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*J Immunol* 2011; 187:6120-6129; Prepublished online 28 October 2011; doi: 10.4049/jimmunol.1101225

http://www.jimmunol.org/content/187/11/6120
Adenosinergic Regulation of the Expansion and Immunosuppressive Activity of CD11b+Gr1+ Cells

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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+Gr1+ cells in a mouse Lewis lung carcinoma model in vivo and demonstrated that these receptors promote preferential expansion of the granulocytic CD11b+Gr1+ 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 [MFI] of 118.5 ± 16.8), followed by CD11b+Gr1low (ΔMFI of 57.9 ± 6.8) and CD11b-Gr1− cells (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+Gr1+ 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.

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-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 subtypes, play an important role in regulation of immune cell functions.
types, promote preferential expansion of granulocytic CD11b+ Gr1dim 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

Endonorbomaran-2-yl-9-methyldenine (N-0861) was a gift from Whitby Research (Richmond, VA), and 5-amino-7-(phenethyl)-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 (Scherin Plough Research Institute, Milan, Italy). 3-Isobutyl-8-pyridiloxindoxine (IPOX) was synthesized, as previously described (24). 3-Ethyl-1-propyl-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). N5-cyclonucleosladenosine, 5'-N-ethylcarboxamidoadenosine (NECA), IB-MECA, 4-[(n-ethyl-5'-carbamoyladenosin-2-y1)-aminoethyl]-phenyl-proionic acid (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine, 3-ethyl-5-benzyl-2-methyl-4-phenophenyl-5-n-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (MRS1191), 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 by the institutional Animal 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). A1 adenosine receptor knockout (A1KO) mice were obtained from Dr. Jürgen Scherermann (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD); A2A receptor knockout (A2AKO) mice were obtained from Dr. Jiang-Fan Chen (Boston University, Boston, MA); A3 adenosine receptor knockout (A3KO) mice were obtained from Marlene Jacobson (Merck Research Laboratories, West Point, PA); and A2BKO mice were obtained from Deltagen (San Mateo, CA). All of the knockout mice used in these studies were backcrossed to the C57BL/6 background for >10 generations.

Bone marrow transplantation and LLC tumor model

For generation of bone marrow chimeric mice, 8-week-old WT recipient mice were maintained on acidified water containing antibiotics for 3 d before and after transplantation. Bone marrow single-cell suspensions were prepared from WT and A2BKO donor mice, as previously described (25). Four to six hours before transplantation, recipient mice received lethal whole-body irradiation (9 Gy) using cesium 137, as previously described (25). 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 WT or adenosine receptor knockout mice. Hematopoietic progenitor cells (Lin-) were isolated using lineage cell depletion kit and LS columns from Miltenyi Biotec (Auburn, CA), according to the manufacturer’s instructions. Resulting cells were >0.95% CD11b+ and >0.95% Gr1+ when assayed by flow cytometry. Hematopoietic progenitor cells were cultured on 24-well plates at 5 × 10^4 cells/ml concentration in RPMI 1640 medium containing 10% FBS, 20 μM HEPES, 50 μM 2-ME, 1× Antibiotic-Antimyotic solution (Sigma) and supplemented with GM-CSF (10 ng/ml) and IL-4 (10 ng/ml; both from R&D Systems, Minneapolis, MN) for 5 d under humidified atmosphere of air/CO2 (19:1) at 37°C, as previously described (23).

Magnetic sorting

Enrichment of CD11b+Gr1+high cell subpopulations of MDSCs was carried out according to a previously published protocol (26). In brief, after treatment with FcR Blocking Reagent (Miltenyi Biotec), bone marrow-derived MDSCs (10^6 cells/ml) were stained with 5 μM anti-mouse Ly6G-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 SE columns that had been equilibrated with dilution buffer. Columns were washed three times with 3 ml dilution buffer. The retained Ly6G+ 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 FcR Blocking Reagent, cells (10^6 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 FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ), and the data were analyzed 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+Gr-1+low, CD11b+Gr1+mid, or CD11b+Gr1+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). The following primers were used: murine arginase 1 (Arg1), 5'-GAG GAA ACG TGG TCT GCT G-3' (forward) and 5'-CAC AAT TGT AAA GGA GCT GTC-3' (reverse); inducible NO synthase (iNOS), 5'-GAC AAG CTT CAT GTG AGA TTC-3' (forward) and 5'-CTT CGA GGT CCA AGT AAA ATC-3' (reverse); and β-actin, 5'-AGT AGT AGG TTC AGA TCC GTA-3' (forward) and 5'-GCC AGA AGC GTA ATC TTC TTC CTG-3' (reverse).

