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Original Research

Metabolic reprogramming by adenosine antagonism and implications in non-small cell lung cancer therapy ☆

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Abstract

Non-small cell lung cancer (NSCLC) is a heterogeneous disease with genetic and environmental parameters that influence cell metabolism. Because of the complex interplay of environmental factors within the tumor microenvironment (TME) and the profound impact of these factors on the metabolic activities of tumor and immune cells, there is an emerging interest to advance the understanding of these diverse metabolic phenotypes in the TME. High levels of adenosine are characteristic of the TME, and adenosine can have a significant impact on both tumor cell growth and the immune response. Consistent with this, we showed in NSCLC data from TCGA that high expression of the A2BR leads to worse outcome and that expression of A2BR may be different for different mutation backgrounds. We then investigated the metabolic reprogramming of tumor cells and immune cells (T and dendritic cells) by adenosine. We used A2AR and A2BR antagonism or agonism as well as receptor knockout animals to explore whether these treatments altered specific immune compartments or conferred specific therapeutic vulnerabilities. Using the seahorse assay, we found that an A2BR antagonist modulates oxidative stress homeostasis in NSCLC cell lines. In addition, we found distinct metabolic roles of A2AR and A2BR receptors in T cell activation and dendritic cell maturation. These data suggest potential mechanisms and therapeutic benefits of A2 receptor antagonist therapy in NSCLC.

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Keywords: Non-small cell lung cancer, Tumor and immune cells, Gene mutation, A2AR/A2BR antagonist, Tumor microenvironment, Metabolism

Abbreviations: NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; TME, tumor microenvironment; A2AR, adenosine A2A receptor; A2BR, adenosine A2B receptor; TCGA, the cancer genome atlas; *EGFR*, epidermal growth factor receptor; *LKB1*, liver kinase B1; *STK11*, serine/threonine kinase 11; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *KEAP1*, Kelch-like ECH-associated protein 1; mTORC1, Mechanistic target of rapamycin complex 1; DC, Dentritic cell; ATP, Adenosine triphosphate; CD73, Ecto-5'-nucleotidase which catalyzes the conversion of AMP (adenosine monophosphate) to adenosine; CD39, NTPDase1, ectoenzyme which catalyzes the conversion of ATP to AMP; CD38, NADase, ectoenzyme which catalyzes the conversion of NAD^+ to ADP-ribose and cyclic ADP-ribose; AMPK, adenosine monophosphate-activated protein kinase; OCR, oxygen consumption rate; SRC, spare respiratory capacity; p-S6, phosphorylation of ribosomal protein S6; PER, proton efflux rate; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA cycle, tricarboxylic acid cycle; ARE, antioxidant response element; ICIs, immune checkpoint inhibitors; TILs, tumor infiltrating lymphocytes.

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Introduction

The composition of the tumor microenvironment (TME) plays a considerable role in tumor growth and progression by interfering with the normal function of immune accessory cells. The tumor cells and immune cells release multiple factors including growth factors and various cytokines that drive metabolic programs with accompanying nutrient competition and feedback regulation that control reactive oxygen species, for example [1]. Recently, it was suggested that high levels of adenosine in the TME induce immunosuppression by modulating the metabolic milieu and metabolism-related pathways [2]. In our study, we investigated how adenosine and A2 adenosine receptors, A2AR and A2BR, are involved in regulation of metabolism of tumor and immune cells and how inhibition of A2R signaling with antagonists could be exploited for the modulation of tumor and immune cell metabolism in order to enhance immune responses and the efficacy of immunotherapy.

A role for extracellular adenosine (eAdo) in cancer biology has been established and activation of the adenosine signaling pathway is currently viewed as a significant barrier to the effectiveness of immune therapies, making it an important potential therapeutic target in cancer [3]. However, there is little understanding of the immuno-metabolic regulation mediated by A2AR and A2BR in the TME of NSCLC harboring driver gene-mutations, like *EGFR, TP53, KRAS*, and *LKB1* (also known as *STK11*). External ATP (eATP) exerts immunostimulatory effects on this multitude of cell types. However, eATP can be dephosphorylated to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by CD39. Subsequently, CD73 converts AMP into adenosine and inorganic phosphate. eAdo generated by this pathway binds to adenosine receptors, A2AR and A2BR, and inhibits T cell function leading to immune escape by the tumor [4]. The balance of eATP and eAdo regulates levels of inflammation in the TME and the behavior and functions of multiple immune cell types.

The impact of A2BR on tumor growth and antitumor immunity have been delineated using global A2BR gene targeted mice where the authors showed that the growth of syngeneic tumors was reduced in these mice [5]. In addition, myeloid-specific conditional deletion of A2BR delayed primary tumor growth and metastasis [6]. Pharmacologic blockade of A2BR improved the antitumor effect partly through the enhanced capacity of DCs to evoke anti-tumor T cell responses, bolstering anti-VEGF treatment or adoptive T-cell therapy [6–8]. Conversely, A2BR stimulation boosted myeloid-derived suppressive cells (MDSCs), augmented VEGF production, and caused the polarization of macrophages toward an immunosuppressive M2-like phenotype [9,10]. Ryzhov et al. demonstrated that in vitro treatment with the A2BR antagonist PSB603 could reverse the production of VEGF in lung cancer cell lines [11]. Agents targeting other elements of the adenosine pathway are currently undergoing clinical testing, including inhibitors of A2AR and antagonistic antibodies targeting CD73, CD39 and CD38 [12–15].

