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Adenosinergic regulation of the expansion and immunosuppressive activity of CD11b+Gr1+ cells

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Abstract

Extracellular adenosine and purine nucleotides are elevated in many pathological situations associated with the expansion of $CD11b^+Gr1^+$ myeloid-derived suppressor cells (MDSCs). Therefore, we tested whether adenosinergic pathways play a role in MDSC expansion and functions. We found that A_{2B} adenosine receptors on hematopoietic cells play an important role in accumulation of intratumoral $CD11b^+Gr1^{high}$ cells in a mouse Lewis lung carcinoma (LLC) model *in vivo* and demonstrated that these receptors promote preferential expansion of the granulocytic $CD11b^+Gr1^{high}$ subset of MDSCs *in vitro*. Flow cytometry analysis of MDSCs generated from mouse hematopoietic progenitor cells revealed that the $CD11b^+Gr\text{-}1^{high}$ subset had the highest levels of CD73 (ecto-5′-nucleotidase) expression (ΔMFI of 118.5±16.8), followed by $CD11b^+Gr\text{-}1^{int}$ (ΔMFI of 57.9±6.8) and $CD11b^+Gr\text{-}1^{-/low}$ (ΔMFI of 12.4±1.0). Even lower levels of CD73 expression were found on LLC tumor cells (ΔMFI of 3.2±0.2). The high levels of CD73 expression in granulocytic $CD11b^+Gr\text{-}1^{high}$ cells correlated with high levels of ecto-5′-nucletidase enzymatic activity. We further demonstrated that the ability of granulocytic MDSCs to suppress CD3/CD28-induced T cell proliferation is significantly facilitated in the presence of the ecto-5′-nucletidase substrate 5′-AMP. We propose that generation of adenosine by CD73 expressed at high levels on granulocytic MDSCs may promote their expansion and facilitate their immunosuppressive activity.

INTRODUCTION

Accumulating evidence suggests that the endogenous nucleoside adenosine plays an important role in regulation of inflammation and immunity. Extracellular adenosine exerts its actions via cell surface G protein-coupled adenosine receptors. Four subtypes of adenosine receptors have been cloned and classified as A_1, A_{2A}, A_{2B} and A_3 (1). It has been long recognized that adenosine can suppress T cell activity (2,3) by acting on A_{2A} adenosine receptors (4–9). Recently, it has been proposed that generation of pericellular adenosine by the ecto-5′-nucleotidase (CD73) expressed on regulatory $CD4^+Fox3^+$ T lymphocytes is a

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contributing factor to their immunosuppressive properties (10–12). Furthermore, this adenosinergic mechanism of immune suppression was also suggested to play an important role in the ability of tumors to escape from host immunosurveillance (13,14).

In addition to regulatory T lymphocytes, nonlymphoid cells with immunosuppressive properties have been identified and named myeloid-derived suppressor cells (MDSCs). In mice, these cells represent a heterogeneous population of immature myeloid cells with monocytic and granulocytic morphology that are generally characterized as $CD11b^{+}Gr\text{-}1^{+}$ cells. Accumulation of $CD11b^{+}Gr\text{-}1^{+}$ cells has been documented in mice with cancer, and these cells are considered a major contributor to the tumor immuntolerance (15,16). Expansion of MDSC populations is associated not only with tumors, but also with acute and chronic inflammation, traumatic stress, and transplantation (15,17). Importantly, these pathological conditions are known to be associated with an increased release of purine nucleotides from the affected cells, an event that eventually leads to a rise in extracellular adenosine concentrations (18). However, a potential role of adenosine and adenosinergic mechanisms in the expansion of MDSCs and their functions has not been studied.

Among the adenosine receptors, the A_{2B} subtype has the lowest affinity for adenosine. In contrast to other adenosine receptor subtypes, A_{2B} receptors are thought to remain silent under normal physiological conditions when interstitial adenosine levels are low and become active in pathological conditions when local adenosine levels can reach micromolar concentrations (19). The A_{2B} adenosine receptor has recently emerged as an important regulator of immune cell differentiation (20). We have demonstrated that A_{2B} adenosine receptors skew differentiation of dendritic cells from hematopoietic progenitors and monocytes into cells with tolerogenic and pro-angiogenic phenotype (21). Our recent studies in a Lewis lung carcinoma (LLC) isograft model showed that, compared to wild type (WT) controls, A_{2B} receptor knockout (A_{2B}KO) mice exhibited significantly attenuated tumor growth and longer survival times after inoculation with LLC cells (22). In the present study, we used the same tumor model *in vivo* and an established model of MDSC generation *in vitro* (23) to demonstrate that A_{2B} receptors but not other adenosine receptor subtypes promote preferential expansion of granulocytic $CD11b^{+}Gr1^{high}$ MDSCs. Furthermore, our new data suggest that generation of pericellular adenosine by the ecto-5′-nucleotidase (CD73), which is highly expressed on these cells may contribute to their immunosuppressive properties.

MATERIALS AND METHODS

Reagents

Endonorbornan-2-yl-9-methyladenine (N-0861) was a gift from Whitby Research, Inc. (Richmond, VA) and 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine (SCH58261) was a gift from Drs C. Zocchi and E. Ongini (Schering Plough Research Institute, Milan, Italy). 3-isobutyl-8-pyrrolidinoxanthine (IPDX) was synthesized as previously described (24). 3-Ethyl-1-propyl-8-{1-[3-(trifluoromethyl)benzyl]-1*H*-pyrazol-4-yl}-3,7-dihydro-1*H*-purine-2,6-dione (CVT-6883) was provided by CV Therapeutics (Palo Alto, CA). N^6-cyclopentyladenosine (CPA), 5′-N-ethylcarboxamidoadenosine (NECA), 4-((N-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl)-phenyl-propionic acid (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (MRS1191), adenosine-5′-monophosphate (AMP), adenosine 5′-(α, β-methylene) diphosphate (APCP), and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). When used as a solvent, final DMSO concentrations in all assays did not exceed 0.1% and the same DMSO concentrations were used in vehicle controls.

Mice

All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Animal studies were reviewed and approved by the institutional animal care and use committee of Vanderbilt University. Eight- to twelve-week-old age- and sex-matched mice were used. C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). A_1 adenosine receptor knockout (A_1KO) mice were obtained from Dr. Jürgen Schnermann, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD); A_{2A}KO mice were obtained from Dr. Jiang-Fan Chen, Boston University (Boston, MA); A_3KO mice were obtained from Marlene Jacobson, Merck Research Laboratories (West Point, PA); and A_{2B}KO mice were obtained from Deltagen (San Mateo, CA). All of the knockout mice used in these studies were back-crossed to the C57BL/6 genetic background for more than 10 generations.

Bone marrow transplantation and LLC tumor model

For generation of bone marrow chimeric mice, eight-week-old WT recipient mice were maintained on acidified water containing antibiotics for 3 days before, and 14 days after transplantation. Bone marrow single cell suspensions were prepared from WT and A_{2B}KO donor mice as previously described (25). Four-six hours before transplantation, recipient mice received lethal whole body irradiation (9 Gy) using cesium gamma source. Donor bone marrow cells (5×10^6 in 100 μl of sterile PBS) were injected into the retro-orbital venous plexus of the recipient mice. Eight weeks after transplantation, A_{2B} receptor bone marrow-chymeric mice had greater than 90% of the hematopoietic cells replaced as assessed by the expression of A_{2B} receptors in blood cells using RT-PCR. Bone marrow chymeric or normal mice were used as hosts for LLC tumors.

