Adenosine receptors in regulation of dendritic cell differentiation and function

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Abbreviations used: DC, dendritic cell; HPC, hematopoietic progenitor cell; NECA, 5’-N-Ethylcarboxamide adenosine; MLR, mixed lymphocyte reaction; LPS, lipopolysaccharide; VEGF, vascular endothelial growth factor
ABSTRACT

Differentiation of functional dendritic cells (DC) critically depends on the microenvironment. DCs differentiate in hypoxic tumor sites, and inflamed or damaged tissue. Because local concentrations of adenosine reach high physiologically relevant levels in these conditions, we assessed the expression of adenosine receptors and the effect of their activation on differentiation of human monocytes and mouse peritoneal macrophages and hematopoietic progenitor cells (HPCs) into myeloid DCs. Stimulation of adenosine receptors skews DC differentiation toward a distinct cell population characterized by expression of both DC and monocyte/macrophage cell surface markers. Pharmacological analysis and experiments with cells from A2B adenosine receptor knockout mice, identified A2B receptor as the mediator of adenosine effects on DCs. Unlike normal myeloid DCs, adenosine-differentiated DCs have impaired allostimulatory activity and express high levels of angiogenic, pro-inflammatory, immune suppressor and tolerogenic factors, including VEGF, IL-8, IL-6, IL-10, COX-2, TGF-β, and IDO. They promoted tumor growth if injected into tumors implanted in mice. Using adenosine desaminase knockout animals, we demonstrated that DCs with pro-angiogenic phenotype are highly abundant under conditions associated with elevated levels of extracellular adenosine in vivo. Adenosine signaling through A2B receptor is an important factor of aberrant DC differentiation and generation of tolerogenic, angiogenic, and pro-inflammatory cells.
INTRODUCTION

The endogenous adenine nucleotides and adenosine are normally present at low concentrations in the extracellular milieu. However, metabolically stressful conditions, including inflammation and hypoxia characteristic of asthma, solid tumors, and other pathological conditions, result in dramatic increases in extracellular concentrations of adenosine. There are also mechanisms of non-lytic secretion of adenosine during hypoxic conditions.

There is growing evidence that adenosine can actively modulate differentiation and function of myeloid cells. Circulating cells of myeloid lineage, including monocytes and dendritic cell (DC) precursors migrate, to tissues where they differentiate into macrophages or DCs. DCs show impressive interaction with the adjacent microenvironment, which regulates formation of DC subtypes and their functional properties, including expression of cytokines and growth factors. Due to rapid growth, solid tumors routinely experience severe hypoxia and necrosis, which causes adenine nucleotide degradation and adenosine release. Therefore, high levels of extracellular adenosine contribute to the local tumor microenvironment and may greatly influence differentiation of DCs from monocyte/macrophages and DC precursors migrating into tumor tissue. Adenosine acts through four subtypes of adenosine receptors, A₁, A₂A, A₂B, and A₃, which are members of G-protein-coupled family of receptors. A₂A adenosine receptors are generally anti-inflammatory, whereas A₂B and A₃ receptors are implicated in pro-inflammatory action of adenosine. Adenosine receptors are expressed abundantly on monocytes and through these receptors adenosine exerts substantial modulatory effects on monocyte function and further differentiation. A₁ receptors were shown to stimulate formation of giant multinucleated cells from monocytes whereas A₂ receptors inhibited this process. A₂B receptors were implicated in mediating the inhibitory effect of adenosine on macrophage proliferation induced by M-CSF. Exogenous adenosine can prevent monocytes from differentiating into macrophages, leading them to an intermediate differentiation stage between immature DCs and monocytes. Cyclic nucleotides including cAMP, which intracellular level increases in response to stimulation of adenosine A₂ receptors, regulate certain steps of monocyte differentiation and promote...
their differentiation toward a CD1a<sup>low</sup>CD14<sup>+/low</sup>CD209<sup>+</sup> intermediate cell but impair differentiation into functional DCs<sup>12</sup>. Up-regulation of DC-specific ICAM-3-grabbing nonintegrin (CD209) was not affected by cyclic nucleotides<sup>12</sup> indicating that DC development was not blocked at the monocyte stage. The expression of all four adenosine receptor subtypes has been reported in human monocytes and myeloid DCs<sup>9,13-15</sup>. However, the effects of adenosine on differentiation of myeloid DCs from monocytes, macrophages, and hematopoietic progenitor cells (HPCs) and the roles of specific adenosine receptor subtypes involved in this process have not been investigated.

Here we demonstrate that adenosine is an important factor affecting differentiation of myeloid DCs. Signaling through A<sub>2B</sub> adenosine receptors diverts human blood monocytes, mouse bone marrow HPCs and peritoneal monocyte/macrophages from normal differentiation into myeloid DCs toward the formation of a distinct DC population that produces high levels of angiogenic factors and Th2 type cytokines, phenotype associated with promotion of angiogenesis, tumor growth, immune suppression and tolerance. We also demonstrate that these cells appear <i>in vivo</i> under conditions associated with elevated levels of extracellular adenosine and that they can enhance vascularization and growth of tumor. Generation of these adenosine-differentiated DCs would have an adverse effect in cancer, asthma, and inflammatory diseases through a process that could be prevented by A<sub>2B</sub> antagonists.
MATERIALS AND METHODS

Mice

Male 6- to 8-weeks-old C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN). Homozygous $A_{2B}$ adenosine receptor knockout mice were obtained from Deltagen, Inc. (San Mateo, CA). These mice were back-crossed to C57BL/6 genetic background for more than 10 generations. Adenosine desaminase (ADA) knockout mice were described previously. Homozygous $ADA^{-/-}$ knockout newborn mice received polyethylene glycol–modified ADA therapy after birth, until 6 weeks of age. Therapy was then discontinued and mice were left untreated for additional 15 days to allow the signs of respiratory distress to develop. Control mice were wild-type littermates. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals 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 the Vanderbilt University.