Although immune checkpoint inhibitors (ICIs) have dramatically changed the treatment landscape and prognosis of advanced NSCLC, blocking the programmed-death ligand 1 (PD-L1) or its receptor (PD-1) has demonstrated less success in patients with *LKB1* loss or other driver mutant tumors, such as EGFR, for unclear reasons [16,17]. Exploration of potentially targetable immunosuppression pathways is thus clearly indicated in these subsets. Because of the limited nutrient resources in the tumor microenvironment and competition between tumor and immune cells, it is conceivable that targeting TME metabolism could benefit antitumor immunity in these subsets [18].

While immune cell and direct anti-tumor effects of inhibiting or activating A2 receptors have been intensively studied, we uncovered an additional level of TME regulation by adenosine via modulation of cellular metabolism in both tumor and immune cell subsets. Here, we investigate the metabolic reprogramming function of A2 antagonism in both tumor and immune cells. We show that inhibiting A2BR with an antagonist could be an approach to modulate T-cell effector function. Inhibition of A2BR signaling demonstrated profound effects on dendritic cell (DC) metabolism by supporting glycolysis instead of oxidative phosphorylation. Our data reveal differential effects of A2AR and A2BR antagonists on metabolic characteristics in T cells and DCs.

Materials and methods

TCGA RNA expression data

We analyzed *ADORA2B* and *ADORA2A* RNA expression of *EGFR* mutant and wild-type, *STK11* mutant and wild-type, *KEAP1* mutant and wild-type, *ROS1* mutant and wild-type, *KRAS* mutant and wild-type, *BRAF* mutant and wild-type, *ALK* mutant and wild-type, *TP53* mutant and wild-type in lung adenocarcinoma patients from The Cancer Genome Atlas database by Mann-Whitney test. **(Supplementary Table 1)**.

Gene set enrichment analyses

To explore the potential difference in metabolic pattern between *EGFR* mutant and wild-type cancer patients, GSEA (Gene Set Enrichment Analyses) was performed to find enriched terms predicted to have a correlation with the 70 metabolic Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways **(Supplementary Table 2)**.

Cell culture, reagents, and gene transduction

The PC9, H1650, HCC4006, HCC827, H2122, A549 cell lines were either purchased from ATCC or were a generous gift from the laboratory of John Minna and authenticated using STR analysis (Promega). The cell lines were routinely tested for mycoplasma using the MycoAlert Plus kit (Lonza). Cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100µg/ml) in humidified CO_2 incubator at 37°C. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. NECA (pan-specific adenosine receptor agonist) and ZM 241385 (A2AR antagonist) were obtained from Sigma-Aldrich, PSB 603 (A2BR antagonist), BAY 60-6583 (A2BR agonist), CGS 21680 (A2AR agonist) were obtained from Tocris Bioscience.

Viability assay

Cells were seeded at 4×10^3 per well in 96-well plates and incubated in antibiotic-containing RPMI 1640 with 10% FBS. After 24 hours of incubation, various concentrations of PSB 603 were added to each well, and incubation was continued for a further 3 days. These cells were then used for the viability assay, which was performed using alamarBlue (ThermoFisher Scientific). An aliquot of $1/10^{th}$ volume of alamarBlue reagent was added to each well, followed by incubation for 2 hours at 37°C. Fluorescence was measured with a Synergy HT Multi-Mode Microplate Reader (Bio Tek Instruments, Winooski, VT) at excitation wavelength of 560 nm, emission wavelength of 590 nm. The percentage of growth is shown relative to that of the controls. Each sample was assayed in quadruplicate, with each experiment repeated at least two times independently. Nonlinear regression was used to determine IC50 (the half maximal inhibitory concentration) of the cell lines.

Metabolic assay

NSCLC cell lines were treated with either vehicle control, optimized PSB 603 or BAY 60-6583 at optimized concentrations. For all cell lines,

the optimized cell density and carbonyl cyanide-4 phenylhydrazone (FCCP) concentration were determined. Magnetically isolated T cells or splenocytes were treated with vehicle, NECA, ZM 241385 or PSB 603 during activation with CD3/CD28 beads or differentiation toward DCs, respectively, as described below and the seahorse assay was performed. For the extracellular flux assay, the sensor cartridge was hydrated overnight in Seahorse XF Calibrant at 37°C in a non-CO_2 incubator. A total of 10^6 T cells or 5×10^5 DCs with different treatment conditions were seeded in Seahorse culture plate for 30 min. OCR and PER were then measured by an XFe24 Seahorse Extracellular Flux Analyzer following the manufacturer's instructions (Agilent Technologies). During the seahorse assay, cells were treated with oligomycin (0.5μM), FCCP (2μM), rotenone (0.5μM), antimycin A (0.5μM) and 2-DG (50mM). Each condition was performed in 3-5 replicates. Statistical significance was determined using a two-way ANOVA.