Reactive oxygen species production

The oxidation-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen-Molecular Probes, Eugene, OR) was used for the measurement of reactive oxygen species (ROS) production. Cells (10^6/ml) were incubated in serum-free RPMI 1640 medium containing 2 μM CM-H2DCFDA 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 μM HEPES (pH 7.4) buffer containing 2 μM MgCl2, 120 mM NaCl, 5 mM KCl, 10 mM glucose, and 5 mM tetramisole at a concentration of 10^6 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).
nongenomic P, released from nucleotide into assay medium without cells and P, released from cells incubated without nucleotide was subtracted from the total P, released during incubation, giving net values for enzymatic activity. All samples were run in triplicate. Specific activity is expressed as μmol P, released/min/10^6 cells.

T cell-proliferation assay

T cells were isolated from the spleen of naive C57BL/6 mice using T cell enrichment 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+ Gr1+ 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+ cells were described previously on their expression of the myeloid differentiation Ag Gr-1. 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, 28, 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 tumor-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 (18.4 ± 3.0% versus 8.6 ± 3.0%, respectively, p < 0.05, n = 3), whereas the percentage of CD11b+Gr1low cells was significantly lower (55.5 ± 1.7% versus 63.3 ± 2.2%, respectively, p < 0.05, n = 3). The decrease in proportion of mononuclear 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+Gr1high subpopulations of tumor-infiltrating CD45 high
immune cells. We found that the percentage of CD11b\(^+\)Gr-1\(^{high}\) cells was higher in chimeric WT mice given WT bone marrow compared with chimeric WT mice given A\(_{3B}\)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\(^+\)Gr-1\(^{high}\) subsets (25.2 ± 0.7% versus 17.4 ± 0.5%, respectively, \(p < 0.05, n = 3\)). In contrast, the percentage of CD11b\(^+\)Gr-1\(^{-}/low\) cells was significantly lower in chimeric WT mice given WT bone marrow compared with chimeric WT mice given A\(_{2B}\)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\(_{2B}\) adenosine receptors located on WT hematopoietic cells may promote the expansion of CD11b\(^+\)Gr-1\(^{high}\) 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 nonselective adenosine receptor agonist NECA at a concentration of 10 \(\mu\)M. We specifically stimulated A\(_1\) receptors with N\(^6\)-cyclopentyladenosine, A\(_{2A}\) receptors with CGS21680, and A\(_3\) 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 nonselective adenosine receptor agonist NECA, and not the selective A\(_1\), A\(_{2A}\) or A\(_3\) agonists, promoted the expansion of CD11b\(^+\)Gr-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 \(\times 10^6\) cells, respectively, \(p = 0.9, n = 8\)), an increase in the percentage of CD11b\(^+\)Gr-1\(^{high}\) cells in the presence of NECA corresponded to an increase in absolute CD11b\(^+\)Gr-1\(^{high}\) cell numbers. There was no significant difference between CD11b\(^+\)Gr-1\(^{high}\) 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\(^+\)Gr-1\(^{high}\) MDSCs (Fig. 2B).