Reagents

Adenosine receptor antagonists were from the following sources: $A_1$ receptor antagonists $N^\text{6}$-endoonorboran-2-yl-9-methyladenine (N-0861) was a gift from Whitby Research, Inc. (Richmond, VA); $A_{2A}$ receptor antagonist 5-amino-7-(phenylethyl)-2-(1-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) was a generous gift from Drs. C. Zocchi and E. Ongini (Schering Plough Research Institute, Milan, Italy), $A_{2B}$ receptor antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX) was synthesized as previously described; $A_{2B}$ receptor antagonist CVT-6883 was provided by CV Therapeutics Inc. (Palo Alto, CA). $A_3$ receptor antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191) was purchased from Sigma-RBI (Natick, MS). Inhibitor of adenosine deaminase erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), lipopolysaccharide (LPS, E. coli 055:B5), and dimethyl sulfoxide (DMSO) were purchased from Sigma.
(St. Louis, MO). Final concentrations of DMSO did not exceed 0.1% and the same concentrations were used in controls.

**Cell Purification**

Monocytes were purified from peripheral blood mononuclear cells from consented healthy donors. Mononuclear cells were isolated from blood on Ficoll-Hypaque (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) gradient. CD14⁺ monocytes were then immunomagnetically purified using CD14 monoclonal antibody-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Mouse HPCs were isolated from bone marrow of 6 to 8-weeks old mice using immunomagnetic negative selection with biotinylated antibodies against a panel of lineage antigens CD5, CD45R (B220), CD11b, Gr-1, 7-4, and Ter-119 and anti-biotin microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. The purity of CD14⁺ or Lin⁻ cells exceeded 95% by flow cytometric analysis. Mouse peritoneal macrophages were elicited by injecting 2.5 ml of 3% thioglycolate into the peritoneal cavities of mice. Four days later, peritoneal exudate cells were harvested by lavage, washed and resuspended in culture medium (RPMI 1640 medium with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum). Cells were seeded in 24-well culture plates at 10⁶ cells/well in a final volume of 1 ml and cultured at 37 °C for 90 min to allow macrophages to adhere. Non-adherent cells were removed by washing with warm Hank’s balanced salt solution and adherent cells were cultured in the above-mentioned medium.

Murine lung DCs were purified as described previously¹⁸. Briefly, mice were euthanized and the pulmonary cavities were opened. The blood circulatory system in the lungs was cleared by perfusion through the pulmonary artery with 3 ml of saline containing 50 U/ml of heparin (Sigma-Aldrich). Lungs were aseptically removed and cut into small pieces in cold RPMI medium. The dissected tissue was then incubated in RPMI medium containing collagenase XI (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic DNase (30 µg/ml; Sigma-Aldrich) for 30 to 45 min at 37°C. After that 10 ml of cold RPMI were added and digested lungs were further disrupted by gently pushing
the tissue through a nylon screen. The single-cell suspension was washed and centrifuged at 200xg.

To lyse red blood cells, the cell pellet was incubated for 5 min at room temperature with 5 ml of ACK Lysing Buffer (Biosource, Rockville, MD) and washed with PBS containing 0.5% fetal bovine serum (FBS). Cells were then incubated with fluorochrome-conjugated monoclonal antibodies CD11b-PE, CD11c-FITC, and CD45-PerCP or APC, washed and analyzed or sorted using FACSCalibur flow cytometer.

For separation of human adenosine-differentiated DCs into CD1a^−CD14^+ and CD1a^+CD14^− fractions, cells were incubated with fluorochrome-conjugated monoclonal antibodies CD1a-PE and CD14-FITC, washed and flow sorted.

**DC differentiation**

Purified human monocytes, mouse HPCs or peritoneal monocytes/macrophages were cultured in RPMI-1640 medium containing 10% FBS (Invitrogen Corp., Carlsbad, CA) at concentration of 0.5 x 10^6/ml in the presence of 20 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D Systems) in 24-well plate. Monocytes and mouse cells were cultured for 5 and 7 days, respectively; human or mouse cytokines were used as appropriate. For maturation of DCs, 20 ng/ml TNF-α or 1 μg/ml LPS were added for additional 24-48 hrs of culture. Half of the medium was replenished with fresh medium with cytokines every 3 days. Concentrated solutions of adenosine receptor agonists and antagonists were prepared in DMSO (vehicle) and added to cells at indicated concentrations. The maximal concentration of DMSO in culture did not exceed 0.1% and was found to have no significant effect.

**Flow Cytometry**

Human and mouse DCs were characterized by flow cytometry according to the manufacturer's recommendation (Becton-Dickinson, Mountain View, CA). Aliquots of cells (10^5 cells in 100 μl buffer) were incubated with the respective fluorochrome-conjugated monoclonal antibodies (CD1a-PE, CD11b-PE or PerCP, CD11c-FITC or PE, CD14-FITC, CD45-PerCP or APC, CD83-FITC, CD86-
FITC, CD205-FITC, CD209-FITC, MHCII-FITC or PE; all from BD Pharmingen, San Diego, CA) for 30 min on ice, washed and analyzed using a FACSCalibur flow cytometer; data were collected by analyzing 10-50,000 events using Cell Quest software (Becton-Dickinson).

