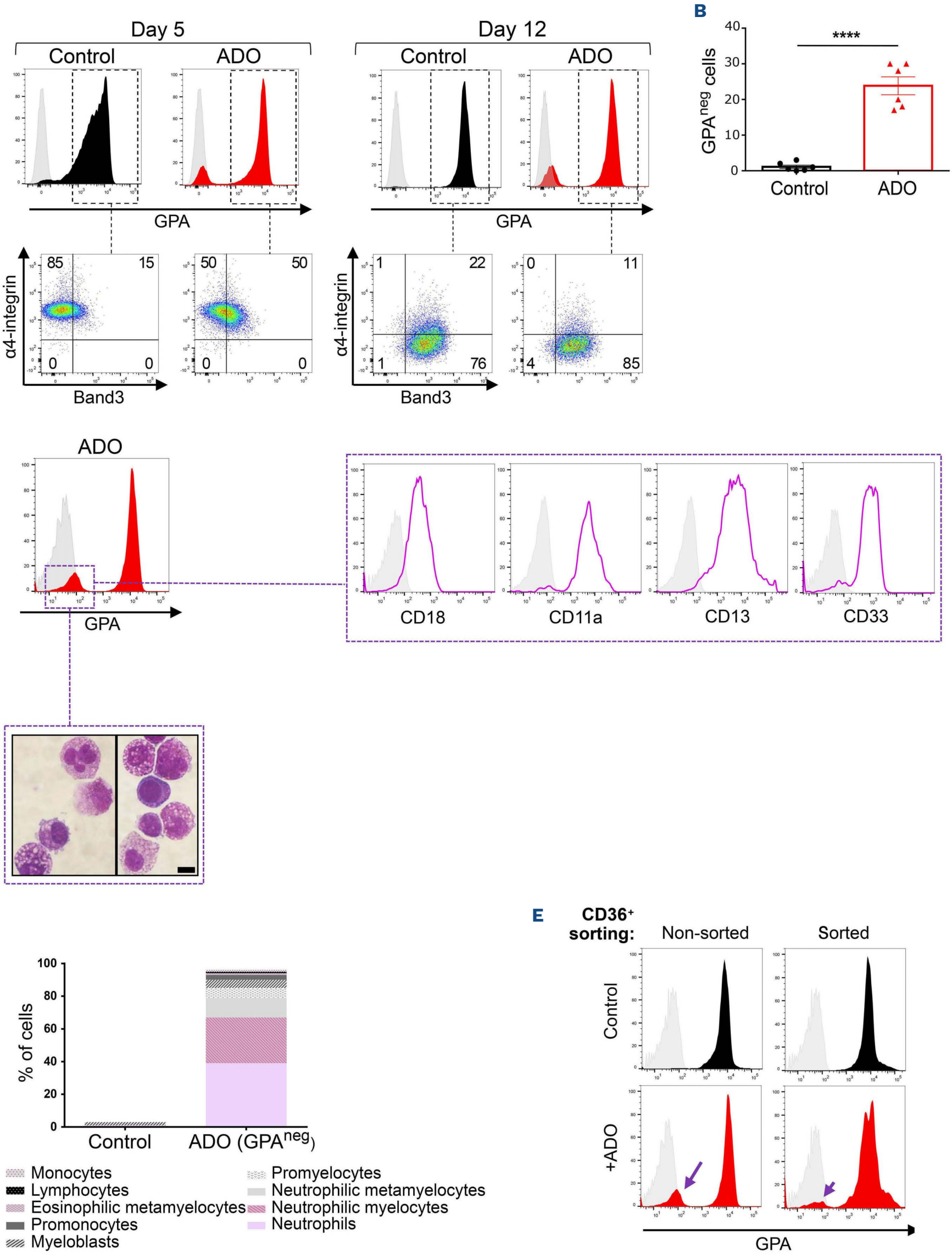

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Figure 2. High level of adenosine induces myeloid differentiation even in presence of erythropoietin. (A) Flow cytometric analyses of GPA were performed to follow the erythroid-lineage specification and erythroid differentiation. GPA expression was analyzed inside 7-AAD^{neg} viable cells. Data are shown as peak histograms at indicated days (erythropoietin [EPO] control, black; adenosine-treated cells, red). Erythroblast maturation was monitored via α4-integrin and Band3 levels of GPA^{pos} cells. α4-integrin^{high}/Band3^{low} cells represent less mature erythroblasts, whereas α4-integrin^{low}/Band3^{high} cells are further differentiated. Frames represent gating hierarchy. (B) Percentage of GPA^{neg} cells at day 12 of differentiation in cells cultured with or without adenosine. Data shown as mean cell % ± Standard Error of Mean. Unpaired *t* test: ****P<0.0001; N=6 independent experiments. (C) At day 12 of EPO-induced erythroid differentiation, adenosine-treated cells show a GPA^{neg} subpopulation (outlined by the purple dotted square below). These GPA^{neg} cells were FACS-sorted and analyzed by flow cytometry for expression levels of myeloid surface markers. Corresponding histograms are shown for unstained controls (shaded gray) and specific stainings (purple line) as indicated. GPA^{neg} subpopulation was also MGG-stained; representative pictures are shown. Bar represents 10μm. (D) Characterization and count of MGG-stained adenosine-induced GPA^{neg} cells and control cells plotted in histograms. Percentages of the respective identified-cell types are shown. (E) CD34^{+} cells were FACS-sorted on the basis of CD36^{+} at day 7 of the expansion phase, then replaced in culture and stimulated by EPO. At day 12 of EPO-induced erythroid differentiation, expression levels of GPA were compared between the previously CD36^{+}-sorted and non-sorted cells in both control and adenosine-treated cells. Adenosine-induced GPA^{neg} subpopulation is indicated by the bigger purple arrow in non-sorted cells, and the smaller purple arrow in CD36^{+}-sorted cells.

Extracellular adenosine impacts the erythroid differentiation of hematopoietic stem and progenitor cells and promotes myelopoiesis in the presence of erythropoietin

To further characterize the effect of high dose of adenosine on erythropoiesis, we monitored the erythroid surface markers of viable cells during erythroblast maturation. Interestingly, adenosine treatment led to the persistence of a non-erythroid subpopulation of GPA^{neg} cells, despite the presence of erythropoietin (EPO) in the culture medium (Figure 2A). Notably, at day 12 of the culture, whereas all control cells expressed GPA, 24±6% of adenosine-treated cells remained GPA^{neg} (P<0.0001) (Figure 2B). Band3 and α4-integrin markers showed normal erythroid differentiation of GPA^{pos} cells in both conditions (Figure 2A). To further characterize the non-erythroid population found upon adenosine treatment, GPA^{neg} population was FACS-sorted at day 12 of differentiation, and cells were analyzed for the expression of several surface hematopoietic markers. Surprisingly, these cells up-regulated a wide range of myeloid surface markers, such as CD18, CD11a, CD13, and