For generation of tumor isografts, Lewis lung carcinoma (LLC) cells (American Type Culture Collection, Manassas, VA; Catalog No. CRL-1642) were propagated in ATCC-formulated Dulbecco's Modified Eagle's Medium (Catalog No. 30-2002) supplemented with 10% FBS and 1X Antibiotic-Antimycotic mixture (Sigma) under humidified atmosphere of air/CO_2 (19:1) at 37°C. LLC cells were dislodged from cell culture plates by repetitive pipetting with sterile PBS, and then pelleted by centrifugation at 300 × g for 10 min. Cells were resuspended in PBS and counted using a hemocytometer. A final concentration was adjusted to 5×10^6 cells/ml, and 100 μl of cell suspension was injected subcutaneously into the right flank using a tuberculin syringe and a 27-gauge needle. Animals were inspected daily; in all events, tumors did not exceed 2 cm in diameter or 10 % of animal weight. Mice were killed on day 14 after LLC cell inoculation. To prepare single cell suspensions, extracted tumors were chopped into small pieces, incubated in DMEM medium with 10% FBS, 1500 U/ml collagenase (Sigma) and 1000 U/ml hyaluronidase (Sigma) for 1 hour at 37° C, and then passed through a cell strainer. Total cell numbers were counted and $CD45^+$ cell populations that represent tumor-infiltrating host immune cells were analyzed by flow cytometry.

Generation of MDSCs from bone marrow hematopoietic progenitors

Bone marrow cells were harvested from the femurs of wild type or adenosine receptor knockout mice. Hematopoietic progenitor cells (Lin^-) were isolated using lineage cell depletion kit and LS columns from Miltenyi Biotec Inc. (Auburn, CA) according to the manufacturer's instructions. Resulting cells were >50% CD117-positive as assayed by flow cytometry. Hematopoietic progenitor cells were cultured on 24-well plates at 5×10^4 cells/mL concentration in RPMI medium containing 10% FBS, 20 mM Hepes, 50 μM 2-mercaptoethanol, 1X antibiotic-antimycotic solution (Sigma, St. Louis, MO) and supplemented with granulocyte-macrophage colony stimulating factor (GM-CSF; 10 ng/mL)

and IL-4 (10 ng/ml; both from R&D systems, Inc., Minneapolis, MN) for 5 days under humidified atmosphere of air/CO_2 (19:1)at 37° C as previously described (23).

Magnetic sorting

Enrichment of $CD11b^+Gr\text{-}1^{high}$ cell subpopulations of MDSCs was carried according to a previously published protocol (26). In brief, after treatment with FcR Blocking reagent (Miltenyi Biotec Inc.), bone marrow-derived MDSCs (10^7 cells/ml) were stained with 5 μl per ml of anti-mouse Ly-6G-biotin antibody (Clone 1A8, Biolegend, San Diego, CA) for 10 min followed by washing and incubation with 20 μl per ml of anti-biotin microbeads (Miltenyi Biotec Inc.) for 15 min at 4°C. The cells were then washed and resuspended in dilution buffer for magnetic cell separation. The labeled cells were passed through MS separation columns that had been equilibrated with dilution buffer. Columns were washed three times with 3 ml of dilution buffer. The retained Ly-6G positive cells were eluted from the column outside the magnetic field by pipetting 5 ml of dilution buffer onto the column. Resulting cell preparations were analyzed for CD11b and Gr-1 cell surface expression by flow cytometry.

Flow cytometry

After treatment with FcR Blocking Reagent, cells (10^6 cells/ml) were incubated with the relevant antibodies for 20 minutes at 4°C. If not stated otherwise, all antibodies were obtained from eBioscience, Inc. (San Diego, CA). Data acquisition was performed on a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and the data were analyzed with WinList 5.0 software. Antigen negativity was defined as having the same fluorescent intensity as the isotype control. FACSAria cell sorter (BD Biosciences) was used to isolate $CD11b^+Gr\text{-}1^{-/low}$, $CD11b^+Gr\text{-}1^{int}$ or $CD11b^+Gr\text{-}1^{high}$ cell subpopulations.

Real-time RT-PCR

Total RNA was isolated from cells with the RNeasy Mini kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed on an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primers for murine arginase 1 (Arg1) were: 5′-GAG GAA AGC TGG TCT GCT G-3′ (forward) and 5′-CAC AAT TTG AAA GGA GCT GTC-3′ (reverse), for inducible nitric oxide synthase (iNOS) were: 5′-GAC AAG CTG CAT GTG ACA TC-3′ (forward) and 5′-CTT GGA CTT GCA AGT GAA ATC-3′ (reverse), and for β-actin were: 5′-AGT GTG ACG TTG ACA TCC GTA-3′ (forward) and 5′-GCC AGA GCA GTA ATC TCC TTC T-3′ (reverse).

Reactive oxygen species (ROS) production

The oxidation-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen-Molecular Probes, Eugene, OR) was used for the measurement of ROS production. Cells (10^6/ml) were incubated in serum-free RPMI medium containing 2 μM CM-H_2DCFDA in the absence or presence of increasing concentrations of PMA at 37°C for 30 min, washed with PBS, and then labeled with anti-CD11b-PE and anti-Gr-1-PE-Cy7 (clone RB6-8C5) antibodies. After incubation for 20 min at 4°C, cells were washed with PBS and analyzed using flow cytometry.

Ecto-5′-nucleotidase assay

Ecto-5′-nucleotidase activity was measured in MDSC subpopulations isolated by cell sorting on FACSAria cell sorter (BD Biosciences). Cells were washed twice in cold phosphate-free buffer and resuspended in 20 mM Hepes, pH 7.4 buffer containing 2 mM $MgCl_2$, 120 mM NaCI, 5 mM KCI, 10 mM glucose and 5 mM tetramisole at a concentration of 10^5 cells/ml. Reaction was started by addition of AMP to a final concentration of 1 mM and carried out at

37°C for 40 min. Reaction was stopped with the addition of trichloroacetic acid to a final concentration of 5% and immediately put on ice. The release of inorganic phosphate (P_i) was measured by the malachite green method as described by Baykov et al (27). The non-enzymatic P_i released from nucleotide into assay medium without cells and P_i released from cells incubated without nucleotide was subtracted from the total P_i released during incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity is expressed as μmol P_i released/min/10^6 cells.

T cell proliferation assay

T cells were isolated from the spleen of naïve C57BL6 mice by using T cell enrichment columns (R&D system, Minneapolis, MN). T cells were seeded in triplicates at a concentration of 10^5 cells/well in U-bottom 96-well plates containing CD3/CD28 Dynabeads (Invitrogen, Carlsbad, CA) and cultured in the absence or presence of AMP together with bone marrow-derived $CD11b^+Gr1^{high}$ cells at concentrations indicated in the Results section. After 72 h of incubation, ^{3}[H]-thymidine was added at 1 μCi per well for an additional 18 hours of incubation followed by cell harvesting and radioactivity count using a liquid scintillation counter.

Statistical Analysis

Data were analyzed using the GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA) and presented as mean ± SEM. Comparisons between several treatment groups were performed using one-way ANOVA followed by appropriate post-tests. Comparisons between two groups were performed using two-tailed unpaired *t* tests. A P value < 0.05 was considered significant.

RESULTS

Stimulation of A_{2B} but not other adenosine receptor subtypes promotes expansion of $CD11b^+Gr\text{-}1^{high}$ cells

