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Adenosinergic Regulation of the Expansion and Immunosuppressive Activity of CD11b+Gr1+ Cells

Sergey Ryzhov,*† Sergey V. Novitskiy,‡ Anna E. Goldstein,*† Asel Biktasova,‡ Michael R. Blackburn,§ Italo Biagioni,†,*‖ Mikhail M. Dikov,‡ and Igor Feoktistov*†‖

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 A2B adenosine receptors on hematopoietic cells play an important role in accumulation of intratumoral CD11b+Gr1high cells in a mouse Lewis lung carcinoma model in vivo and demonstrated that these receptors promote preferential expansion of the granulocytic CD11b+Gr1high subset of MDSCs in vitro. Flow cytometry analysis of MDSCs generated from mouse hematopoietic progenitor cells revealed that the CD11b+Gr1high subset had the highest levels of CD73 (ecto-5'-nucleotidase) expression (Δmean fluorescence intensity [ΔMFI] of 118.5 ± 16.8), followed by CD11b+Gr1low (ΔMFI of 57.9 ± 6.8) and CD11b+Gr1− (ΔMFI of 12.4 ± 1.0) subsets. Even lower levels of CD73 expression were found on Lewis lung carcinoma tumor cells (ΔMFI of 3.2 ± 0.2). The high levels of CD73 expression in granulocytic CD11b+Gr1high cells correlated with high levels of ecto-5'-nucleotidase enzymatic activity. We further demonstrated that the ability of granulocytic MDSCs to suppress CD3/CD28-induced T cell proliferation was significantly facilitated in the presence of the ecto-5'-nucleotidase 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. The Journal of Immunology, 2011, 187: 6120–6129.

A ccumulating 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 A1, A2A, A2B, and A3 (1). It has long been recognized that adenosine can suppress T cell activity (2, 3) by acting on A2A adenosine receptors (4–9). Recently, it was proposed that generation of pericellular adenosine by the ecto-5'-nucleotidase (CD73) expressed on regulatory CD4+Fox3+ T lymphocytes is a contributing factor to their immunosuppressive properties (10–12). Furthermore, this adenosinergic mechanism of immune suppression was 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 monocyctic and granulocytic morphology that are generally characterized as CD11b+Gr-1− cells. Accumulation of CD11b+Gr-1− cells has been documented in mice with cancer, and these cells are considered a major contributor to the tumor immunotolerance (15, 16). Expansion of MDSC populations is associated with tumors, as well as 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 an increase in extracellular adenosine concentrations (18). However, a potential role for adenosine and adenosinergic mechanisms in the expansion of MDSCs and their functions has not been studied.

Among the adenosine receptors, the A2B subtype has the lowest affinity for adenosine. In contrast to other adenosine receptor subtypes, A2B 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 A2B adenosine receptor has recently emerged as an important regulator of immune cell differentiation (20). We demonstrated that A2B adenosine receptors skew differentiation of dendritic cells from hematopoietic progenitors and monocytes into cells with tolerogenic and proangiogenic phenotypes (21). Our recent studies in a Lewis lung carcinoma (LLC) isograft model showed that, compared with wild-type (WT) controls, A2B receptor knockout (A2BKO) mice exhibited significantly attenuated tumor growth and longer survival times after inoculation with LLC cells (22). In the current study, we used the same tumor model in vivo and an established model of MDSC generation in vitro (23) to demonstrate that A2B receptors, but not other adenosine receptor sub-

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Abbreviations used in this article: A1KO, A1 adenosine receptor knockout; A2AKO, A2A adenosine receptor knockout; A2BKO, A2B adenosine receptor knockout; A3KO, A3 adenosine receptor knockout; APCR, adenosine 5'- (α,β-methylene) diphosphate; Arg1, arginase 1; CM-H2DCFDA, 5-(and-6)-chloromethyl-2',7'- dichlorodihydrofluorescein diacetate; nNOS, inducible NO synthase; IPDX, 3-isobutyl-1-methylxanthine; LLC, Lewis lung carcinoma; MDSC, myeloid-derived suppressor cell; MFI, mean fluorescence intensity; NECA, 5'-N-ethylcarboxamidoadenosine; Pi, inorganic phosphate; ROS, reactive oxygen species; WT, wild-type.