CD33 (Figure 2C). Moreover, May-Grünwald-Giemsa (MGG) staining of this sorted population revealed granular cells at different stages of differentiation (Figure 2D). These findings indicate that extracellular adenosine allows myeloid differentiation even in an erythroid-favoring environment. Next, to determine the nature of the progenitors resulting in the adenosine-induced myeloid cells in the presence of EPO, erythroid progenitors were FACS-sorted on the basis of CD36 at day 7 of the expansion phase. CD36^{+} cells were then differentiated in the presence of EPO and adenosine. Interestingly, the percentage of GPA^{neg} cells decreased from 30% to 8%, indicating that adenosine induces the myeloid differentiation of CD36^{neg} progenitors (Figure 2E). This finding was further confirmed by the absence of myeloid cells when adenosine was added in the culture three days after the EPO-induced terminal differentiation (*data not shown*). Altogether, we demonstrate that, at a higher concentration, adenosine inhibits erythroid proliferation and promotes myeloid differentiation of CD36^{neg} progenitors.

Activation of A3 adenosine receptor by Cl-IB-MECA perturbs the balance of erythroid *versus* myeloid differentiation of hematopoietic stem and progenitor cells

In order to understand how extracellular adenosine disturbs erythropoiesis, we first performed similar experiments with other nucleosides, including guanosine, cytidine or uridine. Neither cytidine nor uridine had any effect on cell proliferation, while guanosine slightly attenuated cell growth (Figure 3A). However, differentiation of erythroid precursors was not impacted by any of these nucleosides (*data not shown*). Exogenously added adenosine can either enter cells through specific nucleoside transporters (primarily via ENT1) or bind to cell membrane adenosinergic receptors.[17] To address the pathway by which adenosine perturbs hematopoiesis, we co-treated cells with adenosine and nitrobenzylthioinosine (NTBI, also called NBMPR), a potent bidirectional inhibitor of ENT1. Accordingly, cell proliferation was further decreased in co-treated cells as compared to untreated or adenosine-only-treated cells (Figure 3B), suggesting that the observed effects are mediated through extracellular adenosine signaling. To confirm this finding, we investigated the pharmacological activation of AR among CD34^{+} human erythroid progenitor cells cultured in the presence of EPO, using selective agonists. Recently published transcriptomic data showed that only A_{2B} and A_3 receptors are expressed in CD34^{+} progenitors and erythroid precursors.[18] We first tested the Cl-IB-MECA (2-chloro-N6-[3-iodobenzyl]-adenosine-5'-N-methyluronamide), a highly selective agonist of the A_3AR.[12,19] In accordance with its known anti-proliferative action, CI-IB-MECA decreased the number of erythroid precursors, as shown by a significant decrease in expansion (P<0.01) (Figure 3C) with an increase in cell death (Figure 3D). Notably, at day 12, only 32±2% of CI-IB-MECA-treated cells were viable. The erythroid commitment potential of hematopoietic progenitors was also significantly decreased, as monitored by the analysis of GPA expression (Figure 4A, B). Furthermore, CI-IB-MECA significantly delayed erythroid maturation of the GPA^{pos}-erythroid-committed