Distinct subpopulations of $CD11b^+Gr\text{-}1^+$ cells have been previously described based on their expression of the myeloid differentiation antigen Gr-1. Three subsets of $CD11b^+Gr\text{-}1^+$ cells, i.e. $CD11b^+Gr\text{-}1^{low}$, $CD11b^+Gr\text{-}1^{int}$ and $CD11b^+Gr\text{-}1^{high}$ have been recently characterized morphologically, phenotypically and functionally in several murine tumor models (23,26,28,29). We analyzed $CD45^+$ immune cells in LLC tumors grown in A_{2B}KO and WT mice using antibodies against CD11b and Gr-1. Flow cytometric analysis of tumor single cell suspensions shows that the proportion of tumor-infiltrating $CD45^+$ host immune cells was similar in tumors extracted from A_{2B}KO and WT mice (Figure 1A, B). However, the percentage of $CD11b^+Gr\text{-}1^{high}$ cells was significantly higher in WT compared to A_{2B}KO mice (18.4±1.2 vs. 8.6±3.0%, respectively; P<0.05, n=3), whereas the percentage of $CD11b^+Gr\text{-}1^{-/low}$ cells was significantly lower (55.5±1.7 vs. 63.3+2.2%, respectively; P<0.05, n=3). The decrease in proportion of monocytic $CD11b^+Gr\text{-}1^{-/low}$ cells correlated with lower frequency of cells expressing F4/80, CD11c, and MHC II, cell surface markers characteristic for the differentiated myeloid cells macrophages and dendritic cells (Supplementary Figure). Although the percentage of $CD11b^+Gr\text{-}1^{int}$ cells tended to be higher in WT compared to A_{2B}KO mice, the difference between these subsets (22.6±1.3 vs. 19.4+1.3%, respectively; n=3) did not reach statistical significance (Figure 1C, D). To determine whether the lack of A_{2B} receptors on hematopoietic or non-hematopoietic host cells is primarily responsible for a decrease in populations of $CD11b^+Gr\text{-}1^{high}$ cells in LLC tumors, we generated bone marrow–chimeric mice and analyzed $CD11b^+Gr\text{-}1^+$ subpopulations of tumor-infiltrating $CD45^+$ host immune cells. We found that the percentage of $CD11b^+Gr\text{-}1^{high}$ was higher in chimeric wild-type mice given wild-type bone marrow compared to chimeric wild-type mice given A_{2B}KO bone marrow (18.7±0.3 vs.

11.6±1.5%, respectively; $P<0.01$, n=3) and a similar difference was also observed between $CD11b^+Gr\text{-}1^{int}$ subsets (25.2±0.7 vs. 17.4+0.5%, respectively; $P<0.05$, n=3). In contrast, the percentage of $CD11b^+Gr\text{-}1^{-/low}$ cells was significantly lower in chimeric wild-type mice given wild-type bone marrow compared to chimeric wild-type mice given A_{2B}KO bone marrow (45.6±0.4 vs. 57.3+1.4%, respectively; $P<0.01$, n=3) (Figure 1E, F). Taken together, these *in vivo* data imply that A_{2B} adenosine receptors located on WT hematopoietic cells may promote the expansion of $CD11b^+Gr\text{-}1^{high}$ cells.

To test this hypothesis, we employed a previously established model of MDSC generation from mouse bone marrow hematopoietic progenitors *in vitro* (23). Bone marrow hematopoietic progenitor cells isolated from WT mice were cultured for 5 days with GM-CSF and IL-4 in the absence or presence of adenosine receptor agonists. We stimulated all adenosine receptors with the non-selective adenosine receptor agonist NECA at a concentration of 10 μM. We specifically stimulated A_1 receptors with CPA, A_{2A} receptors with CGS21680 and A_3 with IB-MECA at their selective concentrations (30) of 100 nM, 1 μM and 1 μM, respectively. As seen in Fgure 2A, only the non-selective adenosine receptor agonist NECA, but not the selective A_1, A_{2A} or A_3 agonists promoted the expansion of $CD11b^+Gr\text{-}1^{high}$ cells. Because there was no significant difference between total numbers of MDSCs generated in the absence and presence of NECA (1.45±0.24 and 1.42±0.14 × 10^6 cells, respectively; $P=0.9$, n=8), an increase in the percentage of $CD11b^+Gr\text{-}1^{high}$ cells in the presence of NECA corresponded to an increase in absolute $CD11b^+Gr\text{-}1^{high}$ cell numbers. There was no significant difference between $CD11b^+Gr\text{-}1^{high}$ subsets in MDSC populations generated in the presence of NECA added either at the beginning or up to 72 hours after starting the culture of hematopoietic progenitors with GM-CSF and IL-4. However, addition of NECA at later time points resulted in significant decrease in generated $CD11b^+Gr\text{-}1^{high}$ MDSCs (Figure 2B).

Further pharmacological analysis showed that only selective concentrations of A_{2B} antagonists IPDX (10 μM) and CVT-6883 (100 nM) but not those of A_1, A_{2A} or A_3 antagonists (1 μM N0861, 100 nM SCH58261 or 1 μM MRS1191, respectively) (30) inhibited the NECA-induced expansion of $CD11b^+Gr\text{-}1^{high}$ cell population (Figure 2C). Finally, NECA promoted $CD11b^+Gr\text{-}1^{high}$ cell expansion in cultures of WT bone marrow hematopoietic progenitor cells with an estimated EC_{50} value of 62 nM ($-\log EC_{50}$ = 7.21±0.28; Figure 2D). NECA also potently promoted $CD11b^+Gr\text{-}1^{high}$ cell expansion in cultures of cells isolated from mice deficient in A_1, A_{2A} and A_3 receptors, but not in A_{2B} receptors (Figure 2D). Taken together, these results demonstrate that A_{2B} receptors are responsible for the observed adenosine-dependent expansion of $CD11b^+Gr\text{-}1^{high}$ cells.

Stimulation of adenosine receptors promotes preferential expansion of the granulocytic subpopulation of $CD11b^+Gr\text{-}1^{high}$ cells

$CD11b^+Gr\text{-}1^{low}$, $CD11b^+Gr\text{-}1^{int}$ and $CD11b^+Gr\text{-}1^{high}$ subsets have been previously described in mouse bone marrow-derived MDSCs (31,32). We evaluated how these subpopulations are affected in cells generated in the presence of 1 μM NECA. Although we found no difference in total cell numbers between cells cultured in the absence and presence of NECA, the percentage of $CD11b^+Gr\text{-}1^{-/low}$ cells was significantly decreased from 56.5±2.5 to 42.3±3.6%, whereas the percentage of $CD11b^+Gr\text{-}1^{high}$ cells was significantly increased from 14.0±1.0 to 26.6±2.5% in cells generated in the presence of NECA, compared to control cells. No significant difference in $CD11b^+Gr\text{-}1^{int}$ subsets was found between cells generated in the absence and presence of NECA (Figure 3A, B). Morphological evaluation of these subsets showed that $CD11b^+Gr\text{-}1^{-/low}$ subset was composed by mononuclear cells, $CD11b^+Gr\text{-}1^{int}$ subset presented a heterogeneous pattern comprising cells with monocyte-like and polymorphonuclear-like morphology, and $CD11b^+Gr\text{-}1^{high}$ subset was represented by cells with mainly polymorphonuclear-like

morphology. No substantial morphological difference was found between cells generated in the absence and presence of NECA (Figure 3C).

Because $CD11^+Ly\text{-}6C^{high}Ly\text{-}6G^{low}$ and $CD11^+Ly\text{-}6C^{low}Ly\text{-}6G^{high}$ MDSC subpopulations have been previously shown to closely match $CD11b^+Gr\text{-}1^{int}$ and $CD11b^+Gr\text{-}1^{high}$ subsets, respectively (23,26), we used anti-Ly-6C and anti-Ly-6G antibodies as a complementary approach to differentiate between MDSC subsets. Again, we determined that the percentage of $CD11^+Ly\text{-}6C^{low}Ly\text{-}6G^{high}$ cells was significantly increased, from 12.0±0.6 to 24.3±1.8%, in the cell population generated in the presence of NECA, compared to control cells, whereas no significant difference between $CD11^+Ly\text{-}6C^{high}Ly\text{-}6G^{low}$ subsets was found (Figure 3D, E). Taken together, our results suggest that stimulation of A_{2B} receptors promotes preferential expansion of the granulocytic subpopulation of $CD11b^+Gr\text{-}1^+$ cells.