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types, promote preferential expansion of granulocytic CD11b<sup>+</sup> Gr1<sup>low</sup> MDSCs. Furthermore, our new data suggested 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-methylenedine (N-8061) was a gift from Whitby Research (Richmond, VA), and 5-amino-7-((phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-c]1,2,4-triazolo-[1,5-c]pyrimidine (SCH58261) was a gift from Drs. C. Zocchi and E. Ongini (Scherer Plough Research Institute, Milan, Italy). 3-Isobutyl-8-pyrrolidinoxanthine (IPDX) was synthesized, as previously described (24). 3-Ethyl-1-proplyl-8-[1-[3-trifluoromethyl]benzyl]-1H-pyrazol-4-yl]-3,7-dihydro-1H-purine-2,6-dione (CVT-6883) was provided by CV Therapeutics (Palo Alto, CA). N<sup>9</sup>-cyclopentyladenosine, 5'-N-ethylcarboxamidoadenosine (NECA), 1H-DMEM, 4-((N-ethyl-5'-carbamoyl-2-yl)-aminoethyl)-phenyl-propionic acid (CGS21680), 8-cyclopentyl-1,3-dipropionylthiine, 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-n-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (MSR1191), AMP, adenosine 5'-([α,β-methylene]) diphosphate (APCP), and 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 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<sub>2</sub> adenosine receptor knockout (A2AKO) mice were obtained from Dr. Jürgen Schnermann (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD); A<sub>1</sub> adenosine receptor knockout (A1KO) mice were obtained from Drs. C. Zocchi and E. Ongini (Schering Plough Research Institute, Milan, Italy). All of the knockout mice used in these studies were backcrossed to their genetic background for >10 generations.

Bone marrow transplantation and LLC tumor model

For generation of bone marrow chimeric mice, 5- to 8-week-old WT recipient mice were maintained on acidic water containing antibiotics for 3 d before and after transplantation. Bone marrow single-cell suspensions were prepared from WT and A<sub>2</sub>AKO donor mice, as previously described (25). Four to six hours before transplantation, recipient mice received lethal whole-body irradiation (9 Gy) using cesium-137. Bone marrow cells (5 × 10<sup>6</sup> in 100 μl sterile PBS) were injected into the retro-orbital venous plexus of the recipient mice. Eight weeks after transplantation, A<sub>2</sub>A receptor bone marrow-chimeric mice had >90% of the hematopoietic cells replaced, as assessed by the expression of A<sub>2</sub>A receptors in blood cells using RT-PCR. Bone marrow chimeric or normal mice were used as hosts for LLC tumors.

For generation of tumor isoagrafts, LLC cells (American Type Culture Collection, Manassas, VA; Catalog No. CRL-1642) were propagated in American Type Culture Collection-formulated DMEM (Catalog No. 30-2044) supplemented with 10% FBS and 1% antibiotic-antimycotic mixture (Sigma) under humidified atmosphere of air/CO<sub>2</sub> (19:1) at 37°C. Cell suspensions were counted using a hemocytometer. A final concentration was adjusted to 5 × 10<sup>6</sup> cells/ml, and 100 μl cell suspension was injected s.c. into the right flank using a tuberculin syringe. LLC tumors were measured every 2-3 d using calipers, and tumor size was calculated by the formula: width<sup>2</sup> × length × 0.5236. All studies were conducted in accordance with the institutional animal care and use committee of Vanderbilt University. All studies were conducted in accordance with the Animal Welfare Act and as adopted and promulgated by the U.S. National Institutes of Health. Animal studies were reviewed and approved by the institutional animal care and use committee of Vanderbilt University.