Dendritic cell differentiation

Hematopoietic cells were isolated (Miltenyi Biotec) from spleen of wild type C57BL/6J [19], cultured in RPMI 1640 medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in humidified CO_2 incubator at 37°C, at concentration of 0.5×10^6 / mL in the presence of 20 ng/mL GM-CSF (PeproTech) and 10 ng/mL IL-4 (PeproTech) in 24-well plate. Cells were cultured for 7 days, half of the medium was replenished with fresh medium with cytokines every 3 days. For maturation of DCs, both spleen derived DCs and bone marrow derived DCs were prepared. Bone marrows were collected from femur and tibia of C57BL/6J mice [20]. The collected cells were treated with ACK buffer (ThermoFisher Scientific) to remove red blood cells. Remaining single cell suspension was cultured in the complete RPMI 1640 medium, which was refreshed with GM-CSF and IL-4 every two days and cultured for ten days. At day 10, DCs were stimulated with 100 ng/mL LPS (Sigma-Aldrich) in the presence or absence of NECA, ZM 241385 or PSB 603 for 18h. Suspension and low attached cells were harvested to be used in the experiments.

Immunoblotting

Equal amounts of protein from cell lysates were mixed with sodium dodecyl sulfate (SDS) sample buffer and separated on SDS–polyacrylamide gel electrophoresis gels (BioRad, Hercules, CA) before Western blot analysis. The primary antibodies used were A2BR (Sigma-Aldrich), A2AR (Sigma-Aldrich), phospho-$S6^{Ser235/236}$, GAPDH and β-actin (Cell Signaling Technology, Danvers, MA). Quantification of the Western blot data were performed by measuring the intensity of the hybridization signals by using ImageJ analysis software (National Institutes of Health, Bethesda, MD). Cells were cultured in 10 mL of RPMI-1640 medium with 10% FBS in the presence or absence of NECA, ZM 241385 or PSB 603. Cells were lysed in RIPA buffer supplemented with Complete Mini Ethyl-enediaminetetraacetic Acid–Free Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche), and their protein concentrations were determined by using a BCA protein assay kit (Pierce; ThermoFisher Scientific).

Flow cytometry analysis

For evaluation of cell surface markers, cells were incubated with the relevant antibodies (CD11b, CD11c, MHCII) at the manufacture's recommended concentrations for 20 minutes at 4°C. Fluorochrome-labeled antibodies were obtained from Biolegend. Flow cytometry acquisition was performed with an LSRFortessa flow cytometer (BD Biosciences). Flow cytometry analysis was performed with FlowJo software. Data were analyzed using a one-way or two-way ANOVA by Prism software (Version 9, GraphPad).

Statistical analysis

Statistical analysis was performed with Prism software (Version 9, GraphPad) using nonparametric and parametric paired (Friedman or Wilcoxon or t test) and unpaired (Kruskal-Wallis or Mann-Whitney) tests as indicated. For multiple comparisons, adjusted *P*-values were calculated by one-way ANOVA or two-way ANOVA. ($^*P<0.033$, $^{**}P<0.002$, $^{***}P<0.0002$, $^{****}P<0.0001$)

Results

Worse overall survival of NSCLC patients whose tumors express high levels of A2BR

To determine if adenosine receptor expression has an effect on NSCLC outcomes, we examined the overall survival (OS) of lung cancer patients with low and high expression of A2AR and A2BR. We examined 1925 NSCLC tumors with publicly available data, including 865 patients with LUADs and 675 with LUSCs. When we looked at all NSCLC, we found that OS was significantly worse in patients with high expression of A2BR, with a HR of 1.2 (95% CI, 1.06 to 1.36, p=0.0049) (Figure 1A). Patients with low expression of A2BR had an OS of 79 months (6.6 years) while patients with high A2BR expression had an OS of 63 months (5.3 years). We did not see a significant difference in the median survival time between patients with high and low expression of A2AR, which was 70 months (5.83 years) and 69 months (5.75 years), respectively, with a HR ratio of 0.96 (95% CI 0.85-1.09, p=0.55). When we looked at adenocarcinoma and squamous cell carcinoma separately, it became clear that outcome was driven by adenocarcinoma histology with a HR of 1.29 (95% CI, 1.02 to 1.62, p=0.034) (Figure 1B). The outcome in squamous cell patient population was not significant. Patients with adenocarcinoma and low expression of A2BR had an OS of 107 months (8.92 years) while patients with high expression of A2BR had an OS of 92.97 months (7.75 years). These data suggest that high expression of the A2BR leads to worse outcomes in lung cancer patients with adenocarcinoma histology.