cells, as monitored by Band3 and α4-integrin surface markers. Notably, at day 7, only 15±6% of CI-IB-MECA-treated erythroblasts acquired Band3 expression, while 51±2% of untreated-cells expressed Band3 (Figure 4B). Also, at day 12 of differentiation, CI-IB-MECA-treated cells failed to acquire Band3 marker or lose α4-integrin marker and up-regulated c-kit surface markers, which is consistent with the block in erythroid maturation (Figure 4C, D). Moreover, the activation of A_3 receptor resulted in the differentiation of myeloid cells, even in the presence of EPO, as indicated by the expression of CD33 marker on CI-IB-MECA-treated cells (Figure 4D). Repre-

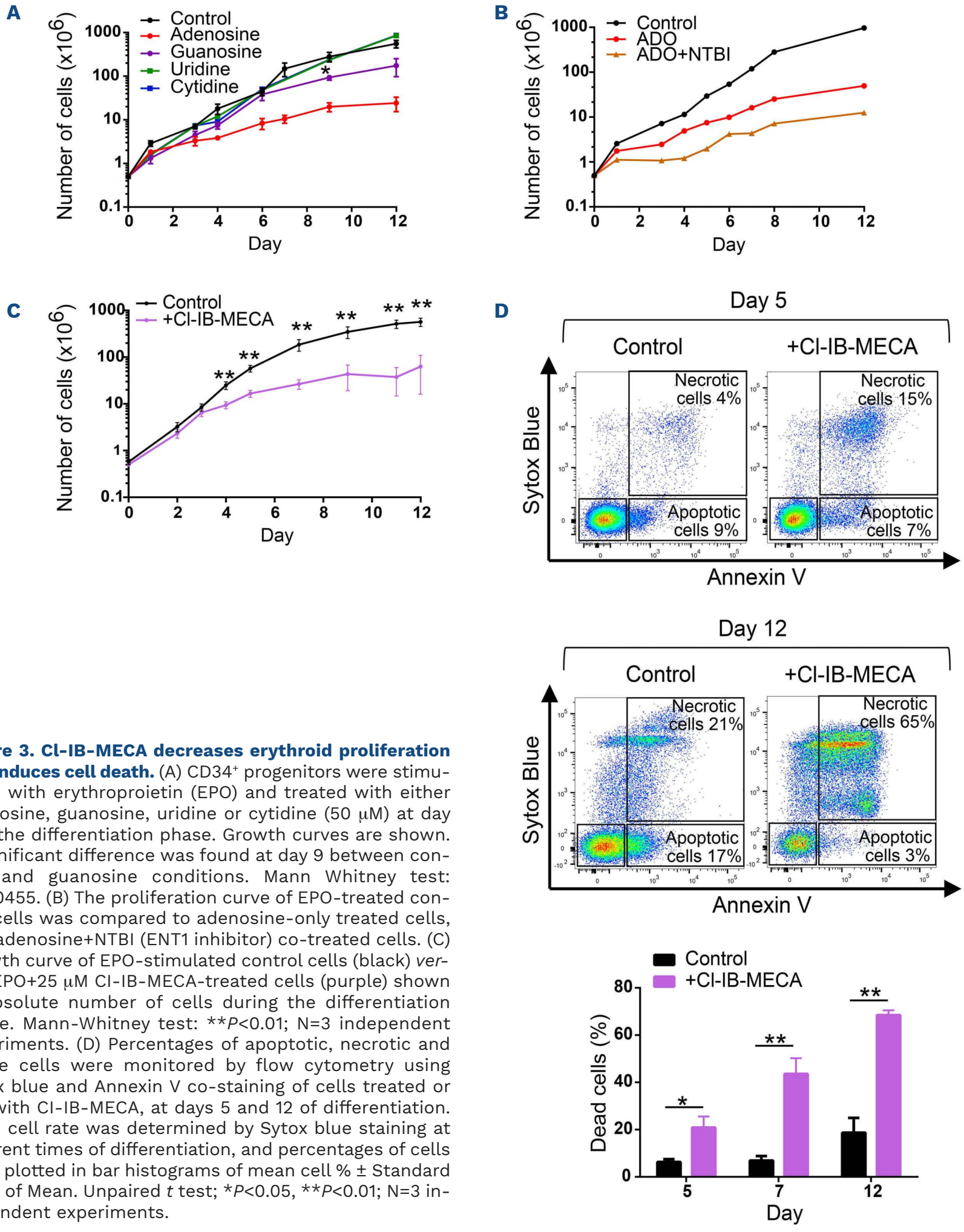


Figure 3. Cl-IB-MECA decreases erythroid proliferation and induces cell death. (A) CD34⁺ progenitors were stimulated with erythroproietin (EPO) and treated with either adenosine, guanosine, uridine or cytidine (50 μM) at day 0 of the differentiation phase. Growth curves are shown. A significant difference was found at day 9 between control and guanosine conditions. Mann Whitney test: *P*=0.0455. (B) The proliferation curve of EPO-treated control cells was compared to adenosine-only treated cells, and adenosine+NTBI (ENT1 inhibitor) co-treated cells. (C) Growth curve of EPO-stimulated control cells (black) *versus* EPO+25 μM CI-IB-MECA-treated cells (purple) shown in absolute number of cells during the differentiation phase. Mann-Whitney test: **P<0.01; N=3 independent experiments. (D) Percentages of apoptotic, necrotic and viable cells were monitored by flow cytometry using Sytox blue and Annexin V co-staining of cells treated or not with CI-IB-MECA, at days 5 and 12 of differentiation. Dead cell rate was determined by Sytox blue staining at different times of differentiation, and percentages of cells were plotted in bar histograms of mean cell % ± Standard Error of Mean. Unpaired *t* test; *P<0.05, **P<0.01; N=3 independent experiments.

sentative images of MGG staining indicate the presence of a mix of immature erythroblasts and myeloblasts among CI-IB-MECA-treated cells, whereas untreated cells present exclusively erythroblasts at mature stages of differentiation and reticulocytes (Figure 4E). Altogether, these findings indicate that A_3AR activation on hematopoietic progenitors leads to myeloid differentiation and erythropoiesis inhibition.