Generation of MDSCs from bone marrow hematopoietic progenitors

Bone marrow cells were harvested from 10-week-old WT or A<sub>2</sub>A receptor knockout mice. Hematopoietic progenitor cells (Lin<sup>−</sup>) were isolated using lineage cell depletion kit and LS columns from Miltenyi Biotec (Auburn, CA), according to the manufacturer’s instructions. Resulting cells were >95% CD117<sup>+</sup>, as assayed by flow cytometry. Hematopoietic progenitor cells were cultured on 24-well plates at 5 × 10<sup>4</sup> cells/ml concentration in RPMI 1640 medium containing 10% FBS, 20 mM HEPES, 50 μM 2-ME, 1× Antibiotic-Antimycotic solution (Sigma) and supplemented with GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) from R&D Systems, Minneapolis, MN) for 5 d under humidified atmosphere of air/CO<sub>2</sub> (19:1) at 37°C, as previously described (23).

Magnetic sorting

Enrichment of CD11b<sup>+</sup>Gr1<sup>low</sup> cell subpopulations of MDSCs was carried out according to a previously published protocol (26). In brief, after treatment with Fe<sup>3+</sup>Cl<sub>3</sub> Blocking Reagent (Miltenyi Biotec), bone marrow-derived MDSCs (10<sup>7</sup> cells/ml) were stained with 5 μM anti-mouse Ly<sup>6G</sup>-biotin Ab (clone 1A8; BioLegend, San Diego, CA) for 10 min, followed by washing and incubation with 20 μM anti-biotin microbeads (Miltenyi Biotec) 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 dilution buffer. The retained Ly<sup>6G</sup> cells were eluted from the column outside the magnetic field by pipetting 5 ml 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 Fe<sup>3+</sup>Cl<sub>3</sub> Blocking Reagent, cells (10<sup>7</sup> cells/ml) were incubated with the relevant Abs for 20 min at 4°C. If not stated otherwise, all Abs were obtained from eBioscience (San Diego, CA). Data acquisition was performed on a FACSAria cell sorter (BD Biosciences) with WinList 5.0 software. Ag negativity was defined as having the same fluorescent intensity as the isotype control. FACSaria cell sorter (BD Biosciences) was used to isolate CD11b<sup>+</sup>Gr1<sup>−/low</sup>, CD11b<sup>+</sup>Gr1<sup>int</sup>, or CD11b<sup>+</sup>Gr1<sup>high</sup> cell subpopulations.

Real-time RT-PCR

Total RNA was isolated from cells with the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Real-time RT-PCR was performed on an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). The following primers were used: murine arginase 1 (Arg1), 5'-GAG GAA AGC TGG TCT GCT G-3' (forward) and 5'-CAC AAT TGG AAA GTA GCT GTC-3' (reverse); inducible NO synthase (iNOS), 5'-GAC AAG CTT CAT GTG ATC AAC TCT GCT GAA AAC GAT GTA AAT CTC-3' (reverse); and β-actin, 5'-AGT AGG TCC ATG TAC ACC TCT GCC GGA GTA ATT CTA-3' (forward).

Reactive oxygen species production

The oxidation-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM<sub>H<sub>2</sub>DCFDA; Invitrogen-Molecular Probes, Eugene, OR) was used for the measurement of reactive oxygen species (ROS) production. Cells (10<sup>6</sup> cells/ml) were incubated in serum-free RPMI 1640 medium containing 2 μM CM<sub>H<sub>2</sub>DCFDA 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) Abs. 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<sub>2</sub>, 120 mM NaCl, 5 mM KCl, 10 mM glucose, and 5 mM tetramisole at a concentration of 10<sup>6</sup> 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. Reactions were stopped by the addition of trichloroacetic acid to a final concentration of 5% and immediately put on ice. The release of inorganic phosphate (P<sub>i</sub>) was measured by the malachite green method, as described by Baykov et al. (27).
noneszymatic Pi, released from nucleotide into assay medium without cells and Pi released from cells incubated without nucleotide was subtracted from the total Pi released during incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity is expressed as μmol Pi released/min/10^6 cells.

**T cell-proliferation assay**

T cells were isolated from the spleen of naive C57BL/6 mice using T cell enrichtment columns (R&D Systems). 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+Gr1high cells at concentrations indicated in the Results. After 72 h of incubation, [3H]thymidine was added at 1 μCi/well for an additional 18 h of incubation, followed by cell harvesting and radioactivity count using a liquid scintillation counter.