Expression of the A2 receptors is altered in the presence of selected gene mutations in NSCLC

The determination that A2BR expression is associated with worse outcome led us to examine if A2BR expression was associated with many of the known driver mutations that are known to occur almost exclusively in adenocarcinomas. We investigated the TCGA data set of surgically resected stage I to III LUADs and identified 64 tumors with *EGFR* mutations and 436 tumors without *EGFR* driver mutations (*EGFR* WT). We also identified tumors with other oncogenic alterations, including *KRAS, TP53, STK11, KEAP1, ALK, ROS1,* and *BRAF*. Interestingly, *EGFR*-mutant tumors revealed higher mRNA expression of *A2BR* compared to *EGFR* WT (Figure 1C). Conversely, the tumors with a *KEAP1, ROS1* or *STK11* mutation demonstrated lower expression of *A2BR* mRNA. No significant correlation was found between expression of *A2BR* and other mutations. Expression of *A2AR* showed significant changes only with *KRAS* mutation, where tumors with mutant *KRAS* expressed *A2AR* at a lower level (**Figure S1A**). Since we are interested in the metabolic impact of A2BR signaling, we compared RNAseq data from the TCGA for *EGFR* wild type and *EGFR* mutant samples to identify differentially expressed genes involved in cancer metabolism. When we queried the 2605 genes from the 70 metabolic pathways in KEGG, GSEA revealed that altered genes from *EGFR* mutant group were significantly enriched in the peroxisome pathway, valine, leucine and isoleucine degradation, glycan biosynthesis, type I diabetes, primary bile acid biosynthesis, and panthotenate and coenzyme A biosynthesis, suggesting that the *EGFR* mutation has effects on cellular metabolism (**Figure S1B**).

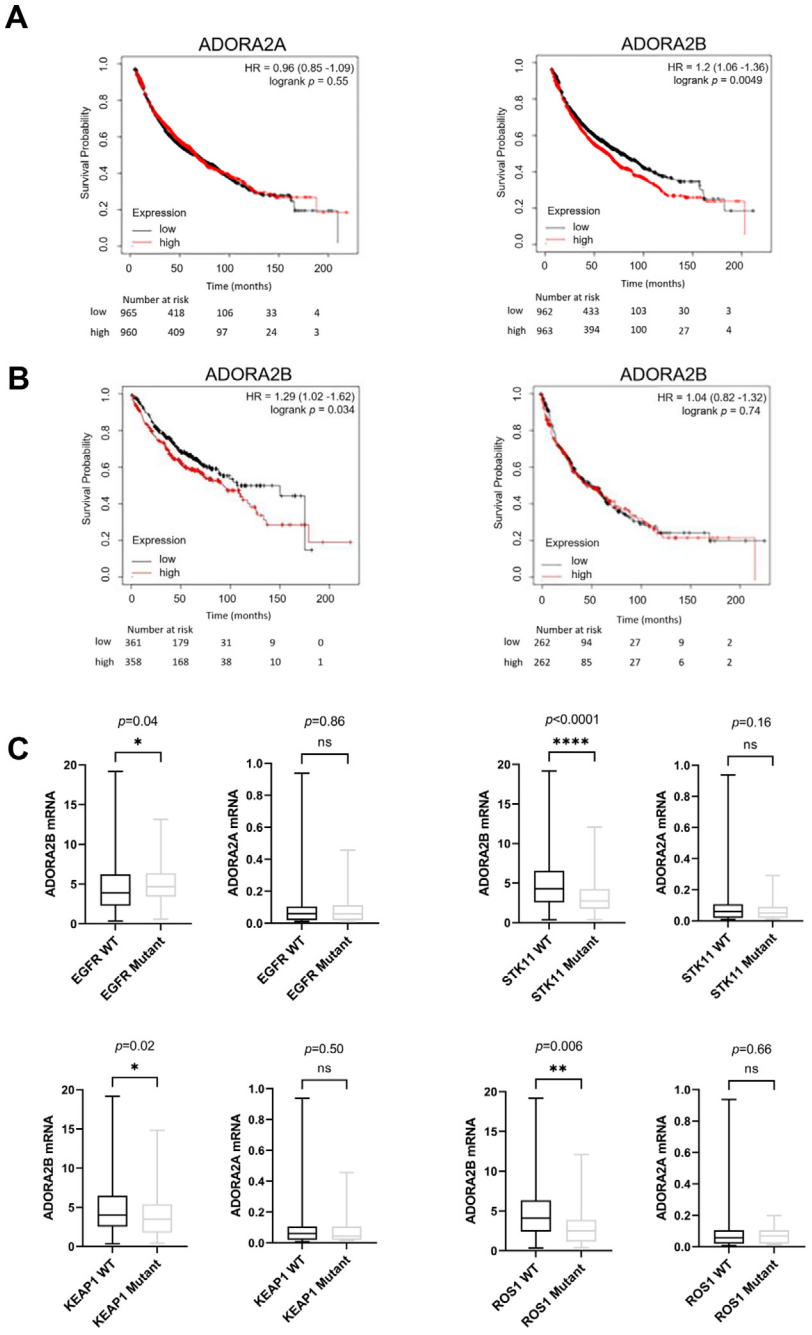

Figure 1. A2 receptor expression in NSCLC and the relationship with overall survival and mutations associated with NSCLC. (A) Kaplan-Meier plot was used to evaluate A2AR and A2BR expression and their relationship to OS of total 1925 lung cancer patients. (https://kmplot.com) **(B)** A Kaplan-Meier plot shows A2BR expression and its relationship to OS of LUAD and LUSC lung cancer patients. **(C)** A2BR and A2AR mRNA expression in of 500 LUAD patient tumor samples from TCGA were analyzed in tumors containing specific gene mutations and compared to their wild type counterparts. A Mann-Whitney test was used to determine significance. *EGFR* gene (WT 436 vs Mutant 64); *KEAP1* gene (WT 416 vs Mutant 84); *ROS1* gene (WT 477 vs mutant 23); *STK11* gene (WT 434 vs Mutant 66). (*$P<0.033$, **$P<0.002$, ***$P<0.0002$, ****$P<0.0001$)