A_{2B} receptor-mediated expansion of granulocytic $CD11b^+Gr\text{-}1^{high}$ cells does not change their ROS production or Arg1 and iNOS expression

Previous studies suggested that the suppressive activity of MDSCs is associated with the production of ROS and with the expression of Arg1 and iNOS (23,26,28). Therefore, we compared the expression of these genes in subpopulations of $CD11b^+Gr\text{-}1^+$ cells generated in the absence or presence of 1 μM NECA (Figure 4A, B). We found that $CD11b^+Gr\text{-}1^{-/low}$ cells expressed the highest levels of Arg1 and iNOS transcripts, compared to $CD11b^+Gr\text{-}1^{int}$ and $CD11b^+Gr\text{-}1^{high}$ subsets. The only statistical significant difference was an increase in Arg1 expression in $CD11b^+Gr\text{-}1^{-/low}$ cells generated in the presence of NECA compared to control, but not in $CD11b^+Gr\text{-}1^{int}$ or $CD11b^+Gr\text{-}1^{high}$ subsets (Figure 4A). We also observed a 3-fold increase in iNOS expression in $CD11b^+Gr\text{-}1^{high}$ cells generated in the presence of NECA compared to control (Figure 4B). However, this difference did not reach statistical significance (P=0.07, n=4) possibly due to the low expression of iNOS in these subsets, hence, the higher amount of intra-sample variability.

In contrast to their low expression of Arg1 and iNOS, $CD11b^+Gr\text{-}1^{high}$ subsets are characterized by the highest levels of ROS production in response to stimulation with 100 nM PMA (Figure 4C). However, we found no difference in PMA-induced ROS generation between $CD11b^+Gr\text{-}1^{high}$ subsets of cells generated in the absence and presence of NECA (Figure 4D). Thus, we concluded that A_{2B} receptor-mediated expansion of granulocytic $CD11b^+Gr\text{-}1^{high}$ cells does not change their ability to produce ROS or their expression of Arg1 and iNOS.

Granulocytic $CD11b^+Gr\text{-}1^{high}$ cells are characterized by high levels of ecto-5′-nucletidase activity

Recent evidence suggests that ecto-5′-nucleotidase (CD73) is expressed at high levels on the regulatory T lymphocyte subset (Tregs) and plays an important role in their immunosuppressive properties by generating extracellular adenosine, which then suppresses effector T cell responses via A_{2A} adenosine receptors (10–12). This mechanism, however, has not been explored in nonlymphoid suppressor cells. Therefore, we next analyzed the expression of CD73 in $CD11b^+Gr\text{-}1^+$ cell subpopulations using flow cytometry. We found that the $CD11b^+Gr\text{-}1^{high}$ subset had the highest levels of CD73 expression (ΔMFI of 118.5±16.8), followed by $CD11b^+Gr\text{-}1^{int}$ (ΔMFI of 57.9±6.8) and $CD11b^+Gr\text{-}1^{-/low}$ (ΔMFI of 12.4±1.0). Even lower levels of CD73 expression were found on LLC tumor cells (ΔMFI of 3.2±0.2; Figure 5A, B).

The high levels of CD73 expression correlated with high levels of ecto-5′-nucletidase enzymatic activity in granulocytic $CD11b^+Gr\text{-}1^{high}$ cells. No significant differences in ecto-5′-nucletidase activities were found between cells generated in the absence and

presence of NECA (Figure 5C). To determine if adenosine produced as a result of ecto-5′-nucletidase activity could contribute to the expansion of $CD11b^{+}Gr\text{-}1^{high}$ cells, we generated MDSCs in the presence of the ecto-5′-nucletidase substrate AMP. As seen in Figure 5D, 100 μM AMP significantly increased the population of generated $CD11b^{+}Gr\text{-}1^{high}$ MDSCs and this effect was inhibited by the ecto-5′-nucletidase inhibitor APCP (100 μM).

Ecto-5′-nucletidase activity potentiates the immunosuppressive properties of $CD11b^{+}Gr\text{-}1^{+}$ cells

To determine if CD73 highly expressed on granulocytic $CD11b^{+}Gr\text{-}1^{+}$ cells could contribute to their immunosuppressive properties, we initially used a previously published method of enrichment of granulocytic $CD11b^{+}Gr\text{-}1^{high}$ cells by immunomagnetic sorting with anti-Ly-6G antibody (26). These cells were then co-cultured at different proportions with T cells stimulated with anti-CD3–anti-CD28–coupled microbeads in the absence or presence of increasing concentrations of the ecto-5′-nucletidase substrate AMP. In the absence of AMP, inhibition of T cell proliferation by $Ly\text{-}6G^{+}$ cells was observed only at their highest concentration of 12%, but not at 6 or 3%. With increasing concentrations of AMP, however, even 3% $Ly\text{-}6G^{+}$ cells became capable of inhibiting T cell proliferation (Figure 6A).

In complementary set of experiments, granulocytic $CD11b^{+}Gr\text{-}1^{high}$ subsets were isolated directly by flow cytometry sorting from cells generated in the absence or presence of NECA. $CD11b^{+}Gr\text{-}1^{high}$ subsets were co-cultured with T cells in the absence or presence of AMP. Figure 6B shows that AMP facilitated the suppression of T cell proliferation by either $CD11b^{+}Gr\text{-}1^{high}$ subsets isolated from cells generated in the absence or presence of NECA. Taken together, these results suggest that ecto-5′-nucletidase activity of CD73 can potentiate the immunosuppressive properties of $CD11b^{+}Gr\text{-}1^{+}$ cells.

DISCUSSION

Myeloid-derived suppressor cells were initially described as natural suppressor cells without lymphocyte-lineage markers that could suppress lymphocyte response to immunogens and mitogens (33). The population of these cells is expanded in various pathologic conditions, including infections, inflammatory diseases, trauma and neoplastic diseases, presumably with the purpose of limiting T cell responses (15–17). Various factors produced in these conditions, including growth factors and inflammatory cytokines, have been proposed to induce the expansion of MDSCs (reviewed in (15)). Adenosine and purine nucleotides are released into the interstitium under conditions of hypoxia, cell stress or injury (18), and become part of the microenvironment in most if not all the conditions associated with MDSC accumulation. Therefore, we hypothesized that adenosinergic pathways play a role in MDSC accumulation and functions.

In this study, we reproduced a previously described LLC isograft tumor model in $A_{2B}KO$ and WT mice (22) and analyzed the expression of myeloid cell surface markers CD11b and Gr-1 characteristic for MDSCs in the population of tumor-infiltrating $CD45^{+}$ immune cells. We observed significantly lower frequency of $CD11b^{+}Gr\text{-}1^{high}$ cells in LLC tumors growing in $A_{2B}KO$ mice compared to WT control, indicating that A_{2B} adenosine receptors may play a role in accumulation of $CD11b^{+}Gr\text{-}1^{high}$ cells in LLC tumors *in vivo*. Importantly, the proportion of $CD11b^{+}Gr\text{-}1^{high}$ cells was also lower in LLC tumors growing in WT chimeric mice with transplanted $A_{2B}KO$ bone marrow compared to WT mice given WT bone marrow, suggesting that A_{2B} adenosine receptors located on hematopoietic cells may regulate the expansion of $CD11b^{+}Gr\text{-}1^{high}$ cells.

Indeed, our *in vitro* studies demonstrated that A_{2B} receptors promote preferential expansion of granulocytic ($CD11b^{+}Gr\text{-}1^{high}/Ly\text{-}6G^{+}Ly\text{-}6C^{low}$) subpopulations of MDSCs. Using

genetic and pharmacological approaches we determined that the A_{2B} receptor, but not the other adenosine receptor subtypes can promote the expansion of $CD11b^{+}Gr\text{-}1^{high}$ cells generated from bone marrow hematopoietic progenitors *in vitro*. Several lines of evidence support our conclusion. First, only the nonselective adenosine receptor agonist NECA, but not agonists at other adenosine receptor subtypes promoted the expansion of $CD11b^{+}Gr\text{-}1^{high}$ cells. Second, this effect was inhibited by selective A_{2B} antagonists, but not by selective antagonists at other adenosine receptor subtypes. Finally, NECA had no effect in cell cultures derived from A_{2B}KO mice but potently promoted the expansion of $CD11b^{+}Gr\text{-}1^{high}$ cells lacking any other adenosine receptor subtype.