**Statistical analysis**

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

**Results**

*Stimulation of A2B, but not other adenosine receptor subtypes, promotes expansion of CD11b+Gr1high cells*

Distinct subpopulations of CD11b+Gr1+ subpopulations of tumor-infiltrating CD45+ host immune cells were described previously based on their expression of the myeloid differentiation Ag Gr1. Three subsets of CD11b+Gr1+ cells (CD11b+Gr1low, CD11b+Gr1int, and CD11b+Gr1high) have been recently characterized morphologically, phenotypically, and functionally in several murine tumor models (23, 26, 29). We analyzed CD45+ immune cells in LLC tumors grown in A2BKO and WT mice using Abs against CD11b and Gr-1. Flow cytometric analysis of tumor single-cell suspensions showed that the proportion of tumour-infiltrating CD45+ host immune cells was similar in tumors extracted from A2BKO and WT mice (Fig. 1A, 1B). However, the percentage of CD11b+Gr1high cells was significantly higher in WT mice compared with A2BKO mice (18.2 ± 1.2% versus 8.6 ± 3.0%, respectively, p < 0.05, n = 3), whereas the percentage of CD11b+Gr1low cells was significantly lower (55.2 ± 1.7% versus 63.3 ± 2.2%, respectively, p < 0.05, n = 3). The decrease in proportion of monocytic CD11b+Gr1low cells correlated with a lower frequency of cells expressing F4/80, CD11c, and MHC class II, cell surface markers characteristic for the differentiated myeloid cells, macrophages, and dendritic cells (Supplemental Fig. 1). Although the percentage of CD11b+Gr1int cells tended to be higher in WT mice compared with A2BKO mice, the difference between these subsets (22.6 ± 1.3% versus 19.4 ± 1.3%, respectively, n = 3) did not reach statistical significance (Fig. 1C, 1D). To determine whether the lack of A2B receptors on hematopoietic or nonhematopoietic host cells is primarily responsible for a decrease in populations of CD11b+Gr1high cells in LLC tumors, we generated bone marrow-chimeric mice and analyzed CD11b+Gr1+ subpopulations of tumor-infiltrating CD45+ host...
immune cells. We found that the percentage of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells was higher in chimeric WT mice given WT bone marrow compared with chimeric WT mice given A\textsubscript{2B}KO bone marrow (18.7 ± 0.3% versus 11.6 ± 1.5%, respectively, \(p < 0.01, n = 3\)), and a similar difference was also observed between CD11b\textsuperscript{+}Gr-1\textsuperscript{high} subsets (25.2 ± 0.7% versus 17.4 ± 0.5%, respectively, \(p < 0.05, n = 3\)). In contrast, the percentage of CD11b\textsuperscript{+}Gr-1\textsuperscript{low} cells was significantly lower in chimeric WT mice given WT bone marrow compared with chimeric WT mice given A\textsubscript{2B}KO bone marrow (45.6 ± 0.4% versus 57.3 ± 1.4%, respectively, \(p < 0.01, n = 3\)). Taken together, these in vivo data implied that A\textsubscript{2B} adenosine receptors located on WT hematopoietic cells may promote the expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells.

To test this hypothesis, we used 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 d with GM-CSF and IL-4 in the absence or presence of adenosine receptor agonists. We stimulated all adenosine receptors with the nonsel ective adenosine receptor agonist NECA at a concentration of 10 \(\mu\)M. We specifically stimulated A1 receptors with N6-cyclopentyladenosine, A2A receptors with CGS21680, and A3 receptors with IB-MECA at their selective concentrations (30) of 100 nM, 1 \(\mu\)M, and 1 \(\mu\)M, respectively. As seen in Fig. 2A, only the nonsel ective adenosine receptor agonist NECA, and not the selective A1, A2A, or A3 agonists, promoted the expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{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 \(\times 10^6\) cells, respectively, \(p = 0.9, n = 8\)), an increase in the percentage of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells in the presence of NECA corresponded to an increase in absolute CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cell numbers. There was no significant difference between CD11b\textsuperscript{+}Gr-1\textsuperscript{high} subsets in MDSC populations generated in the presence of NECA added either at the beginning or up to 72 h after starting the culture of hematopoietic progenitors with GM-CSF and IL-4. However, addition of NECA at later time points resulted in a significant decrease in generated CD11b\textsuperscript{+}Gr-1\textsuperscript{high} MDSCs (Fig. 2B).