A2BR blockade alters oxidative phosphorylation in NSCLC cell lines

Using the Seahorse assay, we found that blockade of the A2BR with a receptor specific antagonist, PSB 603, affected oxygen consumption rates in NSCLC cell lines. In light of the increased A2BR expression we saw in *EGFR* mutant tumor samples, we evaluated adenosine signaling modulation in a panel of *EGFR* mutant cell lines (PC9, H1650, HCC4006, HCC827) with PSB 603 and noted a tendency to increase basal respiration, maximal respiration, ATP production and spare respiratory capacity (SRC) in the presence of PBS 603 (Figure 2A-B, Figure 3A-B; Figure S2A-E). However, the only significant changes occurred in maximal respiration in PC9 and HCC4006 cells, basal respiration in PC9, and spare respiratory capacity in HCC4006 (Figure 2A, Figure 3A). As expected, treatment of the PC9 and H1650 cell lines with the A2BR agonist, BAY 60-6583, showed the opposite effect, and showed significant changes in maximal respiration and spare respiratory capacity in both cell lines. In PC9 cells, basal respiration

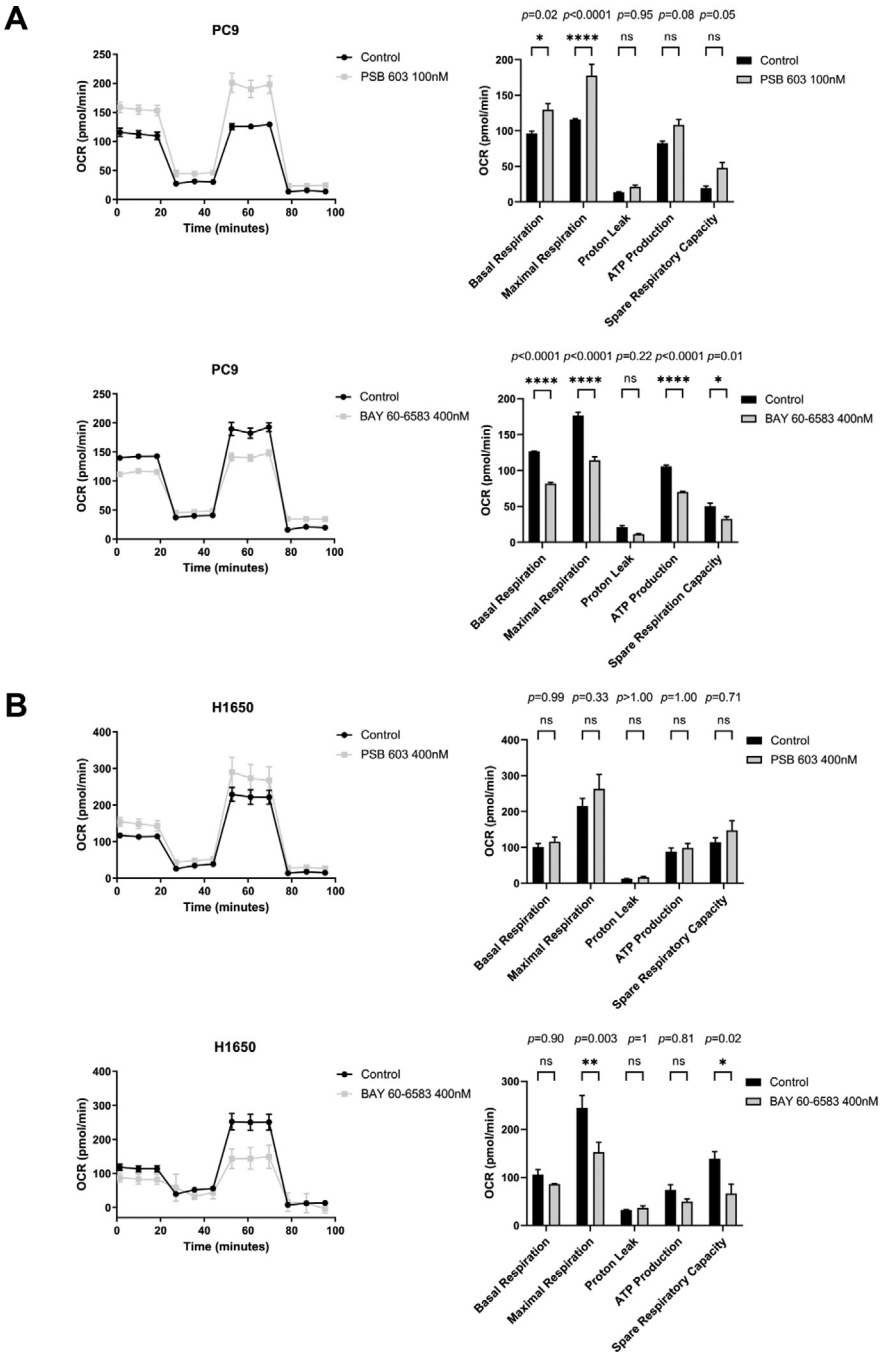

Figure 2. Antagonism and agonism of A2BR alters oxidative phosphorylation in NSCLC cell lines. (A-B) OCR was assessed in *EGFR* mutant cell lines (PC9, H1650) basally and in response to the mitochondrial inhibitors oligomycin, FCCP, and rotenone and antimycin A (Rot/AA). OCR profiles (left panels) and calculation of basal respiration, maximal respiration, proton leak, ATP production and SRC (right panels) for cell lines treated 3 hours with PSB 603/BAY 60-6583. ($^{*}P<0.033$, $^{**}P<0.002$, $^{***}P<0.0002$, $^{****}P<0.0001$)

and ATP production were also significant (Figure 2A-B). A2BR antagonist had similar effect on oxidative consumption rate (OCR) in the H2122 and A549, which carry mutated *LKB1* and *KRAS* genes, mutations also associated with immune suppression (Figure 3C-D; Figure S2F-G). In these cell lines the effects of A2BR inhibition were significant in most of the categories of oxidative phosphorylation including basal respiration, maximal respiration, ATP production, and spare respiratory capacity. Collectively, our data show that oxidative phosphorylation is affected by A2BR signaling modulation.

Effects of adenosine receptor blockade on metabolic pattern of CD4^{+} T cells

It is well known that the key nutrients like glucose, which are consumed by tumor cells, also modulate T cell activity [21]. CD38, CD39 and CD73 acting as the ectoenzymes which promote adenosine generation are expressed on T cells and have also been identified as immune checkpoints [4]. Therefore, we examined the effect of adenosine receptor inhibition on T cell metabolic pattern, in particular, in the CD4^{+} T cell.