Analysis of MDSC fractions generated in the absence or presence of NECA demonstrated that stimulation of A_{2B} receptors favored accumulation of granulocytic MDSCs at the expense of monocytic MDSCs. In agreement with previous reports (26,28), Gr-1/Ly-6G brightness positively correlated with polymorphonuclear-like morphology, and negatively with monocyte-like morphology. Preferential expansion of granulocytic MDSCs is often observed in various tumor models (23) and our study demonstrated that stimulation of A_{2B} adenosine receptors promoted preferential expansion of MDSCs with granulocytic $CD11b^{+}Gr\text{-}1^{high}/Ly\text{-}6G^{+}Ly\text{-}6C^{low}$ phenotype. Because extracellular levels of adenosine have been shown to increase during tumor growth (34,35), it is possible that this adenosinergic mechanism contribute to the tumor-associated expansion of granulocytic MDSCs.

We found that granulocytic MDSCs generated in the presence of NECA were morphologically similar to those generated in the absence of NECA. Furthermore, we found no difference in their ability to generate ROS, a putative mediator of their immunosupressive properties (23). The suppressive activity of MDSCs has been also associated with the metabolism of L-arginine (36). This amino acid serves as a substrate for two enzymes, iNOS (which generates NO) and arginase 1 (which converts L-arginine to urea and L-ornithine). In agreement with previous reports (26), we found that the expression of these enzymes was negatively correlated with Gr-1 brightness. Moreover, no significant difference in the expression of Arg1 and iNOS genes was seen between granulocytic MDSCs generated in the presence and absence of NECA.

The role of granulocytic MDSCs in the regulation of immune responses has long been a subject of controversy; even though the expansion of granulocytic MDSCs has been documented at many pathological conditions, their suppressive activity is only moderate in conventional *in vitro* assays (23,26,28,29). One possible explanation is that these *in vitro* conditions do not reflect the pathological microenvironment generated by the same disease processes that lead to the expansion of MDSCs, with accumulation of factors that induce their immunosuppressive activity (15). Purine nucleotides including AMP are known to accumulate in the interstitium following cell stress/damage (18) and may constitute such factors. An important novel aspect of our studies, therefore, is the demonstration of the very high levels of CD73 expression in granulocytic MDSCs. We found that the expression of CD73 and ecto-5′-nucletidase enzymatic activities in MDSC subsets are positively correlated with Gr-1 brightness. This finding may help us understand the biological significance of the A_{2B} receptor-dependent expansion of granulocytic MDSCs. The role of CD73 in these conditions becomes very important; our study demonstrated that *in vitro* ability of granulocytic MDSCs to suppress CD3/CD28-induced T cell proliferation is significantly facilitated in the presence of the ecto-5′-nucletidase substrate AMP.

Thus, our study indicated that generation of adenosine by CD73 may be a novel mechanism of immunosuppression by granulocytic MDSCs. In this study we focused specifically on the expression of CD73 given its key role as the pacemaker of adenosine generation from

adenine nucleotides (37). Tumor cells including LLC release high levels of ATP (38). High extracellular ATP concentrations in the hundreds micromolar range were detected in tumor sites *in vivo* (39). Generation of adenosine from ATP depends also on the ecto-nucleoside triphosphate diphosphohydrolase activity of CD39. Myeloid cells are known to express CD39 (40) and we detected the expression of this marker on the surface of MDSCs (data not shown). It would be interesting to determine whether the enzymatic activity of CD39 on MDSCs is sufficient to efficiently generate adenosine from ATP, or they would require partner cells with higher ecto-nucleoside triphosphate diphosphohydrolase activity on their surface.

Immunosuppressive activities of adenosine have been long recognized (2,3), and multiple studies attributed these properties mainly to inhibition of T cell responses via A_{2A} adenosine receptors (4–9). In fact, a similar mechanism involving adenosine generation by CD73 has been initially demonstrated in $CD4^+Foxp3^+$ regulatory T lymphocytes (10–12). Furthermore, recent studies suggested that tumors expressing high levels of CD73 may use this adenosinergic mechanism to induce tumor imuunotolerance via A_{2A} adenosine receptor-mediated suppression of T cell responses (14). However, contribution of cancer cells would be minimal in our model because we found very low levels of CD73 expression on LLC tumor cells compared to those in granulocytic MDSCs. That CD73 expression on host cells is crucial for protection of tumors from host immunosurveillance was recently demonstrated in other tumor models generated in CD73 gene-targeted mice; CD73 ablation in hosts significantly suppressed the growth of MC38 colon cancer, EG7 lymphoma, AT-3 mammary tumors and B16F10 melanoma (13).

Based on the previously described adenosine effects on T cell functions (4–9) and our new data obtained in this study, we propose a model of adenosineric regulation of immune responses by MDSCs (Figure 7). According to this model, generation of adenosine by CD73 expressed at high levels on granulocytic MDSCs will have a dual effect both on their expansion and suppressive activity. Acting on A_{2A} receptors, adenosine will suppress the activity of T cells (4–9), thus contributing to the MDSC properties to limit immune responses. Acting on A_{2B} receptors of myeloid precursors, adenosine will promote the expansion of granulocytic MDSCs, a subset with the highest levels of CD73 expression. This positive feedback mechanism would facilitate further generation of adenosine and enhanced immunosupression, until cell stress/damage in the affected area is ameliorated and the levels of interstitial purine nucleotides return to normal.

Although our current study focused primarily on MDSCs, the role of adenosine in the regulation of differentiation and functions of other myeloid cells, e.g. macrophages and dendritic cells has been described (21,41–45). Our new data contribute to the growing evidence that adenosine serves as an important immunomodulating molecule. The proposed model identifies the A_{2B} receptor as a critical modulator of myeloid cell differentiation and the CD73/adenosine A_2 receptor axis as a potential therapeutic target to overcome immunosupression if necessary, e.g. to enhance efficacy of cancer vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

A_1KO	A_1 adenosine receptor knockout
A_{2A}KO	A_{2A} adenosine receptor knockout
A_{2B}KO	A_{2B} adenosine receptor knockout
A_3KO	A_3 adenosine receptor knockout
Arg1	arginase 1
APCP	adenosine 5′-(α,β-methylene) diphosphate
CPA	N^6-cyclopentyladenosine
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
iNOS	inducible nitric oxide synthase
IPDX	3-isobutyl-8-pyrrolidinoxanthine
LLC	Lewis lung carcinoma
MDSC	myeloid-derived suppressor cell
NECA	5′-N-ethylcarboxamidoadenosine
ROS	reactive oxygen species
WT	wild type