Further pharmacological analysis showed that only selective concentrations of A\textsubscript{2B} antagonists IPDX (10 \(\mu\)M) and CVT-6883 (100 nM), but not those of A1, A2A, or A3 antagonists (1 \(\mu\)M N0861, 100 nM SCH58261, or 1 \(\mu\)M MRS1191, respectively) (30), inhibited the NECA-induced expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cell population (Fig. 2C). Finally, NECA promoted CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cell expansion in cultures of WT bone marrow hematopoietic progenitor cells with an estimated EC50 value of 62 nM (\(-\log EC_{50} = 7.21 ± 0.28\); Fig. 2D). NECA also potently promoted CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cell expansion in cultures of cells isolated from mice deficient in A1, A2A, and A3 receptors but not in A2B receptors (Fig. 2D). Taken together, these results demonstrated that A2B receptors are responsible for the observed adenosine-dependent expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells.

**FIGURE 2.** Stimulation of A\textsubscript{2B} adenosine receptors promotes expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells in vitro. A, Cytofluorographic dot plots of MDSCs generated from mouse bone marrow hematopoietic progenitors in the presence of the nonsel ective agonist NECA or selective concentrations of receptor-specific agonists. Representative results of three experiments are shown. NECA, but not the selective agonists to A1, A2A, or A3 adenosine receptors, increased the proportion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cell subpopulation. B, Effect of addition of NECA (1 \(\mu\)M) at different time points during generation of MDSCs (starting in the absence of NECA) on the percentage of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells assessed by flow cytometry on day 5. Values are expressed as mean ± SEM (\(n = 3\)). **\(p < 0.01\), compared with the values obtained with NECA added at the beginning of MDSC generation (time 0), one-way ANOVA with the Dunnett posttest. C, Selective antagonists at the A\textsubscript{2B} receptor (IPDX and CVT-6883), but not selective antagonists at A1, A2A, and A3 adenosine receptors (N0861, SCH58261, and MRS1191, respectively), inhibit NECA-induced expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} subset. MDSCs were generated from mouse bone marrow hematopoietic progenitors in the absence (Basal) or presence of 1 \(\mu\)M NECA and antagonists at their selective concentrations, as indicated in Results. The proportion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} cells was measured by flow cytometry. Values are expressed as mean ± SEM (\(n = 3\)). *\(p < 0.05\), **\(p < 0.01\), compared with NECA, one-way ANOVA with the Dunnett posttest; \(p < 0.05\), ***\(p < 0.01\), compared with basal values, one-way ANOVA with the Dunnett posttest. D, NECA-induced expansion of CD11b\textsuperscript{+}Gr-1\textsuperscript{high} subset is not reproduced only in cells from A\textsubscript{2B}KO animals. MDSCs were generated from mouse bone marrow hematopoietic progenitors obtained from A1KO, A2A KO, A3B KO, A1KO, or WT mice in the absence or presence of increasing concentrations of NECA. Values are expressed as mean ± SEM (\(n = 5\)).
Stimulation of adenosine receptors promotes preferential expansion of the granulocytic subpopulation of CD11b+ Gr-1high cells

CD11b+Gr-1low, CD11b+Gr-1int, and CD11b+Gr-1high subsets were 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-1low cells was significantly decreased from 56.5 ± 2.5% to 42.3 ± 3.6%, whereas the percentage of CD11b+Gr-1high cells was significantly increased from 14.0 ± 1.0% to 26.6 ± 2.5% in cells generated in the presence of NECA, compared with control cells. No significant difference in CD11b+Gr-1int subsets was found between cells generated in the absence and presence of NECA (Fig. 3A, 3B). Morphological evaluation of these subsets showed that the CD11b+Gr-1low subset was composed of mononuclear cells, the CD11b+Gr-1int subset presented a heterogeneous pattern comprising cells with monocyte-like and polymorphonuclear-like morphology, and the CD11b+Gr-1high 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 (Fig. 3C).