**Mixed lymphocyte reaction (MLR)**

For allogeneic MLR human T cells were purified from peripheral blood mononuclear cells from consented healthy donors by negative selection using immunomagnetic Pan T cell Isolation Kit II with a cocktail of biotinylated antibodies CD14, CD16, CD19, CD56, CD36, CD123, and CD235a (Miltenyi Biotec) according to the manufacturer’s protocol. MLR was set up by culturing 50,000 T cells per well for 4 days with varying concentrations of irradiated DCs in 96-well plates. T cell proliferation was assessed after the addition of 1 μCi/well of [³H]-thymidine (Amersham) for the final 18 hrs. All measurements were performed in triplicate and presented as mean CPM ± SE.

**Real-Time PCR Protocol and Primers**

Total RNA was isolated from cells using RNeasy Mini kit (QIAGEN, Valencia, CA). Real-time RT-PCR was performed on ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primer pairs for adenosine receptors (A₁, A₂A, A₂B, A₃) were obtained from Applied Biosystems Co. (catalog numbers Hs00181231-m1, Hs00169123-m1, Hs00386497-m1, and Hs00181232-m1, respectively). The following pairs of primers were used for quantification of mRNA transcripts: human VEGF, forward primer 5'-GGGCAGAATCATCACGAAGTG-3' and reverse primer 5'-ATTGGATGGCAGTAGCTGCG-3'; IL-8, forward primer 5'-TGCCAAGGAGTGCTAAAG-3' and reverse primer 5'-TCCACAACCCCTCTGCAC-3'; COX-2, forward primer 5'-TGCATTCTTTGCCCAGCACT-3' and reverse primer 5'-AAAGGCGCAGTTTACGCTGT-3'; arginase 2, forward primer 5'-TCGGTACCATTAGTGGCCATG-3' and reverse primer 5'-CTGTCCATGGAGATTTCCTGATG-3'; IL-10, forward primer 5'-GGTGATGCCCCAAGCTGA-3' and reverse primer 5'-TCCCCAGGGAGTTCACA-3'; IL-6, forward primer 5'-
CACAGACAGCCACTCACCTC-3’ and reverse primer 5’-TTTCTGCCAGTGTCACCTTCT-3’; IDO, forward primer 5’-AGTCCGTGAGTTGTCCTTTCAA-3’ and reverse primer 5’-TTTCACAGGGGCTATAAGCT-3’; β-actin, forward primer 5’-CGGCCAGGAGGAGCAGG-3’ and reverse primer 5’-GGCTGGGTGTTGAAGGT-3’. RT-PCR was performed using 1 µg of DNase-treated total RNA under conditions recommended by the manufacturer.

Measurements of Secreted Factors
Mouse VEGF, IL-6, and CXCL1, human IL-6, IL-8, IL-10, VEGF, and IFN-γ secretion was quantified using the DuoSet ELISA Development Systems (R&D Systems) according to the manufacturer’s instructions.

Mouse Tumor Model Experiments
C57BL/6 mice were injected s.c. with 0.5x10⁶ of Lewis lung carcinoma cells (LLC) from AATC. Two weeks later when tumors reached about 7 mm in diameter, mice were randomly divided into 3 groups with 5 animals in each. Immature DCs generated from HPCs in the presence or absence of 100 µM NECA were washed and suspended in PBS. Groups of mice received intratumoral injections of 0.25x10⁶ of normal or adenosine-differentiated DCs in 50 µl of PBS or 50 µM of PBS as a control. The number of injected DCs was equal to the average number of tumor-infiltrating CD11c⁺ cells, which accumulate in 2-week old LLC tumor of the same size in C57BL/6 mice, as we determined in our preliminary experiments. Seven days later mice received i.v. injection of FITC-labeled dextran (M.W. 2,000,000, Sigma) for visualization of blood vessels in tissues¹⁹,²⁰. After 15 min, mice were sacrificed; tumors were extracted, weighted, fixed in 10% formalin, paraffin-embedded and sectioned (5 µm). Fluorescent images were taken from tumor sections and the number of tumor blood vessels identified by dextran-FITC fluorescence was counted using Axiophot fluorescent microscope (Zeiss). Tissue slides were also stained with peroxidase-labeled mouse monoclonal antibodies to the endothelial surface marker CD34 (Santa Cruz Biotechnology) or von Willebrand factor (Dako) and the number of
tumor blood vessels identified by peroxidase substrate staining was counted using Eclipse E600 microscope (Nikon) and NIH image software ImageJ.

**Statistical Analysis**

Statistical analyses were performed using Excel software. Differences between mean values were assessed using Student's *t* test. Statistical significance was set at *p*<0.05.
RESULTS

Adenosine receptors on human monocytes, DCs, and adenosine-differentiated DCs

We have assessed the expression of adenosine receptors on human CD14+ monocytes as well as on immature and mature DCs, and on adenosine-differentiated cells by real-time PCR and have found various levels of all four receptor mRNA transcripts (Fig. 1A). A2A and A2B receptors were most abundant on monocytes and adenosine-differentiated cells. Differentiation of immature DCs was associated with a remarkable increase in A1 expression, whereas A2A expression was downregulated. Upon further maturation of DCs in the presence of LPS the receptor profile changed again and demonstrated dramatic upregulation of A2A receptors. Overall, the adenosine receptor profiles for monocytes and adenosine-differentiated DCs were remarkably similar, and characterized by high and almost equal levels of A2A and A2B receptors and lack or low levels of A1. The major difference was a much higher expression of A3 receptor in adenosine-differentiated cells. We believe that such a profile of intermediate cells reflects their immature state and suggests that in terms of differentiation they are closer to monocytes than to DCs yet different from both of those.