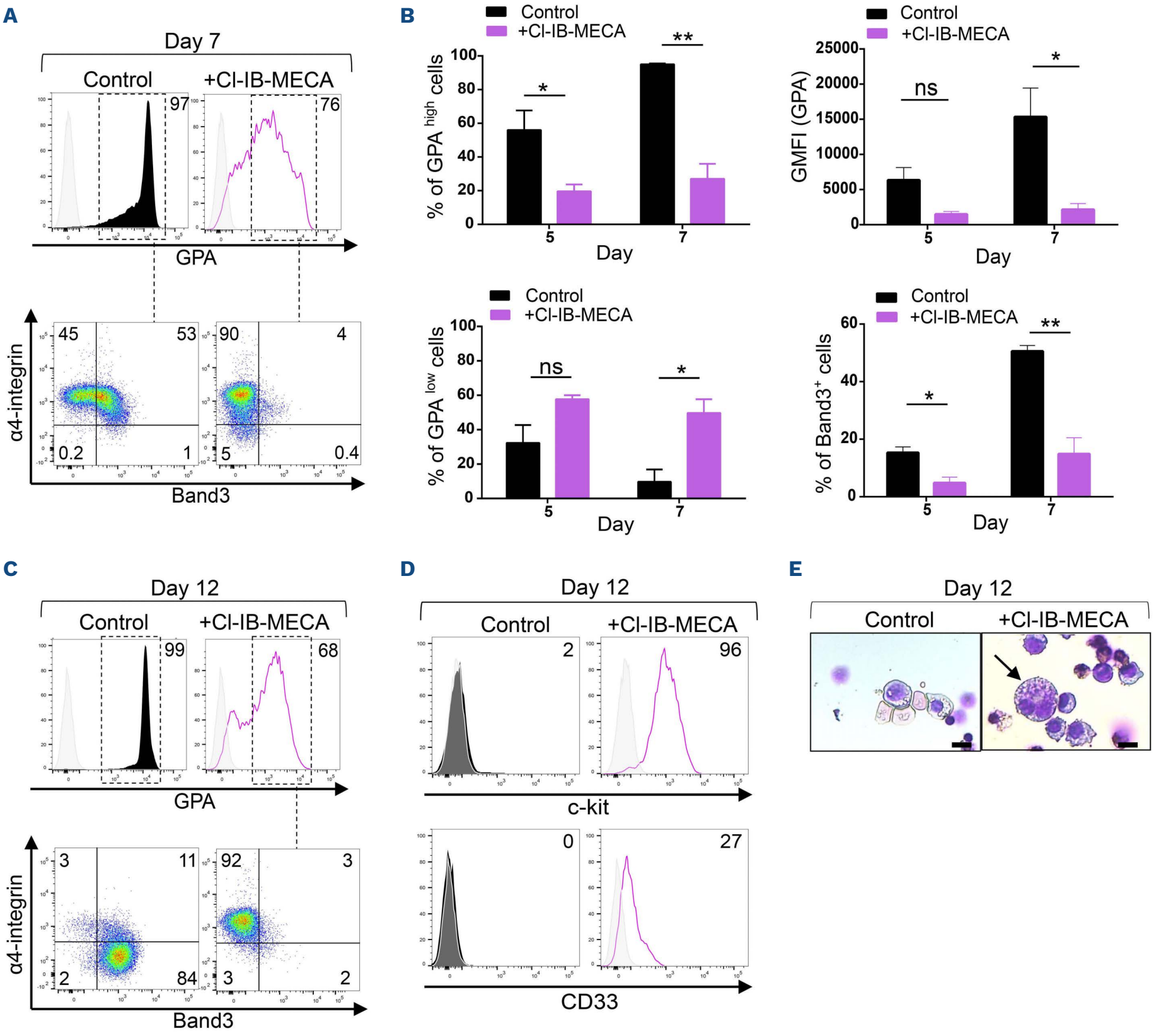
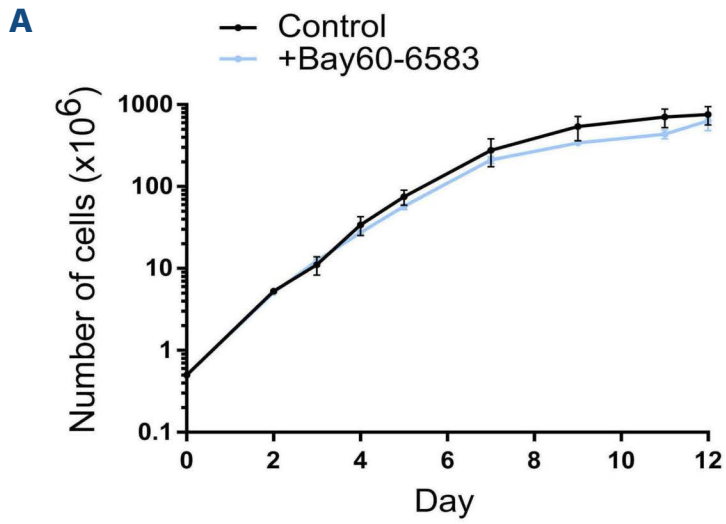