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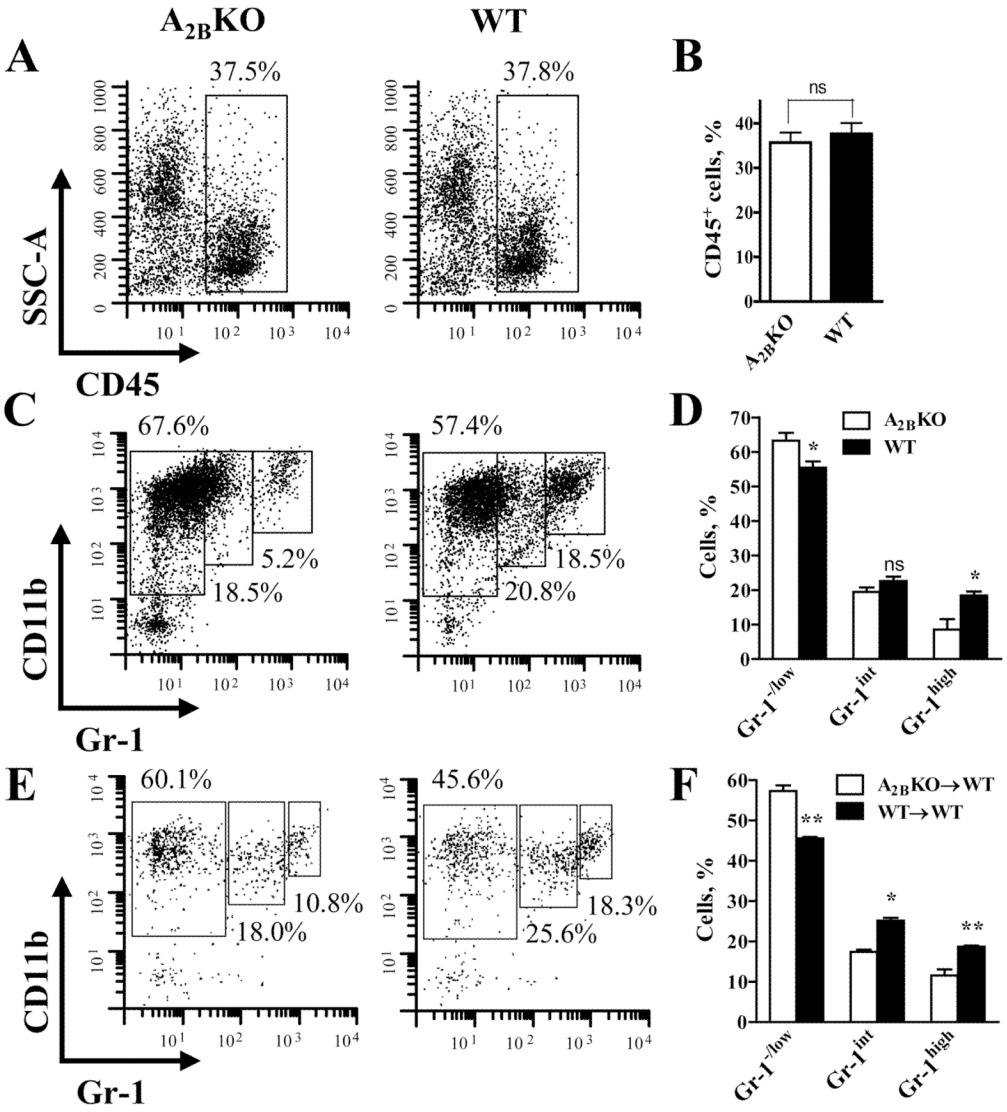

Figure 1. Ablation of A_{2B} adenosine receptors reduces the percentage of $CD11b^{+}Gr\text{-}1^{high}$ cells in the population of tumor-infiltrating host immune cells

(A) Representative cytofluorographic dot plots showing the percentage of immune host cells ($CD45^{+}$) in total tumor cell population. Single cell suspensions were prepared from tumors extracted from A_{2B}KO and WT mice on day 14 after inoculation with LLC cells.

(B) Aggregate data from flow cytometry analysis of $CD45^{+}$ cells obtained from 3 A_{2B}KO and 3 WT animals. Values are expressed as mean±SEM; ns indicates non-significant difference (unpaired two-tail t-tests).

(C) Representative example of flow cytometry analysis of tumor-infiltrating immune host cells (gated for CD45) from A_{2B}KO and WT mice using anti-CD11b and anti-Gr-1 antibodies.

(D) The percentage of $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets in the populations of tumor-infiltrating immune host cells from 3 A_{2B}KO and 3 WT mice measured by flow cytometry. Values are expressed as mean±SEM; the asterisks indicate significant difference (* $P<0.05$, unpaired two-tail t-tests) and ns indicates non-significant difference, compared to corresponding A_{2B}KO values.

(E) Representative example of flow cytometry analysis of tumor-infiltrating immune host cells from WT chimeric mice transplanted with A_{2B}KO bone marrow cells (A_{2B}KO→WT) and with WT bone marrow cells (WT→WT).
(F) The percentage of $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets in the populations of tumor-infiltrating immune host cells from 3 A_{2B}KO→WT and 3 WT→WT bone marrow chimeric mice measured by flow cytometry. Values are expressed as mean ±SEM; the asterisks indicate significant difference (* $P<0.05$, ** $P<0.01$, unpaired two-tail t-tests), compared to corresponding A_{2B}KO→WT values.

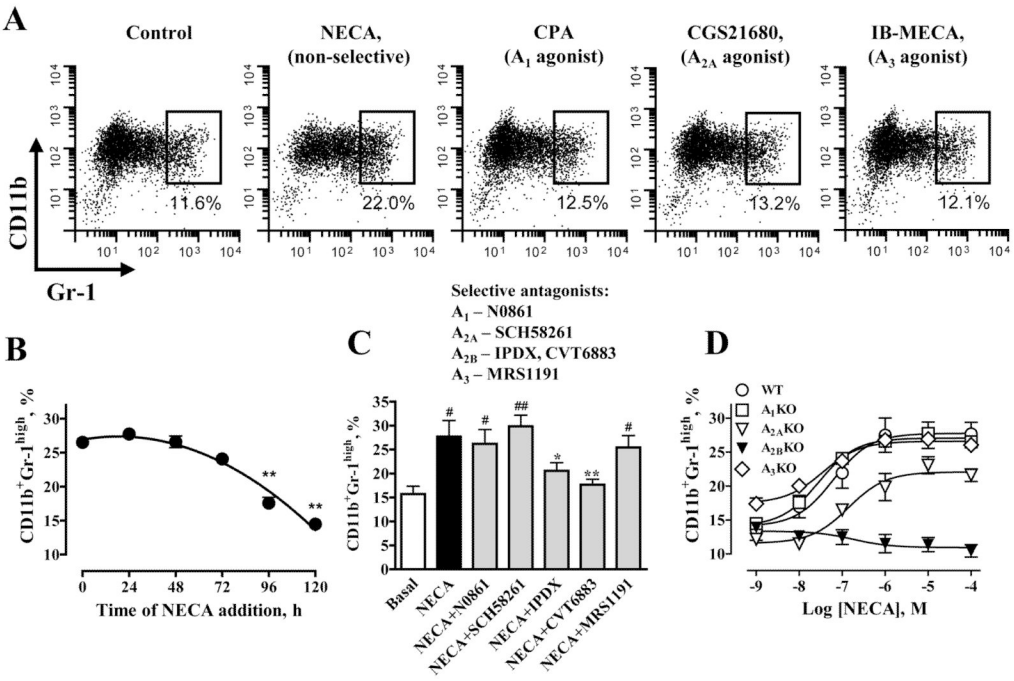

Figure 2. Stimulation of A_{2B} adenosine receptors promotes expansion of $CD11b^{+}Gr\text{-}1^{high}$ cells *in vitro*

(A) Cytofluorographic dot plots of MDSCs generated from mouse bone marrow hematopoietic progenitors in the presence of the non-selective agonist NECA or selective concentrations of receptor-specific agonists. Representative results of three experiments are shown. NECA, but not the selective agonists to A_1, A_{2A}, and A_3 adenosine receptors increased the proportion of $CD11b^{+}Gr\text{-}1^{high}$ cell subpopulation.

(B) Effect of addition of NECA (1 μM) at different time points during generation of MDSCs (starting in the absence of NECA) on the percentage of $CD11b^{+}Gr\text{-}1^{high}$ cells assessed by flow cytometry on day 5. Values are expressed as mean±SEM, n=3. Asterisks indicate significant difference (** P<0.01, one-way ANOVA with Dunnett's postest), compared to the value obtained with NECA added at the beginning of MDSC generation (time 0).

(C) Selective antagonists at the A_{2B} receptor (IPDX and CVT-6883) but not selective antagonists at A_1, A_{2A}, and A_3 adenosine receptors (N0861, SCH58261, and MRS1191, respectively) inhibit NECA-induced expansion of $CD11b^{+}Gr\text{-}1^{high}$ subset. MDSCs were generated from mouse bone marrow hematopoietic progenitors in the absence (Basal) or presence of 1 μM NECA and antagonists at their selective concentrations as indicated in Results. The proportion of $CD11b^{+}Gr\text{-}1^{high}$ cells was measured by flow cytometry. Values are expressed as mean±SEM, n=3. Asterisks indicate significant difference (* P<0.05, ** P<0.01, one-way ANOVA with Dunnett's postest), compared to NECA, and pounds indicate significant difference (# P<0.05, ## P<0.01, one-way ANOVA with Dunnett's postest), compared to basal values.