Because CD11+Ly-6ChighLy-6Glow and CD11+Ly-6ClowLy-6Ghigh MDSC subpopulations were previously shown to closely match CD11b+Gr-1int and CD11b+Gr-1high subsets, respectively (23, 26), we used anti–Ly-6C and anti–Ly-6G Abs as a complementary approach to differentiate between MDSC subsets. Again, we determined that the percentage of CD11+Ly-6ChighLy-6Glow 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 with control cells, whereas no significant difference between CD11+Ly-6ChighLy-6Glow subsets was found (Fig. 3D, 3E). Taken together, our results suggested that stimulation of A2B receptors promotes preferential expansion of the granulocytic subpopulation of CD11b+Gr-1+ cells.

A2B receptor-mediated expansion of granulocytic CD11b+Gr-1high 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-1+ cells generated in the absence or presence of 1 μM NECA (Fig. 4A, 4B). We found that CD11b+Gr-1low cells expressed the highest...
Granulocytic CD11b+Gr-1high cells are characterized by high levels of ecto-5'-nucleotidase activity

Recent evidence suggested that ecto-5'-nucleotidase (CD73) is expressed at high levels on the regulatory T lymphocyte subset and plays an important role in their immunosuppressive properties by generating extracellular adenosine, which then suppresses effector T cell responses via A2A adenosine receptors (10–12). However, this mechanism has not been explored in nonlymphoid suppressor cells. Therefore, we next analyzed the expression of CD73 in CD11b+Gr-1+ cell subpopulations using flow cytometry. We found that the CD11b+Gr-1high subset had the highest levels of CD73 expression (Δmean fluorescence intensity [MFI] of 118.5 ± 16.8), followed by CD11b+Gr-1int (ΔMFI of 57.9 ± 6.8) and CD11b+Gr-1low (ΔMFI of 12.4 ± 1.0) cells. Even lower levels of CD73 expression were found on LLC tumor cells (ΔMFI of 3.2 ± 0.2; Fig. 5A, 5B).

The high levels of CD73 expression correlated with high levels of ecto-5'-nucleotidase enzymatic activity in granulocytic CD11b+Gr-1high cells. No significant differences in ecto-5'-nucleotidase activities were found between cells generated in the absence and presence of NECA (Fig. 5C). To determine whether adenosine produced as a result of ecto-5'-nucleotidase activity could contribute to the expansion of CD11b+Gr-1high cells, we generated MDSCs in the presence of the ecto-5'-nucleotidase substrate AMP. As seen in Fig. 5D, 100 μM AMP significantly increased the population of generated CD11b+Gr-1high MDSCs, and this effect was inhibited by the ecto-5'-nucleotidase inhibitor A6P (100 μM).

**Ecto-5'-nucleotidase activity potentiates the immunosuppressive properties of CD11b+Gr-1+ cells**

To determine whether CD73 highly expressed on granulocytic CD11b+Gr-1+ cells could contribute to their immunosuppressive properties, we initially used a previously published method of enrichment of granulocytic CD11b+Gr-1high cells by immunomagnetic sorting with anti-Ly-6G Ab (26). These cells were then cocultured 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'-nucleotidase substrate AMP. In the absence of AMP, inhibition of T cell proliferation by Ly-6G+ cells was observed only at their highest concentration of 12% and not at 6 or 3%. However, with increasing concentrations of AMP, even 3% of Ly-6G+ cells became capable of inhibiting T cell proliferation (Fig. 6A).

In a complementary set of experiments, granulocytic CD11b+Gr-1high subsets were isolated directly by flow cytometry sorting from cells generated in the absence or presence of NECA. CD11b+Gr-1high subsets were cocultured with T cells in the absence or presence of AMP. Fig. 6B shows that AMP facilitated the suppression of T cell proliferation by either CD11b+Gr-1high subsets isolated from cells generated in the absence or presence of NECA. Taken together, these results suggested that ecto-5'-nucleotidase activity of CD73 can promote the expansion and potentiate the immunosuppressive properties of CD11b+Gr-1+ cells (Fig. 7).