The presence of adenosine leads to formation of phenotypically and functionally distinct DC population

Myeloid DCs can develop from infiltrating tissue monocyte/macrophages and bone marrow-derived DC precursors. We assessed the effect of adenosine on DC differentiation from human blood monocytes and from mouse HPCs and peritoneal macrophages. When cultured in vitro in the presence of GM-CSF and IL-4 human monocytes differentiate into immature DCs. They acquire specific morphological features and markers of immature DCs including CD1a and CD209 but lose marker of monocyte/macrophages (CD14). Subsequent treatment with LPS or TNF-α induces maturation of DCs associated with upregulation of MHC class II and costimulatory molecules and increased T cell stimulatory activity. We differentiated monocytes into DCs in the absence or presence of adenosine and monitored phenotypical changes using CD1a and CD14 cell
surface markers. Monocytes express high levels of CD14 but lack the expression of CD1a, whereas DCs express high level of CD1a and virtually no CD14 (Fig 1B). Adenosine, added to cell culture, affects DC differentiation from monocytes in the presence of GM-CSF and IL-4 and dramatically decreases production of CD1a+ DCs. Differentiation of monocytes in the presence of a stable cell-impermeable adenosine analog NECA has similar effect on DC differentiation as adenosine and is exerted in a concentration-dependent manner (Fig. 1B, C). The population of adenosine-differentiated DCs produced from human monocytes in the presence of NECA resembles classical DCs morphologically but is phenotypically distinct from either monocytes or conventional immature DCs (iDC). These cells are characterized by the expression of CD209, a DC specific marker (Fig. 2A), low or no expression of DC marker CD1a and fail to lose the monocytic marker CD14 (Fig. 1B).

At 30 µM of NECA about equal proportions of CD1a+ and CD1a low CD14+ cells are produced from human monocytes. We assessed the expression of myeloid and DC-specific cell surface markers on CD1a+ and CD1a low CD14+ cells after separating them by flow sorting. CD1a low CD14+ cell fraction of adenosine-differentiated DCs expressed CD40, CD80, and CD209 markers at levels similar to those of CD1a+ cells (Fig. 2A). However, in contrast to normal CD1a+ DCs, they expressed significantly higher levels of MHC class II and CD86 molecules and bear low levels of CD16, a marker present on macrophages. The acquisition of DC specific marker CD209 not present on monocyte/macrophages by CD1a low CD14+ cells indicates that these cells present a phenotypically distinct DC population rather than under differentiated monocytes.

**DCs generated in the presence of adenosine are functionally impaired**

DCs generated from human monocytes in the presence of NECA have impaired ability to induce T cell proliferation, as assessed by allogeneic mixed lymphocyte reaction (MLR, Fig. 2B). Adenosine-differentiated DCs respond to maturation stimuli by LPS or TNF-α by gaining allostimulatory activity but it is much less than in normal DCs. The impaired ability of adenosine-differentiated DCs to induce proliferation of allogeneic T cells coincides with significantly decreased production of IFN-γ by
stimulated T cells (Fig. 2C).

**Time course of DC generation in the presence of adenosine**

Kinetics of DC generation from human monocytes in the presence of NECA follows the same pattern as control cells, but the number of CD1a+ DCs remains lower at all times, and is associated with increased proportion of CD14+ cells (Fig. 2D). Similar data were obtained for DC differentiation from mouse HPCs.

**Adenosine-differentiated DCs are pro-angiogenic**

Skewed differentiation of immune cells in pathological microenvironment may induce them to release a variety of factors that further augment local inflammation and angiogenesis. We assessed the inflammatory and angiogenic properties of adenosine-differentiated DCs and specifically those of CD1a^low^CD14^+^ cells generated from human monocytes. We found high levels of mRNA transcripts and secreted proteins of key angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8 in CD1a^low^CD14^+^ DCs, whereas CD1a^+^CD14^-^ cells barely had any of those (Fig. 3A).

**DCs generated in the presence of adenosine express Th2 type immune response cytokines, pro-inflammatory, immune suppressor and tolerogenic factors**

Adenosine-differentiated DCs, have profoundly altered cytokine expression profile as compared to classical myeloid DCs. It is characterized by a mix of pro-inflammatory and anti-inflammatory cytokines and upregulation of immune suppressor and tolerogenic factors. Using real-time PCR and ELISA we quantified expression of mRNA transcripts and secretion of soluble factors by isolated CD1a^low^CD14^+^ cells generated from human monocytes. These cells secret high levels of IL-6 and IL-10 (Fig. 3B), the cytokines that polarize naïve CD4^+^ T cells towards a Th2 type immune response. In addition to secretion of IL-6 associated with acute phase of inflammation, mature adenosine-differentiated DCs also showed more than 10-fold increase in expression of pro-inflammatory COX-2.
mRNA (Fig. 4A). Significant secretion of IL-10 suggests that adenosine-differentiated DCs are immune suppressive, consistent with their impaired ability to stimulate proliferation and IFN-γ production by allogeneic T cell (Fig. 2B, C), and can contribute to induction of immune tolerance. DCs obtained in the presence of adenosine have also markedly increased expression of TGF-β, another factor promoting generation of regulatory T cells (Fig. 4B).