Further pharmacological analysis showed that only selective concentrations of A\(_{2B}\) antagonists IPDX (10 \(\mu\)M) and CVT-6883 (100 nM), but not those of A\(_1\), A\(_{2A}\), or A\(_3\) antagonists (1 \(\mu\)M N0861, 100 nM SCH58261, or 1 \(\mu\)M MRS1191, respectively) (30), inhibited the NECA-induced expansion of CD11b\(^+\)Gr-1\(^{high}\) cell population (Fig. 2C). Finally, NECA promoted CD11b\(^+\)Gr-1\(^{high}\) cell expansion in cultures of WT bone marrow hematopoietic progenitor cells with an estimated EC\(_{50}\) value of 62 nM (−logEC\(_{50}\) = 7.21 ± 0.28; Fig. 2D). NECA also promoted CD11b\(^+\)Gr-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 (Fig. 2D). Taken together, these results demonstrated that A\(_{2B}\) receptors are responsible for the observed adenosine-dependent expansion of CD11b\(^+\)Gr-1\(^{high}\) cells.

**FIGURE 2.** Stimulation of A\(_{2B}\) adenosine receptors promotes expansion of CD11b\(^+\)Gr-1\(^{high}\) cells in vitro. A, Cytofluorographic dot plots of MDSCs generated from mouse bone marrow hematopoietic progenitors in the presence of the nonselective 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-1\(^{high}\) 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\(^+\)Gr-1\(^{high}\) cells assessed by flow cytometry on day 5. Values are expressed as mean ± SEM (\(n = 3\)). **\(p < 0.01\), compared with the value 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\(_{2B}\) 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-1\(^{high}\) 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\(^+\)Gr-1\(^{high}\) 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; **\(p < 0.01\), compared with NECA and antagonists at their selective concentrations, as indicated in Results. The proportion of CD11b\(^+\)Gr-1\(^{high}\) cells was measured by flow cytometry. Values are expressed as mean ± SEM (\(n = 3\)). **\(p < 0.01\), compared with the value obtained with NECA added at the beginning of MDSC generation (time 0), one-way ANOVA with the Dunnett posttest. D, NECA-induced expansion of CD11b\(^+\)Gr-1\(^{high}\) subset is not reproduced only in cells from A\(_{3B}\)KO animals. MDSCs were generated from mouse bone marrow hematopoietic progenitors obtained from A\(_1\)KO, A\(_{2A}\)KO, A\(_{3B}\)KO, A\(_3\)KO, 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-6ClowLy-6Ghigh 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

**FIGURE 3.** Adenosine receptors promote preferential expansion of granulocytic MDSCs characterized by CD11b+Gr-1high/Ly-6ChighLy-6Glow 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 Abs. B, The percentage of CD11b+Gr-1low, CD11b+Gr-1int, and CD11b+Gr-1high subsets generated in the absence or presence of NECA measured by flow cytometry. Values are expressed as mean ± SEM (n = 8). **p < 0.01, compared with control, unpaired two-tailed t tests. ns, nonsignificant difference. C, Staining with Diff-Quik to evaluate subset morphology. CD11b+Gr-1low, CD11b+Gr-1int, and CD11b+Gr-1high 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 Abs. E, The percentage of Ly-6ChighLy-6Glow and Ly-6ClowLy-6Ghigh subsets generated in the absence or presence of NECA measured by flow cytometry. Values are expressed as mean ± SEM (n = 3). **p < 0.01, compared with control, unpaired two-tailed t tests. ns, nonsignificant difference.
levels of Arg1 and iNOS transcripts compared with CD11b\(^+\)Gr-1\(^{\text{int}}\) and CD11b\(^+\)Gr-1\(^{\text{high}}\) subsets. The only statistically significant difference was an increase in Arg1 expression in CD11b\(^+\)Gr-1\(^{\text{high}}\) cells generated in the presence of NECA compared with control; none was noted in CD11b\(^+\)Gr-1\(^{\text{int}}\) or CD11b\(^+\)Gr-1\(^{\text{low}}\) subsets (Fig. 4A). We also observed a 3-fold increase in iNOS expression in CD11b\(^+\)Gr-1\(^{\text{high}}\) cells generated in the presence of NECA compared with control (Fig. 4B). However, this difference did not reach statistical significance \((p = 0.07, n = 4)\), possibly as a result of the low expression of iNOS in these subsets and hence, the greater intrasample variability.