(D) NECA-induced expansion of $CD11b^{+}Gr\text{-}1^{high}$ subset is not reproduced only in cells from A_{2B}KO animals. MDSCs were generated from mouse bone marrow hematopoietic progenitors obtained from A_1, A_{2A}, A_{2B}, A_3 adenosine receptor KO or WT mice in the absence or presence of increasing concentrations of NECA. Values are expressed as mean ±SEM, n=3.

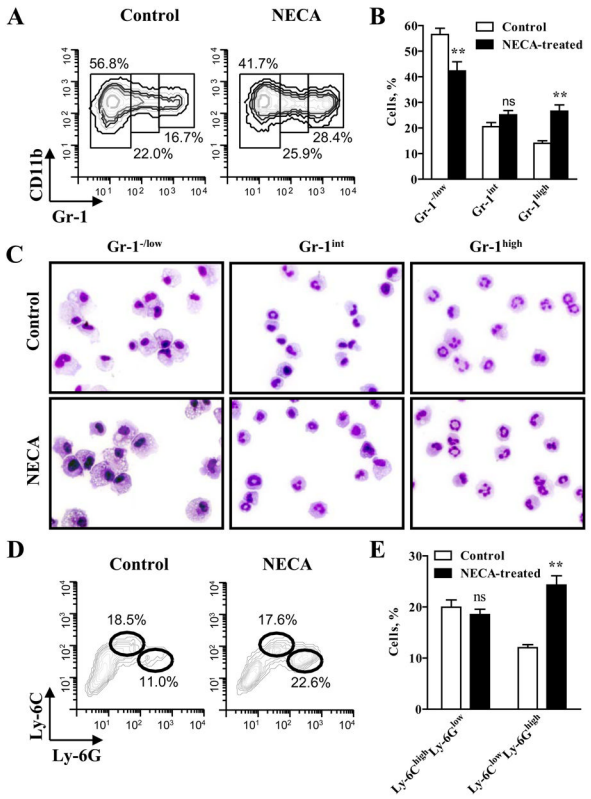

Figure 3. Adenosine receptors promote preferential expansion of granulocytic MDSCs characterized by CD11b^{+}Gr-1^{high}/Ly-6C^{low} Ly-6G^{high} phenotype

MDSCs were generated from mouse bone marrow hematopoietic progenitors in the absence (Control) or presence of 1 μM NECA.

(A) Representative example of flow cytometry analysis using anti-CD11b and anti-Gr-1 antibodies.

(B) The percentage of CD11b^{+}Gr-1$^{-/low}$, CD11b^{+}Gr-1^{int} and CD11b^{+}Gr-1^{high} subsets generated in the absence or presence of NECA measured by flow cytometry. Values are expressed as mean±SEM, n=8. Asterisks indicate significant difference (** P<0.01, unpaired two-tail t-tests) and ns indicates non-significant difference, compared to control.

(C) Staining with Diff-Quik to evaluate subset morphology. CD11b^{+}Gr-1$^{-/low}$, CD11b^{+}Gr-1^{int} and CD11b^{+}Gr-1^{high} subsets were generated in the absence or presence of 1 μM NECA and sorted by flow cytometry.

(D) Representative example of flow cytometry analysis using anti-Ly-6C and anti-Ly-6G antibodies.

(E) The percentage of Ly-6C^{high}Ly-6G^{low} and Ly-6C^{low}Ly-6G^{high} subsets generated in the absence or presence of NECA measured by flow cytometry. Values are expressed as mean ±SEM, n=3. Asterisks indicate significant difference (** P<0.01, unpaired two-tail t-tests) and ns indicates non-significant difference, compared to control.

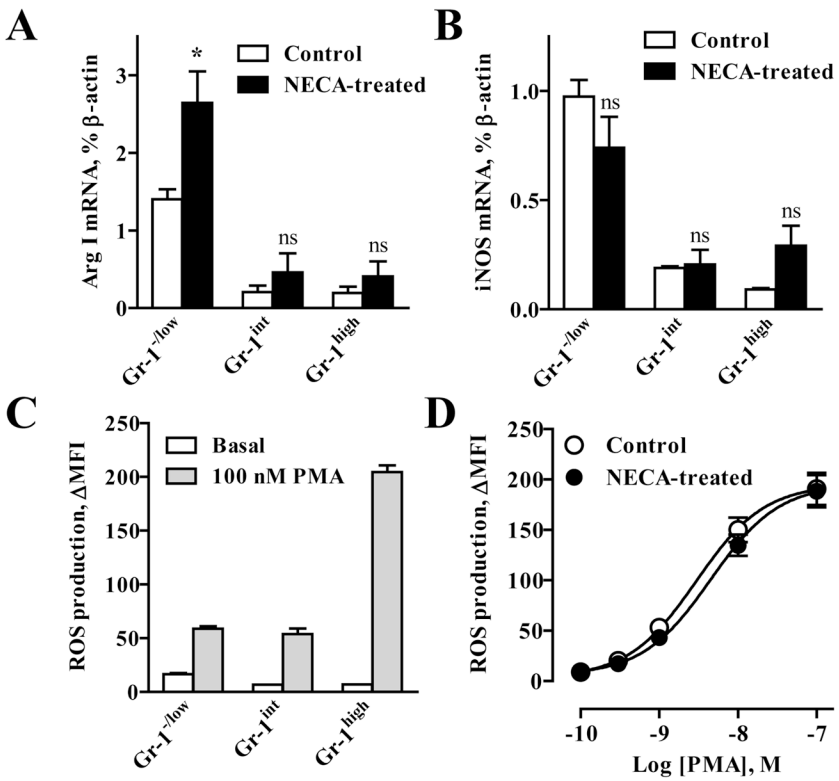

Figure 4. Production of reactive oxygen species in MDSC subsets and the expression of enzymes relevant to their suppressive activity

(A) MDSCs were generated from mouse bone marrow hematopoietic progenitors in the absence (Control) or presence of 1 μM NECA. Real-time RT-PCR analysis of Arg 1 mRNA was performed in $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets isolated by flow cytometry. Values are expressed as mean±SEM, n=3. The asterisk indicates significant difference (* $P<0.05$, unpaired two-tail t-tests) and ns indicates non-significant difference, compared to control.

(B) MDSCs were generated from mouse bone marrow hematopoietic progenitors in the absence (Control) or presence of 1 μM NECA. Real-time RT-PCR analysis of iNOS mRNA was performed in $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets isolated by flow cytometry. Values are expressed as mean±SEM, n=4; ns indicates non-significant difference, compared to control.

(C) ROS production in $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets at rest or after stimulation with 100 nM PMA was evaluated as described in Methods. Values represent a difference between mean fluorescence intensity (ΔMFI) of cells stained with the oxidation-sensitive dye CM-H_2DCFDA and unstained control. Values are expressed as average of two determinations.

(D) Effect of increasing concentrations of PMA on ROS production in $CD11b^{+}Gr\text{-}1^{high}$ subsets of MDSCs generated in the absence (Control) or presence of 1 μM NECA. Values are expressed as mean±SEM, n=3.

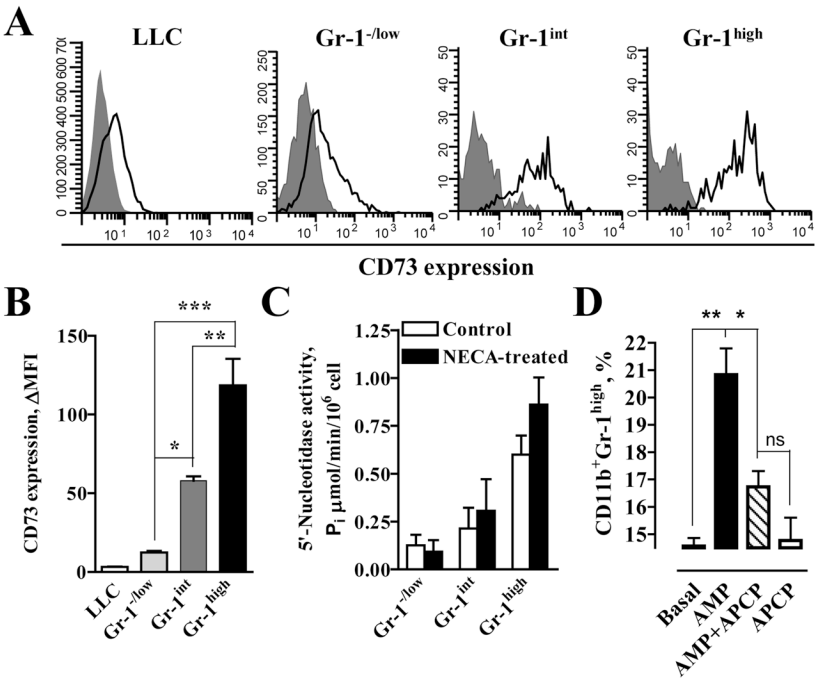

Figure 5. Granulocytic MDSCs express high levels of functional ecto-5′-nucleotidase

(A) Representative flow cytometry histograms of CD73 expression on the surface of LLC tumor cells and $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets of MDSCs generated from mouse bone marrow hematopoietic progenitors.