A subset of DCs expressing indoleamine 2,3-dioxygenase (IDO) has been identified both in human and mice. These cells suppress proliferation of CD4⁺ and CD8⁺ T cells, render the suppressed cells anergic, and are able to induce apoptosis of T cells²³-²⁵. These activities are mediated via increased production of tryptophane catabolites, quinolinic acid and 3-hydroxyanthranilic acid. IDO and tryptophanyl-tRNA-synthetase (TTS) are the enzymes catalyzing degradation and synthesis of tryptophane, respectively. Our data demonstrate that adenosine-differentiated DCs from monocytes have markedly increased expression of IDO (Fig. 4C), whereas TTS expression level does not change (not shown). This indicates that adenosine-differentiated DCs might be an important factor of immune tolerance via production of IDO.

**DCs generated in the presence of adenosine produce tumor growth promoting factor**

DCs generated from human monocytes in the presence of NECA had significantly upregulated levels of arginase 2, an enzyme that catalyses conversion of L-arginine into L-ornithine²⁶,²⁷ and thus provides a substrate for synthesis of polyamines (putrescine, spermidine, spermine), which are essential nutrients utilized for proliferation, neoplastic transformation of mammalian cells, and tumor growth (Fig. 4D). This observation indicates that in addition to generation of angiogenic and pro-inflammatory factors adenosine-differentiated DCs also produce metabolites promoting neoplasia and tumor growth.

**A₂β adenosine receptors mediate effect of adenosine on DC differentiation**

We have several lines of evidence identifying A₂β adenosine receptor as a mediator of adenosine
effects on DC differentiation. 1). The generation of adenosine-differentiated cells by NECA from human monocytes follows a concentration-dependent curve with an estimated EC$_{50}$ value of 12.7 µM suggestive of the involvement of low affinity $A_{2B}$ receptors (Fig. 1C). 2). Receptor-specific agonists are currently available for $A_1$, $A_{2A}$, and $A_3$ adenosine receptors but not for the $A_{2B}$ receptor. None of these selective agonists had any effect on DCs differentiation from human monocytes (Fig. 5A). Lack of effect of $A_1$, $A_{2A}$, and $A_3$ receptor-specific agonists, coupled with a profound effect of the non-selective agonist NECA, indicates the involvement of $A_{2B}$ receptor. 3). The $A_{2B}$ receptor selective antagonists CVT 6883 and IPDX, but not $A_1$, $A_{2A}$, or $A_3$ specific antagonists, were able to efficiently block the effect of NECA on DC differentiation from both human monocytes (Fig. 5B) and mouse peritoneal macrophages (not shown). 4). We assessed the effect of NECA on DC differentiation from HPCs and peritoneal macrophages obtained from wild type and $A_{2B}$ knockout mice. We found that while NECA had minimal effect on DC phenotype, it dramatically influenced the cytokine expression. Mature DCs generated from mouse HPCs or peritoneal macrophages (not shown) in the presence of NECA demonstrated cytokine expression profile similar to that of human DCs produced from monocytes (Fig. 5C). They secrete very high amounts of VEGF and CXCL1, a mouse ortholog of IL-8. They also significantly upregulate secretion of pro-inflammatory IL-6. These effects are lost in cells generated from $A_{2B}$ receptor knockout animals, consistent with the role of this receptor in altered differentiation of DCs.

**Adenosine-differentiated DCs present *in vivo* under conditions associated with elevated levels of extracellular adenosine**

We demonstrated that high physiologically relevant levels of adenosine lead to generation of pro-angiogenic DCs *in vivo* using $ADA^{-/-}$ knockout mice. ADA is a ubiquitous and essential enzyme of purine catabolism that is responsible for hydrolytic deamination of adenosine to inosine and maintaining low concentrations of adenosine in extracellular milieu. Mice deficient in ADA develop pulmonary inflammation and damage in association with adenosine elevations making them a useful
model for assessing the role of adenosine signalling under pathological conditions in lung\textsuperscript{16}.

We characterized CD11b\textsuperscript{low}CD11c\textsuperscript{+}CD45\textsuperscript{+} cells in the lungs of wild type and \textit{ADA}\textsuperscript{-/-} knockout mice. These cells are not lung macrophages, which also express CD11c marker. When flow sorted as indicated in Fig. 6A and stained with Diff-Quick, they demonstrated typical DC morphology with characteristic long projections (Fig. 6B, b and d). These cells express lower levels of CD11b but have high expression of MHCII, CD86, and CD209 markers consistent with the phenotype of DCs (Fig. 6A, C). The total numbers of hematopoietic CD45\textsuperscript{+} cells in the lungs in both types of animals were equal but there were three folds more of CD11b\textsuperscript{low}CD11c\textsuperscript{+} cells in \textit{ADA}\textsuperscript{-/-} knockouts than in wild type mice and they had higher expression of MHCII (Fig. 6C) and were bigger in size (Fig. 6B). We assessed the secretion of VEGF and CXCL1 by these cells without or in the presence of NECA (Fig. 6D). Lung DCs from \textit{ADA}\textsuperscript{-/-} knockout mice had higher basal secretion levels of VEGF and CXCL1 and responded to NECA by a dramatic increase in VEGF production (Fig. 6D). These data directly demonstrate that elevated adenosine affects differentiation of DCs \textit{in vivo} and render them a pro-angiogenic phenotype.