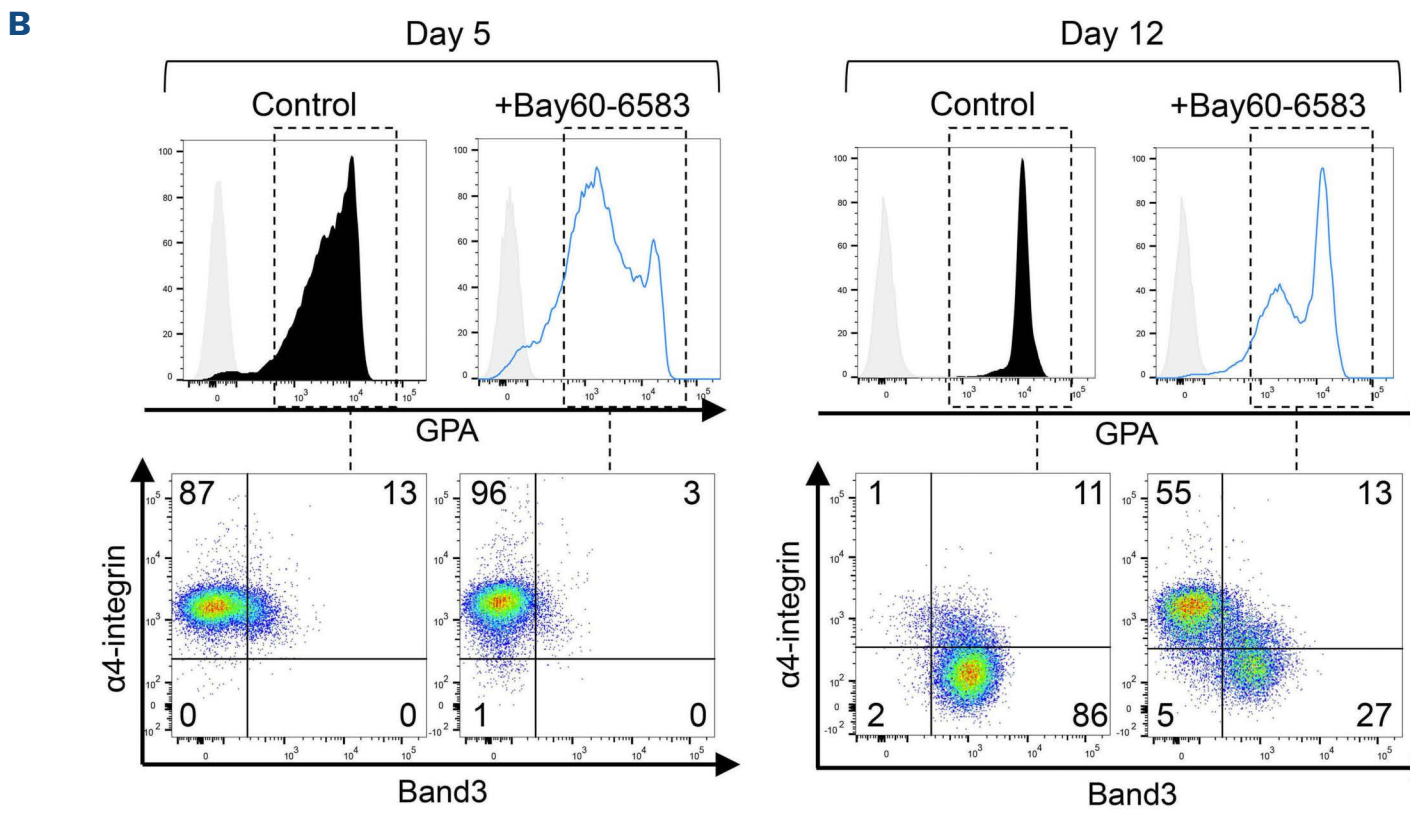