(B) Graphic representation of data from flow cytometry analysis of CD73 expression on the surface of LLC tumor cells and $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets of MDSCs generated from mouse bone marrow hematopoietic progenitors. Values are expressed as mean±SEM, n=4. Asterisks indicate significant difference (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ one-way ANOVA with Bonferroni's postest) between subsets.

(C) Enzymatic activity of ecto-5′-nucleotidase expressed on $CD11b^{+}Gr\text{-}1^{-/low}$, $CD11b^{+}Gr\text{-}1^{int}$ and $CD11b^{+}Gr\text{-}1^{high}$ subsets of MDSCs generated from mouse bone marrow hematopoietic progenitors in the absence (Control) or presence of 1 μM NECA (NECA-treated). Values are expressed as mean±SEM, n=3.

(D) Effect of ecto-5′-nucleotidase inhibition with APCP on AMP-induced expansion of granulocytic MDSCs. The percentage of $CD11b^{+}Gr\text{-}1^{high}$ cells generated in the absence (Basal, APCP) or presence of 100 μM AMP (AMP, AMP+APCP) and in the absence (AMP, Basal) or presence of 100 μM APCP (AMP+APCP, APCP) was measured by flow cytometry. Values are expressed as mean±SEM, n=3. Asterisks indicate significant differences (* $P<0.05$, ** $P<0.01$, one-way ANOVA with Bonferroni's postest) and ns indicates non-significant difference between values.

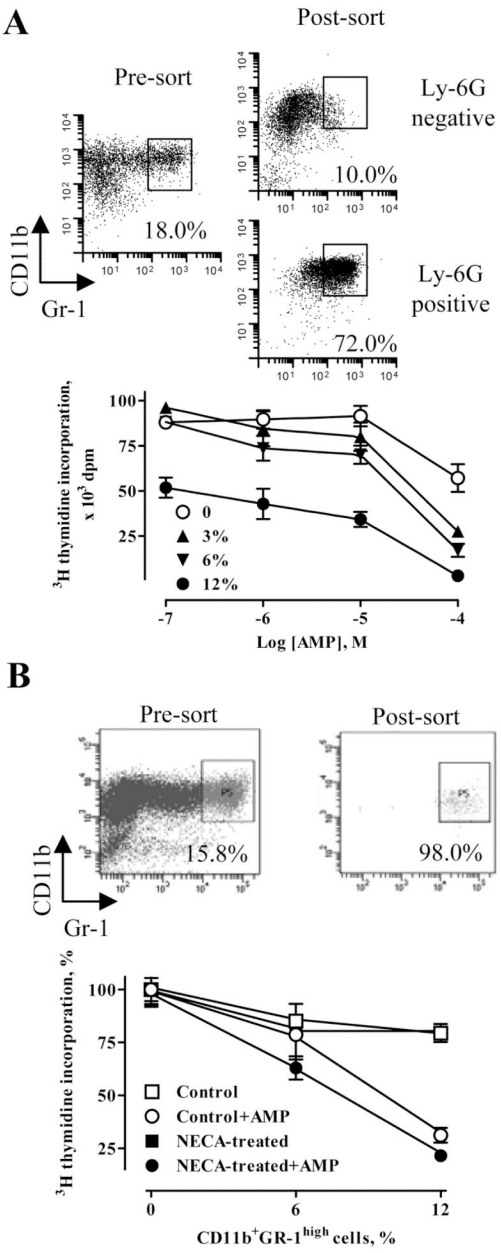

Figure 6. Ecto-5′-nucleotidase activity facilitates the suppression of T cell proliferation by granulocytic MDSCs

(A) MDSCs were generated from mouse bone marrow hematopoietic progenitors and CD11b$^+$Gr-1^{high} cells were enriched (>70%) by positive immunomagnetic selection with anti-Ly-6G antibody. T cells were stimulated with anti-CD3–anti-CD28–coupled microbeads and cultured without (0) or co-cultured together with CD11b$^+$Gr-1^{high} cells added at numbers corresponding to 3, 6 or 12% of T cell numbers in the presence of increasing concentrations of 5′-AMP. Values are expressed as mean±SEM, n=3.

(B) MDSCs were generated from mouse bone marrow hematopoietic progenitors in the absence (Control) or presence of 1 μM NECA (NECA-treated) and CD11b$^+$Gr-1^{high} cells were purified (>95%) by flow cytometry. T cells were stimulated with anti-CD3–anti-CD28–coupled microbeads and cultured without (0) or co-cultured together with

$CD11b^{+}Gr\text{-}1^{high}$ cells added at numbers corresponding to 6 or 12% of T cell numbers in the absence (Control, NECA-treated) or presence of 300 μM 5′-AMP (Control+AMP, NECA-treated+AMP). Data are presented as mean±SEM (n=3) of maximal thymidine incorporation.

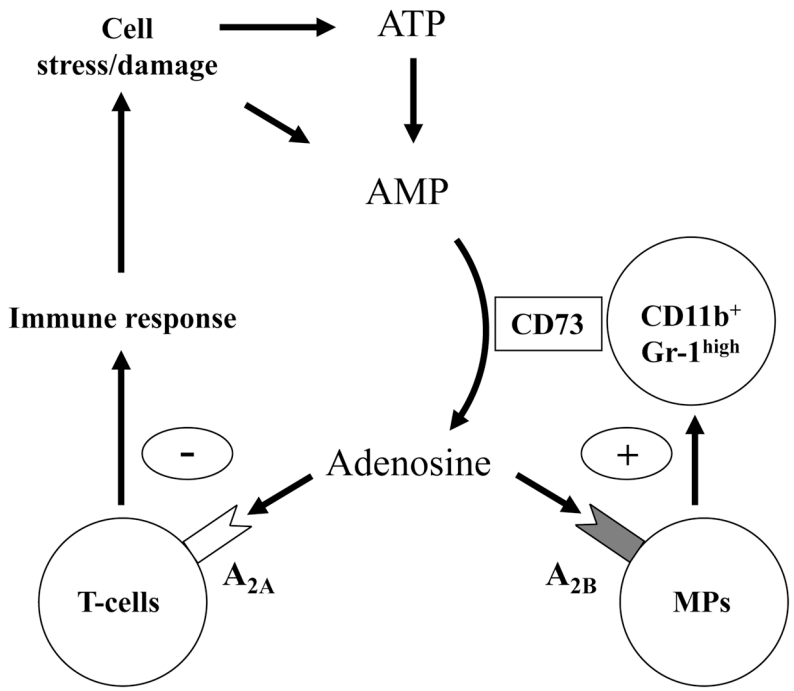

Figure 7. Model of adenosineric regulation of MDSC expansion and function

Generation of adenosine by ecto-5′-nucleotidase (CD73) expressed at high levels on granulocytic MDSCs ($CD11b^{+}Gr\text{-}1^{high}$) may promote their expansion by stimulation of A_{2B} receptors on myeloid progenitors (MPs) and facilitate their suppressive activity by acting on A_{2A} receptors of T cells, thus limiting immune response.