**Discussion**

MDSCs 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 Ref. 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, of 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 A2BKO and WT mice (22) and analyzed the expression of myeloid cell surface markers CD11b and Gr-1 characteristic of MDSCs in the population of tumor-infiltrating CD45+ immune cells. We observed a significantly lower frequency of CD11b+Gr-1high cells in LLC tumors growing in A2BKO mice compared with WT control, indicating that A2B adenosine receptors may play a role in the accumulation of CD11b+Gr-1high cells in LLC tumors in vivo. Importantly, the proportion of CD11b+Gr-1high cells was also lower in LLC tumors growing in WT chimeric mice with transplanted A2BKO bone marrow compared with WT mice given WT bone marrow, suggesting that A2B adenosine receptors located on hematopoietic cells may regulate the expansion of CD11b+Gr-1high cells.

Indeed, our in vitro studies demonstrated that A2B receptors promote preferential expansion of granulocytic (CD11b+Gr-1high/Ly-6G+Ly-6Clow) subpopulations of MDSCs. Using genetic and pharmacological approaches, we determined that the A2B receptor, but not the other adenosine receptor subtypes, can promote the expansion of CD11b+Gr-1high cells generated from bone marrow hematopoietic progenitors in vitro. Several lines of evidence support our conclusion. First, only the nonselective adenosine receptor agonist NECA, and not agonists at other adenosine receptor subtypes, promoted the expansion of CD11b+Gr-1high cells. Second, this effect was inhibited by selective A2B antagonists but not by selective antagonists at other adenosine receptor subtypes. Finally, NECA had no effect in cell cultures derived from A2BKO mice, but it potentely promoted the expansion of CD11b+Gr-1high cells lacking any other adenosine receptor subtype.

Analysis of MDSC fractions generated in the absence or presence of NECA demonstrated that stimulation of A2B receptors favored accumulation of granulocytic MDSCs at the expense of monocytic MDSCs. In agreement with previous reports (26, 28), Gr-1/Ly-6G brightness correlated positively 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 A2B adenosine receptors promoted preferential expansion of MDSCs with granulocytic CD11b+Gr-1high/Ly-6G+Ly-6C+ phenotype. Because extracellular levels of adenosine have been shown to increase during tumor growth (34, 35), it is possible that this adenosinergic mechanism contributes 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 immunosuppressive 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 Arg1 (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; although the expansion of granulocytic MDSCs has been documented in 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. Therefore, an important novel aspect of our studies is the demonstration of the very high levels of CD73 expression in granulocytic MDSCs. We found that the expression of CD73 and ecto-5′-nucleotidase enzymatic activities in MDSC subsets are positively correlated with Gr-1 brightness. This finding may help us to understand the biological significance of the A2B receptor-dependent expansion of granulocytic MDSCs. The role of CD73 in these conditions becomes very important; our study demonstrated that the in vitro ability of granulocytic MDSCs to suppress CD3/CD28-induced T cell proliferation is significantly facilitated in the presence of the ecto-5′-nucleotidase 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 whether 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 A2B adenosine receptors (4–9). In fact, a similar mechanism involving adenosine generation by CD73 was 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 immunotolerance via A3A adenosine receptor-mediated suppression of T cell responses (14). However, the contribution of cancer cells would be minimal in our model because we found very low levels of CD73 expression on LLC tumor cells compared with 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 adenosinergic regulation of immune responses by MDSCs (Fig. 7). According to this model, generation of adenosine by CD73 expressed at high levels on granulocytic MDSCs will have a dual effect on their expansion and suppressive activity. Acting on A2A receptors, adenosine will suppress the activity of T cells (4–9), thus contributing to the MDSC properties to limit immune responses. Acting on A2B 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 immunosuppression 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 A2B receptor as a critical modulator of myeloid cell differentiation and the CD73/adenosine A2 receptor axis as a potential therapeutic target to overcome immunosuppression if necessary (e.g., to enhance efficacy of cancer vaccines).

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Disclosures

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