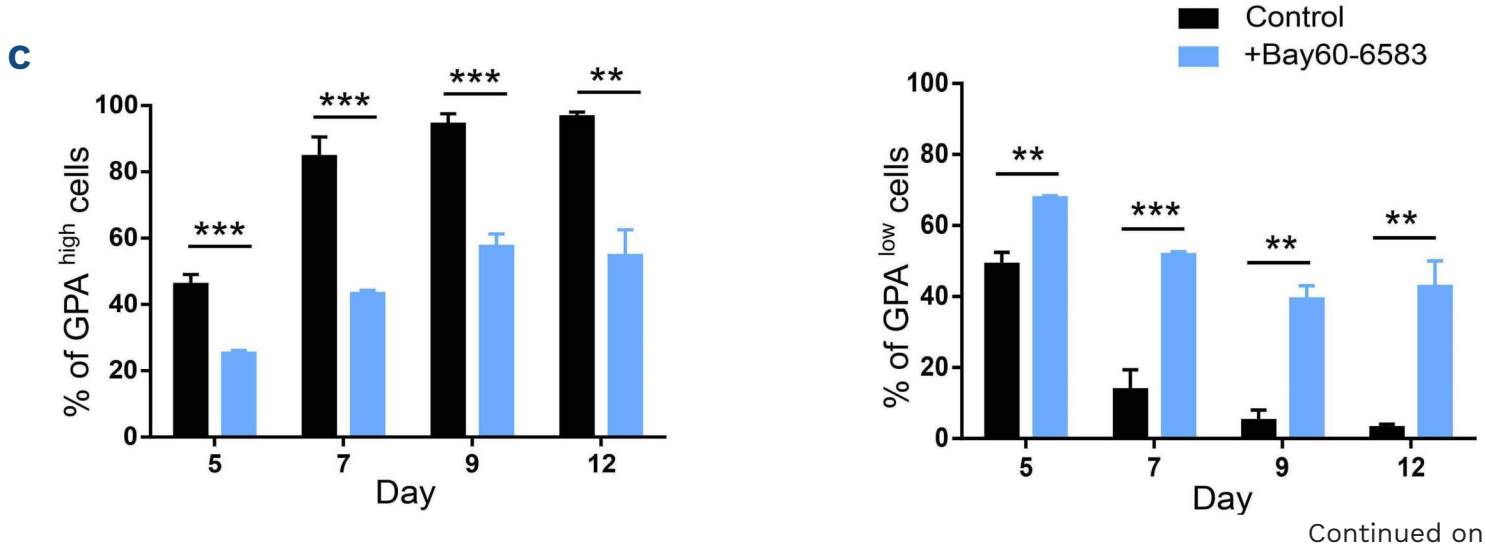
Figure 4. Activation of adenosine receptors by Cl-IB-MECA perturbs the erythroid and myeloid differentiation of hematopoietic stem and progenitor cells. (A) GPA expression was analyzed inside viable cells. Data shown as peak histograms at day 7 of differentiation (erythropoietin [EPO] control, black; CI-IB-MECA-treated cells, purple; unstained control, shaded gray), α4-integrin and Band3 expression levels of GPA^{pos} cells were analyzed to monitor erythroblast maturation. Frames represent gating hierarchy. (B) Percentages of GPA^{high} and GPA^{low} cells, GPA intensity of fluorescence and percentages of $Band3^{+}$ cells in control and CI-IB-MECA-treated cells plotted in bar histograms and presented at indicated days with corresponding colors. Unpaired *t* test: **P*<0.05, ***P*<0.01, ns: not significant; N=3 independent experiments. (C) GPA, and α4-integrin and Band3 expression levels of GPA^{pos} cells, at day 12 of differentiation for EPO-stimulated control cells (black) and CI-IB-MECA treated cells (purple). Gray-shaded peaks represent unstained controls; N=2 independent experiments. (D) c-kit and CD33 were monitored at day 12 of differentiation for EPO-stimulated control cells (black) and CI-IB-MECA-treated cells (purple); N=2 independent experiments. (E) May-Grünwald Giemsa-stained cytospins of control and CI-IB-MECA-treated cells at day 12 of differentiation. Black arrow corresponds to a large eosinophil cell. Bar represents 10 μm.