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

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-1\(^{\text{high}}\) subset had the highest levels of CD73 expression \((\Delta \text{MFI of } 57.9 \pm 6.8)\), followed by CD11b\(^+\)Gr-1\(^{\text{int}}\) \((\Delta \text{MFI of } 12.4 \pm 1.0)\) cells. Even lower levels of CD73 expression were found on LLC tumor cells \((\Delta \text{MFI of } 3.2 \pm 0.2);\) Fig. 5A, 5B).

The high levels of CD73 expression correlated with high levels of ecto-5'-nucleotidase enzymatic activity in granulocytic CD11b\(^+\)Gr-1\(^{\text{high}}\) 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-1\(^{\text{high}}\) cells, we generated MDSCs in the presence of the ecto-5'-nucleotidase substrate AMP. As seen in Fig. 5D, 100 \(\mu\)M AMP significantly increased the population of generated CD11b\(^+\)Gr-1\(^{\text{high}}\) MDSCs, and this effect was inhibited by the ecto-5'-nucleotidase inhibitor APCR (100 \(\mu\)M).

**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-1\(^{\text{high}}\) 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-1\(^{\text{high}}\) subsets were isolated directly by flow cytometry sorting from cells generated in the absence or presence of NECA \((\Delta \text{MFI of } 16.8\pm 5.9)\), followed by CD11b\(^+\)Gr-1\(^{\text{low}}\) \((\Delta \text{MFI of } 57.9 \pm 16.8)\), and CD11b\(^+\)Gr-1\(^{\text{low}}\) \((\Delta \text{MFI of } 12.4 \pm 1.0)\) cells. Even lower levels of CD73 expression were found on LLC tumor cells \((\Delta \text{MFI of } 3.2 \pm 0.2);\) Fig. 5A, 5B).

**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
A nonsignificant difference.

Values are expressed as mean 

compared with WT control, indicating that A 2B adenosine receptors may play a role in the accumulation of CD11b+Gr-1high CD45+ immune cells. We observed a significantly lower frequency of NECA-treated mice, but it potently 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 monocytic-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-6Chigh 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 A2A adenosine receptors (4–9). In fact, a similar mechanism involving adenosine generation by CD73 was initially demonstrated in CD4+Foxp3+ T cells.
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-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 urine 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).

Acknowledgments

We thank Drs. Luiz Belardinelli, Dewan Zeng, and Hongyan Zhong (Gilead Palo Alto, Inc.) for scientific discussion. We also thank Dr. Jürgen Schnermann for providing A1KO mice, Dr. Jiang-Fan Chen for providing A2AKO mice, and Dr. Marlene Jacobson for providing A3KO mice.

Disclosures

I.B. and I.F. are inventors named on a patent licensed to Gilead Palo Alto, Inc. The authors have been recipients of research funding from Gilead Palo Alto, Inc.

References

Supplementary Figure. Ablation of A2B adenosine receptors promotes expansion of differentiated myeloid cells in the population of tumor-infiltrating host immune cells.

(A) Representative cytofluorographic dot plots showing the percentage of cells expressing the macrophage marker 4/80 in CD11b$^+$Gr-1$^-$low subset of tumor-infiltrating immune host cells. Single cell suspensions were prepared from tumors extracted from A2BKO and WT mice on day 14 after inoculation with LLC cells.

(B) Aggregate data from flow cytometry analysis of CD11b$^+$/4/80$^+$ cells obtained from 3 A2BKO and 3 WT animals. Values are expressed as mean±SEM. The asterisk indicates significant difference (* p<0.05, unpaired two-tail t-tests).

(C) Representative cytofluorographic dot plots showing the percentage of cells co-expressing CD11c and MHC II in CD11b$^+$Gr-1$^-$low subset of tumor-infiltrating immune host cells in A2BKO and WT mice.

(D) Aggregate data from flow cytometry analysis of CD11C$^+$/MHC II$^+$ cells obtained from 3 A2BKO and 3 WT animals. Values are expressed as mean±SEM. The asterisk indicates significant difference (* p<0.05, unpaired two-tail t-tests).