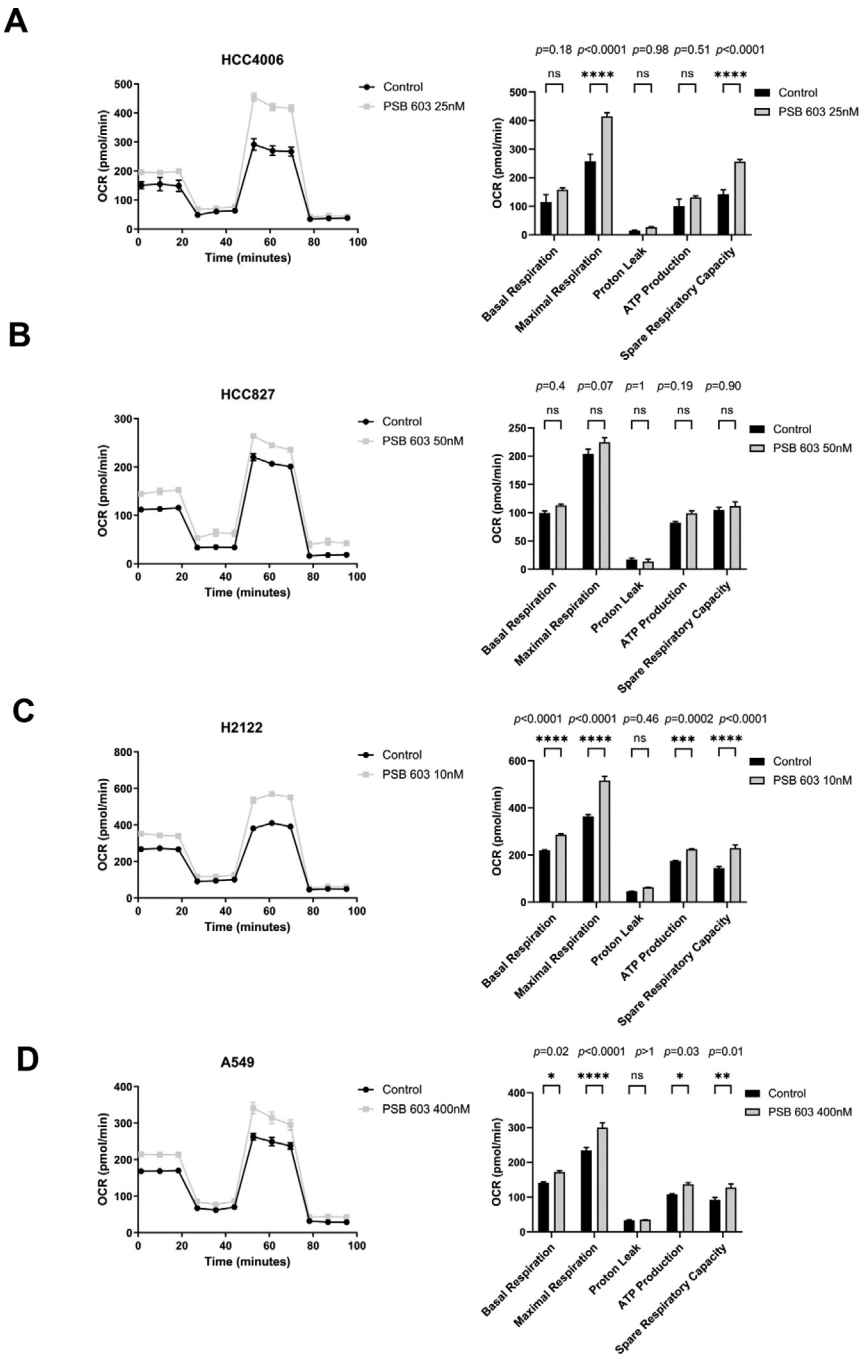

Figure 3. A2BR blockade alters oxidative phosphorylation in NSCLC cell lines. (A-D) OCR was determined in cell lines with *EGFR* mutation (HCC4006, HCC827) and cell lines with *LKB1* mutation (H2122 and A549). OCR profiles (left panel) and calculated values of basal respiration, maximal respiration, proton leak, ATP production and SRC (right panel). Data are shown as mean ± SEM of triplicate samples, representative of at least 3 experiments, analyzed by a two-way ANOVA. (*P<0.033, **P<0.002, ***P<0.0002, ****P<0.0001)

Adenosine is known as an important regulator of T cell function. However, little is known about the functional differences between A2AR and A2BR in the regulation of T cell metabolism. Therefore, we examined the characteristics of both oxidative phosphorylation and glycolysis in T cells in the presence of A2AR and A2BR antagonists ZM 241385 and PSB 603, respectively **(Figure S3A-B)**. We did this in $CD4^+$ T cell populations after activation with anti-CD3/CD28 beads**.** Consistent with the published data, the oxygen consumption rate (OCR) of activated $CD4^+$ T cells was higher than the non-activated T cells (data not shown). Treatment with NECA reduced the OCR in activated CD4+ T cells, which was reversed by the A2AR antagonist ZM 241385 and A2BR antagonist PSB 603 (Figure 4A). PSB 603 increased the maximal respiration, and spare respiratory capacity of the NECA treated $CD4^+$ T cells. ZM 241385 reversed the effects of NECA only with regard to maximal respiration, but not spare respiratory capacity. Mammalian target of rapamycin (mTOR), a central regulator of cell metabolism, has been shown to control T cell memory formation and is