Activation of A2B adenosine receptor impairs erythroid differentiation

Next, we performed similar experiments using the selective A_{2B} agonist BAY 60-6583[20] to investigate the effect of this receptor activation on erythropoiesis. BAY 60-6583 did not affect the erythroblast proliferation (Figure 5A). Although A_{2B} activation did not impact erythroid commitment (since all cultured cells were GPA$^+$), BAY 60-6583 treatment strongly abrogated erythroblast maturation (Figure 5B). Notably, the expression of erythroid markers including GPA and Band3 were delayed in the presence of the BAY 60-6583 (Figure 5C-E). In addition, erythroid differentiation blockage of treated cells was further confirmed by attenuated erythroblast







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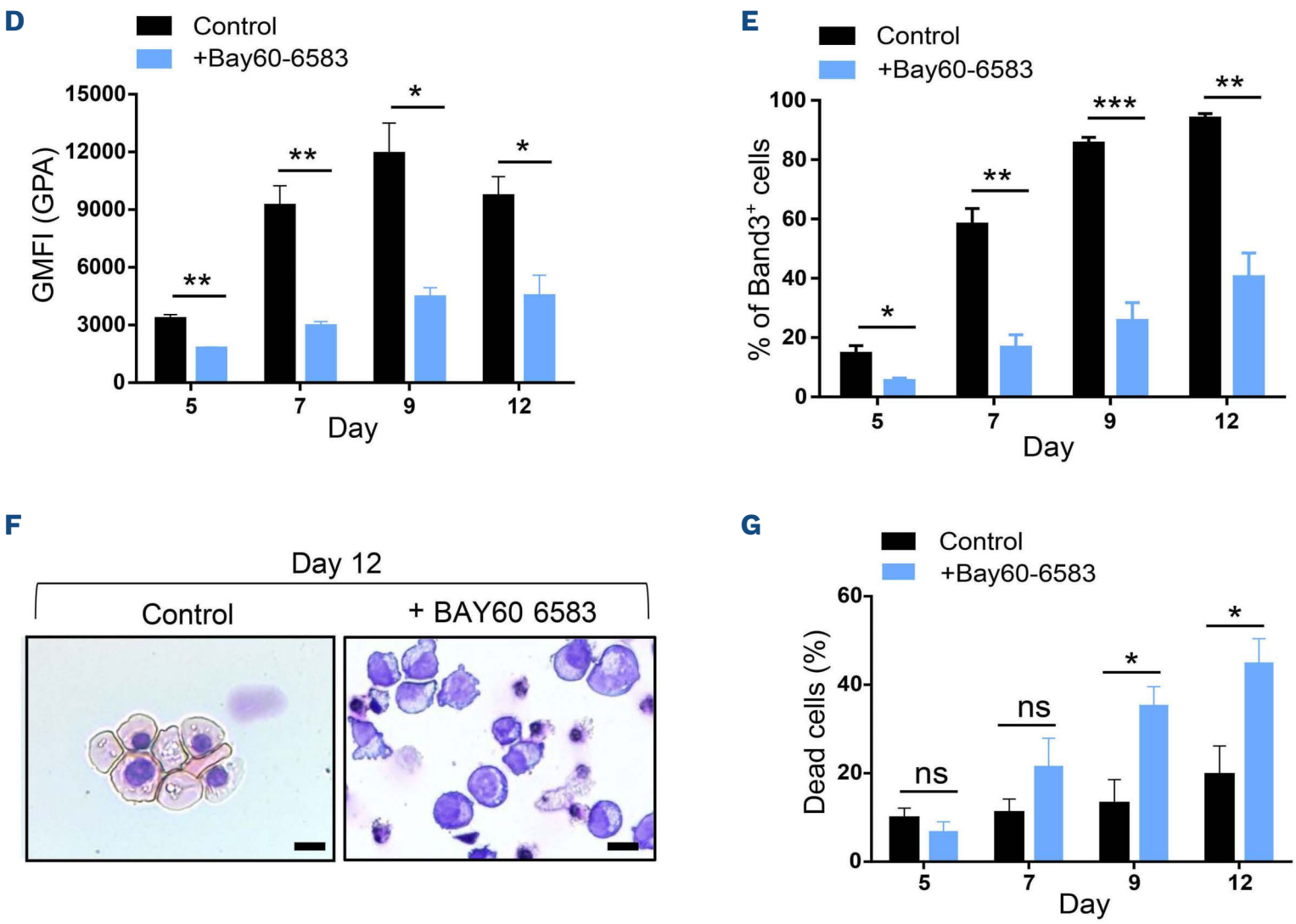