\textbf{Adenosine-differentiated DCs act as angiogenic and tumor growth-promoting factor \textit{in vivo}}

To determine whether our \textit{in vitro} findings are relevant to the regulation of tumor angiogenesis and tumor growth \textit{in vivo}, we conducted mouse tumor model studies with injection of normal or adenosine-differentiated DCs produced from HPCs into subcutaneous tumor. The experiments directly demonstrated that the presence of adenosine-differentiated cells in the tumor could significantly increase its vascularization and promote growth. To determine the tumor microvessel density, we used FITC-dextran method\textsuperscript{19,20} and immunostaining with anti-CD34 and anti-von Willebrand factor antibodies, which allow visualization of both microcapilaries and larger more mature vessels\textsuperscript{28,29}. Figures 7A and 7B-D demonstrate that the tumor weight and the number of blood vessels in tumor tissue sections were significantly increased in the group of mice received adenosine-differentiated immature DCs compared to mice received PBS or normal immature DCs. The fact that there is no difference in tumor growth between PBS and control DC groups indicate that immune reactions have
not fully developed during a period of 7 days in mice received DCs. It is conceivable that the observed differences in tumor growth and vascularization between control DC and NECA DC mice are due to the production of angiogenic and tumor growth-promoting factors by adenosine-differentiated DCs.
DISCUSSION

Recent studies demonstrate a remarkable ability of DC precursors to differentiate into DC subpopulations with various functional features. A set of differentiation/maturation signals determines the properties of resultant DCs. DCs generated under certain conditions may have adverse effect in the pathogenesis of variety of diseases. Limited effectiveness of DC-based cancer vaccines in controlling tumor growth in multiple clinical trials reflects the ability of the pathological microenvironment to significantly affect DC differentiation and function. This underscores the necessity for further identification of pathological mechanisms that alter DC functionality, converting them from a protective to a pathological factor.

In the present study, we have identified an important mechanism of regulation of DC differentiation and properties by adenosine. Our data demonstrate that elevated levels of extracellular adenosine could lead DCs to acquire a specific phenotype associated with pro-angiogenic and pro-inflammatory properties, immune suppression, immune tolerance, and polarization of immune response toward a Th2 type. Both in vitro and in vivo experiments indicate that adenosine induces generation of a phenotypically and functionally distinct subset of DCs rather than blockage of monocyte/macrophage differentiation or modification of their activation. The acquisition of DC-specific cell surface marker (CD209) by adenosine differentiated DCs suggest a role for adenosine in DC differentiation as well as in modulation of their activation.

Adenosine actions are mediated via a P1 family of seven-transmembrane G protein-coupled cell surface receptors\textsuperscript{30-33}. Of the four adenosine receptor subtypes, adenosine binds with high-affinity to the human A\textsubscript{1} and A\textsubscript{3} receptors, with a dissociation constant (K\textsubscript{d}) of 0.3 \( \mu \)M, and to the A\textsubscript{2A} receptors with a K\textsubscript{d} of 0.7 \( \mu \)M. Affinity of A\textsubscript{2B} receptors to adenosine is considerably lower (K\textsubscript{d} of 24 \( \mu \)M)\textsuperscript{34}. Due to this unique feature, A\textsubscript{2B} receptors are likely to remain silent under normal physiological conditions, and become important only during conditions of ischemia or inflammation when interstitial concentrations of adenosine increase. Though all four adenosine receptors are present on human peripheral blood monocytes, immature and mature DCs, results from our experiments convincingly identify the A\textsubscript{2B}
receptor as a mediator of adenosine effects on DCs. Thus, it is conceivable that adenosine becomes a factor affecting DC differentiation in pathological but not in normal tissue. As NECA used in our experiments is cell-impermeable, the observed effects are due to activation of A2B receptor rather than to a metabolic effect of adenosine. We suggest that these effects are mediated by activation of adenylate cyclase and increased levels of intracellular cAMP, as cyclic nucleotides were previously shown to produce similar phenotypical changes during DC differentiation12. However, the exact signaling mechanism is a matter of further investigation. In contrast to other factors of aberrant DC differentiation (IL-10, M-CSF, VEGF, gangliosides), adenosine is a local rather than a systemic factor, which exerts its effect on cells infiltrating inflamed/injured tissue including monocytes, macrophages or DC precursors generated from HPCs. It is worth to mention that in macrophages adenosine strongly up-regulates the expression of VEGF in an A2A receptor-dependent manner35. The expression of A2A receptor increases during DC differentiation. From our experiments, the possibility remains that the mechanism of regulation of VEGF expression in adenosine-differentiated DCs is similar to that in macrophages and involves A2A receptor signaling, whereas A2B receptor regulates differentiation of DCs.