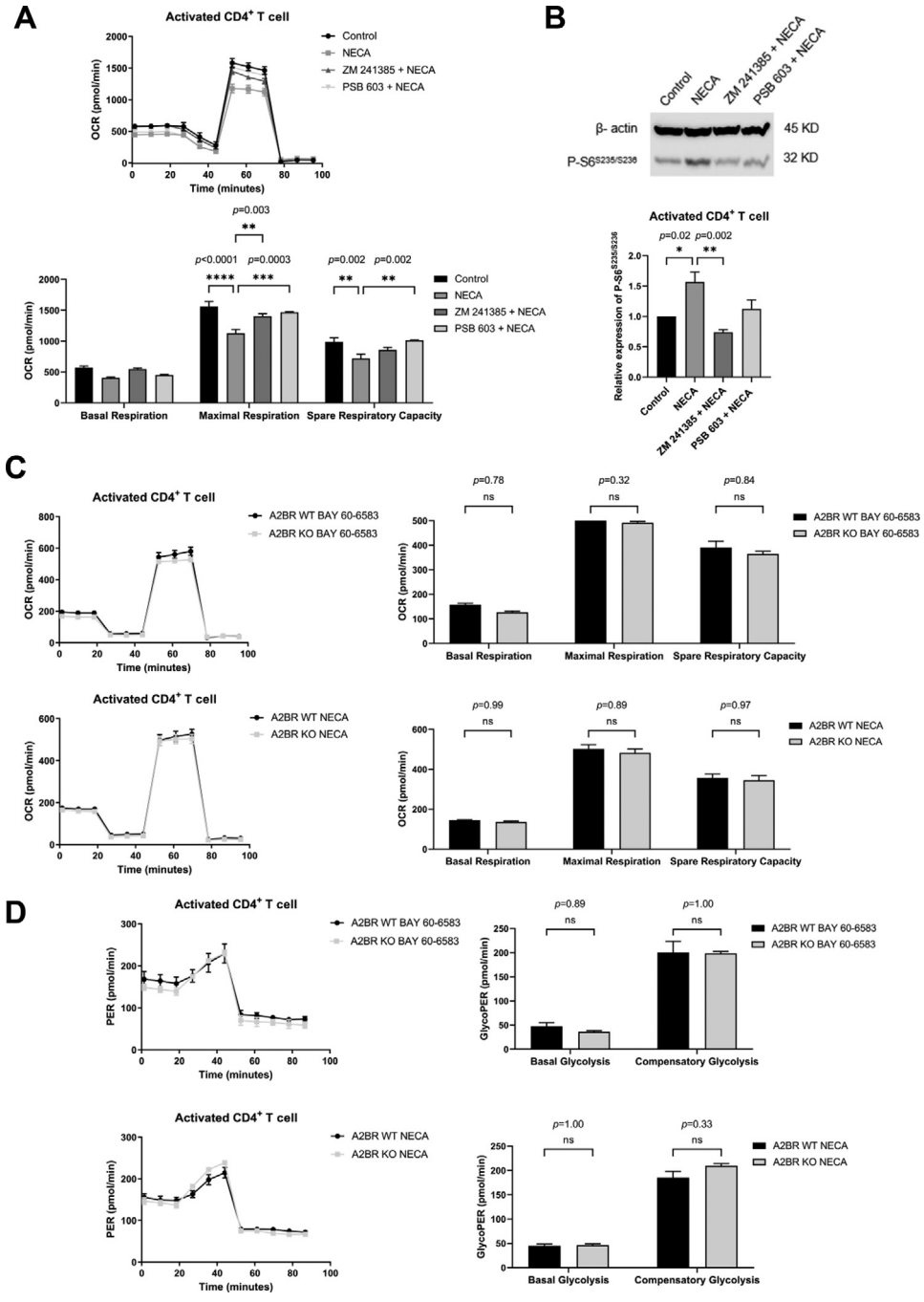

Figure 4. Effects of adenosine receptor blockade on the metabolic activity of CD4+ T cell. (A) CD4+ T cells isolated from spleenocytes were activated by CD3/CD28 beads for 3 days in the presence of vehicle, 30nM NECA alone, and NECA in combination with 500nM ZM 241385 or 30nM PSB 603. Seahorse assay was used to determine OCR. All data are representative of at least 3 experiments, analyzed by a two-way ANOVA. **(B)** Isolated CD4+ T cells from splenocytes were treated as in A. Western blot was probed with antibodies to p-S6 and β-actin. The experiment was done in triplicate and analyzed in a one-way ANOVA. **(C-D)** Seahorse assays were used to determine levels of oxidative phosphorylation and glycolysis in CD4+ T cells isolated from splenocytes of A2BR WT and A2BR KO mice. Assays were performed after cells were activated with CD3/CD28 beads for 3 days in the presence of 30nM NECA or 1μM BAY 60-6583. (*P<0.033, **P<0.002, ***P<0.0002, ****P<0.0001)

known to control lymphoid homing behavior of T cells through regulation of adhesion molecule CD62L [22]. Consistently, NECA increased mTOR activity in the CD4$^+$ T cells as indicated by the phosphorylation of the mTOR target pS6. While both ZM 241385 or PSB 603 tended to reverse this activity, only the A2AR inhibitor did so significantly (Figure 4B). Next, we examined if the metabolic effects depend on the expression of A2BR using CD4+ T cells where the A2BR was genetically deleted **(Figure S3C)**. Treatment of the cells with NECA or the A2BR agonist, BAY 60-6853, had no measurable effect on oxidative phosphorylation or glycolysis as measured by the OCR and PER, respectively (Figure 4C-D). Collectively, these data

suggest that A2 adenosine receptor blockade can reverse the harmful effects of adenosine. In addition, genetic deletion of A2BR eliminated all of the effects of the A2BR agonist, BAY 60-6583, and, most surprisingly, the effects of NECA, implying that the presence of A2BR is necessary for the activity of A2AR.

Adenosine receptor antagonists exert metabolic regulation on immature and mature DCs