Figure 5. Activation of A2B adenosine receptor impairs erythroid differentiation. (A) CD34$^+$ progenitors from peripheral blood of healthy donors were stimulated with erythropoietin (EPO) in the absence or presence of BAY60-6583 (25 μM) at day 0 of the differentiation phase. Growth curves of erythroid precursors from control cells (black) and BAY60-6583-treated cells (blue) are shown in absolute number of cells during the differentiation phase; N=4 independent experiments. (B) Expression levels of GPA are shown for EPO-stimulated control cells (black) and EPO + BAY60-6583-treated cells (blue), as well as α4-integrin/Band3 profile at days 5 and 12 of erythroid terminal differentiation. Gray-shaded histograms correspond to unstained controls. (C) Percentages of GPA^{high} and GPA^{low} cells are plotted in bar histograms and are presented at indicated days with corresponding colors. Unpaired *t* test: ***P*<0.01, ****P*<0.001; N=4 independent experiments. (D) GPA intensity of fluorescence during erythroid differentiation is shown. Unpaired *t* test: **P*<0.05, ***P*<0.01; N=4 independent experiments. (E) Percentages of Band3$^+$ cells are shown at indicated days. Data are presented as mean ± Standard Error of Mean (SEM). Unpaired *t* test: **P*≤0.05, ***P*≤0.007, ****P*≤0.0009; N=4 independent experiments. (F) May-Grünwald Giemsa-stained cytospins of control and BAY60-6583-treated cells at day 12 of differentiation. Bar represents 10 μm. (G) Dead cell rate was determined by Sytox blue staining in flow cytometry and percentages of cells were plotted in bar histograms of mean cell %±SEM; N=4 independent experiments. Unpaired *t* test: **P*<0.05, ns: not significant.

maturation and enucleation (Figure 5F), and increased apoptosis at days 9 and 12 (Figure 5G).

Discussion

Here, we show that adenosine signaling plays an important role in modulating erythroid proliferation and differentiation via the activation of AR. At high concentration, adenosine-mediated decrease in proliferation is most likely due to the activation of A_3, while the attenuation of erythroid maturation is both mediated by A_3 and by A_{2B} receptors. However, A_3 seems to be the only adenosine receptor promoting myeloid differentiation. Our findings indicate that high dose of exogenous adenosine attenuates erythroblast proliferation via cell cycle arrest at G1 and increases apoptosis upon the activation of the p53 pathway. Interestingly, it has been shown that adenosine signaling contributes to p53-induced cell death,[21] as well as G1 cell cycle arrest in breast cancer stem cells[22] and ovarian cancer cell line.[23] Furthermore, adenosine-mediated decreased cell proliferation is partly due to A_3 receptor since the pharmacological activation of this receptor with its highly selective agonist Cl-IB-MECA attenuated erythroid cell proliferation and increased cell death. In addition, we showed that extracellular adenosine induces myeloid differentiation even in the presence of erythropoietin through A_3 receptor. Indeed, myeloid cells at different stages of granular cell differentiation were found upon adenosine treatment and A_3-specific activation led to the presence of CD33$^+$ and ckit$^+$ cells in an erythroid-favoring environment. These findings suggest that adenosine signaling through A_3 influences the survival and differentiation of myeloid progenitors. It is important to note that A_3 activation via its selective agonist IB-MECA has pre-

viously been related to the stimulation of granulopoiesis,[24,25] and A_3 knockout mice display a reduction in mature granulocytes and monocytes compared to wild-type mice.[26]

To conclude, our findings place adenosine signaling as a new player in hematopoiesis regulation by promoting granular cell differentiation, and inhibiting erythroid proliferation and differentiation. Interestingly, adenosine signaling was reported to play a detrimental role in the pathophysiology of SCD, which is characterized by chronic anemia and unexplained high level of neutrophils.[27] Interestingly, an SCD mouse model treated with the polyethylene glycol-modified adenosine deaminase (PEG-ADA) (an FDA-approved drug used for the treatment of human ADA genetic deficiency) exhibited significantly reduced levels of plasma adenosine, decreased leukocyte counts, and increased RBC counts, suggesting that adenosine signaling could contribute to dysregulated hematopoiesis in SCD-like mice.[13] Future studies using cohorts of SCD patients will be necessary to address the question as to whether adenosine signaling is involved in an abnormal hematopoiesis in this disease.

Disclosures

No conflicts of interest to disclose.

Contributions

MM, BK and SA conceived the project and obtained the grant. MM and SA designed the research study. MM, MS, EC, RD, EFG and BK performed the experiments. MM, MS, BK, EC and SA analyzed the data. YL, YC, CLK and TP edited the manuscript. MM, MS and SA wrote the paper.

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Data-sharing statement

The data supporting our findings are available upon request to the corresponding author.

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