During immunological reactions, pro-inflammatory and anti-inflammatory mechanisms and polarization of T cell responses are regulated in a finely tuned manner. Expression of pro-inflammatory and Th1 immune response-promoting cytokines (TNF-α, IL-1, IL-6) are followed by signals associated with type 2-polarized response (IL-4, IL-10, IL-13). Adenosine seems to induce generation of mixed cytokine signals from DCs. Expression of high levels of pro-inflammatory IL-6 and COX-2 associates with the initiation phase of immune reaction. In combination with VEGF and IL-8 (CXCL1 in mice), this might work to mobilize cells from blood. On the other hand, by upregulating IL-10 and TGF-β adenosine-differentiated DCs would affect Th1-mediated immune reactions, induce generation of regulatory T cells, and polarize immune response toward Th2 type. This conclusion is in agreement with published data demonstrating ability of adenosine to induce Th2 polarizing capacity of monocyte-derived DCs36.
The finding that adenosine can drastically alter DC functionality has enormous implications to our understanding of the pathology of diseases such as cancer and asthma. Tumors are known to produce factors skewing normal differentiation of DCs and rendering them incapable of inducing tumor-specific immune responses. Solid tumors have extended areas of necrosis and tissue damage with high local concentrations of adenosine¹. It is likely, therefore, that tumor-associated monocyte/macrophages, immature DCs, and DC precursors are exposed to high concentrations of adenosine. We propose that, under such conditions, DCs differentiate toward cells which not only are unable to elicit potent Th1 type immune response but, on the contrary, induce immune suppression and tolerance by secreting IL-10 and TGF-β. Another mechanism of immune suppression is likely engaged through the significant upregulation of IDO by adenosine-derived DCs. Abnormal numbers of IDO-expressing cells were seen in tumor-bearing animals and in sentinel lymph nodes of cancer patients and associated with significantly worse prognosis²³,²⁵. To make the picture worse, adenosine-differentiated DCs can significantly contribute to tumor angiogenesis and growth by producing high levels of angiogenic factors and provide nutrients for its growth. Thus, exposure of DCs to adenosine might be an important mechanism of tumor escape from immune system and resistance to immune therapies based on DC activation/mobilization. Our data are the first evidence demonstrating the regulation of IDO expression in DCs by adenosine. They identify activation of A₂B receptor by tumor-derived adenosine as a novel mechanism of tumor-associated increase of IDO-producing DCs and induction of immune tolerance.

Adenosine is also known to be highly elevated in asthmatic lung³⁷-³⁹. There is growing evidence that DCs play a critical role not only in allergic sensitization but also in the maintenance of chronic airway inflammation by promoting Th2 responses within the sites of inflammation⁴⁰. Our data suggest the existence a pathological loop in asthmatic lungs in which lung damage and inflammation cause adenosine release with subsequent generation of DCs secreting pro-inflammatory and pro-angiogenic factors, thus inducing tissue remodeling and further augmenting the disease.
The results of this work demonstrate that $A_{2B}$ adenosine receptor could be a valuable therapeutic target. Given the profound effect that adenosine signaling through $A_{2B}$ receptors has on the course of DC differentiation and generation of pro-angiogenic/pro-inflammatory and immune suppressive DC population, it appears likely that targeting $A_{2B}$ adenosine receptor might have enormous potential for therapeutic interventions in cancer, immune and inflammatory diseases.
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Contribution: S.V.N. and S.R. designed and performed the research, analyzed the data. R.Z. and A.E.G. performed some of the research. Y.H. and O.Y.T. discussed the results, analyzed the data, and provided helpful suggestions. M.R.B, I.B., and D.P.C. discussed the results and provided helpful suggestions. I.F. and M.M.D. designed the experiments, supervised their conduct, and analyzed the data. M.M.D. wrote the paper.

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FIGURE LEGENDS

Figure 1. Expression of adenosine receptors on human monocytes, immature, mature, and adenosine-differentiated DCs, and effect of adenosine on DC differentiation.

(A) CD14+ monocytes were purified from peripheral blood; immature DCs (iDC) were generated from monocytes cultured in the presence of GM-CSF and IL-4 for 5 days without (Control iDC) or with NECA (NECA iDC); for maturation 1 µg/ml LPS was added to immature DCs for 2 additional days (DC). Expression of adenosine receptor mRNA transcripts was assessed by real-time PCR as described in Methods. Average values from four different experiments are shown. Error bars denote SE.

(B) Addition of adenosine or its stable analogue NECA skews DC differentiation from monocytes toward a CD1alaowCD14+ cell population. Monocytes were differentiated into immature DCs as above in the absence (Control iDC) or in the presence of 100 µM adenosine plus 10 µM EHNA (Adenosine iDC) or 100 µM NECA (NECA iDC). Combination of adenosine and the adenosine deaminase inhibitor EHNA was used to decelerate adenosine catabolism in cell culture. Expression of CD1a+ and CD14+ markers was assessed by flow cytometry; results are representative of seven experiments.

(C) Concentration-dependence curves of generation of CD1a+ and CD1alaowCD14+ cells in the presence of NECA. Concentrations of NECA corresponding to 50% of maximal effects are shown. Average values from three different experiments are shown. Error bars denote SE.

Figure 2. Phenotype and functional activity of human adenosine-differentiated DCs and time-course of their differentiation.

(A) Immature DCs were generated from human monocytes in the presence of 30 µM NECA to produce about equal proportions of CD1a+ and CD1alaowCD14+ cells; these cells were separated by flow sorting into CD1a+CD14+ and CD1alaCD14+ cell populations (indicated on a dot plot) and expression of myeloid and DC cell surface markers was assessed by flow cytometry (histograms). Results are representative of three experiments.
(B, C) Adenosine-differentiated DCs have impaired ability to stimulate proliferation and IFN-γ production by allogeneic T cells. Immature (iDC) and mature (DC) DCs were generated from human monocytes with or without 100 µM NECA and used as stimulators in MLR with allogeneic donor’s T cells. MLR was performed as described in Methods. IFN-γ concentrations were measured in MLR supernatants by ELISA. Results are representative of three experiments. In (C), average values from three different experiments are shown. Error bars denote SE.

(D) Time course of generation of CD1a+ and CD1a lowCD14+ DCs from human monocytes in the presence of 100 µM NECA. NECA was added to monocytes at day 0 and the proportions of CD1a+ and CD1a lowCD14+ DCs were assessed daily by flow cytometry. Average values from three different experiments are shown. Error bars denote SE.

Figure 3. Adenosine-differentiated CD1a lowCD14+ DCs express VEGF, IL-8, IL-6, and IL-10, a combination of angiogenic, pro-inflammatory, and immune suppressive factors.

(A and B) Immature DCs were generated from human monocytes in the presence of 30 µM NECA and CD1a+ and CD1a−CD14+ cells were separated by immunomagnetic technique. These cells, as well as freshly purified monocytes as control, were cultured under the same conditions with the addition of 20 ng/ml TNF-α. Cells were harvested after 6 hrs for mRNA quantification; culture supernatants for measurements of secreted cytokines were collected from duplicate samples after 24 hrs. Specific mRNA transcripts were quantified by real-time PCR and concentrations of secreted cytokines were measured by ELISA. Average values from three different experiments are shown. Error bars denote SE.

Figure 4. Adenosine-differentiated DCs have upregulated expression of pro-inflammatory COX-2, tolerogenic TGF-β and IDO, and tumor growth promoting ARG2.
(A – D) Immature or mature DCs were generated from human monocytes without or with 100 µM NECA and total RNA was purified from cells as described in Methods. Specific mRNA transcripts for indicated proteins were quantified by real-time PCR. Average values from three different experiments are shown. Error bars denote SE.

Figure 5. \textit{A2B} adenosine receptor mediates effects of adenosine on DC differentiation.

(A) Selective agonists to A1, A2A, and A3 adenosine receptors do not affect DC differentiation. Immature DCs were generated from human monocytes in the presence of indicated concentrations of receptor-selective agonists or NECA. Agonist concentrations were 10 folds higher than their K\textsubscript{d} for the respective receptor. Results are representative of three experiments.

(B) Selective antagonists to A\textsubscript{2B} receptor but not to A1, A2A or A3 adenosine receptors reverse NECA-induced alteration of DC differentiation. Immature DCs were generated from human monocytes in the presence of 100 µM NECA and selective antagonists at indicated concentrations. Antagonist concentrations were 10-fold higher than their K\textsubscript{i} for the respective receptor. The proportion of CD14\textsuperscript{low}CD14\textsuperscript{+} cells was measured by flow cytometry. Average values from three different experiments are shown. Error bars denote SE.

(C) Effect of NECA on cytokine secretion by DCs from mouse HPCs is not reproduced in cells from \textit{A}\textsubscript{2B}\textsuperscript{−/−} knockout animals. Mature DCs were generated from wild type or \textit{A}\textsubscript{2B}\textsuperscript{−/−} knockout mouse HPCs with or without 100 µM NECA with 20 ng/ml TNF-\textalpha added for 24 hrs for maturation. Culture supernatants from maturation step were collected and secreted cytokines measured by ELISA. Average values from three different experiments are shown. Error bars denote SE.

Figure 6. Pro-angiogenic DCs are generated \textit{in vivo} under conditions associated with elevated levels of extracellular adenosine.
(A) Single-cell suspensions from the lungs of wild type or ADA−/− knockout mice were analyzed by multi-color flow cytometry. CD45+ cells were gated and assessed for expression of CD11b and CD11c markers.

(B) Cells gated and sorted as shown in (A, a-d) were spun on glass slides and stained with Diff-Quick to assess their morphology. Note DCs with projections in (b) and (d) and that cells in (d) are bigger. Bar, 10 µm.

(C) CD11blowCD11c+ cells were gated as shown in (A, b and d) and assessed for expression of MHCII, CD86 or CD209 markers.

(D) These cells were cultured for 18 hrs in RPMI with 10% FBS without or with 100 µM NECA. Culture supernatants were collected and secreted cytokines measured by ELISA. Average values from three different experiments are shown. Error bars denote SE.

**Figure 7. Adenosine-differentiated DCs act as angiogenic and tumor growth-promoting factor in vivo.**

Immature DCs were generated with or without 100 µM NECA from mouse HPCs and injected into subcutaneous LLC tumors in mice. 7 days later tumors were isolated and weighted (A) and tumor vascularization was assessed using FITC-dextran (B) or immunostaining with antibodies to CD34 (C) or von Willebrand factor (D). Images were taken from tumor sections and the number of tumor blood vessels identified by FITC-dextran fluorescence (green) or immunostaining (brown) was counted. Images of representative tumor sections demonstrate higher number of blood vessels in tumors received injection of adenosine-differentiated DCs (E). 5 animals per group. Fifteen fields on two non-adjacent sections were counted for each sample. Bar, 100 µm.
Fig. 1
Fig. 2

A

Isotype shaded

- CD14+ cells
- CD1a+ cells

B

Control iDC
NECA iDC
Control DC
NECA DC

C

IFNγ

D

Control iDC
NECA iDC
Fig. 3
Fig. 4
Fig. 5

A. CPA (100 nM, A1), CGS 21680 (1 μM, A2A), IB-MECA (1 μM, A3), NECA (100 μM, non-selective).

B. CD14⁺ cells, %

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<th>Control DC</th>
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<th>SCH</th>
<th>MRS</th>
<th>IPD</th>
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C. VEGF, CXCL1, IL-6

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Fig. 5
Fig. 6
Fig. 7

A. Tumor weight, g

B. Number of blood vessels

C. Number of blood vessels

D. Number of blood vessels

E. Images showing FITC-dextran, CD34, and vWB staining in PBS, Control DC, and NECA DC groups.
Adenosine receptors in regulation of dendritic cell differentiation and function

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