In addition to adenosine receptor inhibitory effects on T cell function and metabolism, adenosine has also been reported to suppress differentiation, maturation and activity of DCs [19]. To assess both effects of adenosine receptor activation and inhibition on dendritic cell differentiation, hematopoietic progenitor cells from mouse spleen were polarized in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4-containing media in the presence of either NECA or the combination of NECA with ZM 241385 or PSB 603. After 7 days in differentiation media immature DCs were detected. While there were fewer $CD11b^{-}CD11c^{+}$ DCs in the presence of NECA, addition of the A2 antagonists ZM 241385 or PSB 603 reversed this effect to some extent with only the A2AR antagonist being significant (Figure 5**A).** Tolerogenic DCs prefer to use oxidative phosphorylation and fatty acid oxidation as their main energy resource while immunogenic DCs metabolism switches to glycolysis [23–25]. However, it remains unknown whether adenosine receptor mediated regulation of DC functionality is linked to the ability to alter metabolic reprogramming. We performed the seahorse assay on immature dendritic cells with A2 adenosine receptor activation or inhibition. NECA promoted OXPHOS, indicative of tolerogenic DCs, by increasing both maximal respiration and spare respiratory capacity. Both ZM 241385 and PSB 603 alleviated the effect with ZM 241385 appearing to be more efficient at significantly reducing both maximal respiration and spare respiratory capacity (Figure 5B). Since DCs undergo metabolic reprogramming from predominantly OXPHOS to glycolysis to mount an immunogenic response, we evaluated the glycolysis rate in mature DCs with LPS stimulation [25]. NECA treatment significantly reduced glycolysis in matured DCs (Figure 5C). Strikingly, ZM 241385 and PSB 603 exhibited opposite effects on the mature DCs. A2AR inhibition reduced both basal and compensatory glycolysis while inhibition of the A2BR increased glycolysis, which suggests there is a role for A2BR blockade in DC metabolic reprogramming to a more immunogenic state (Figure 5C). To further understand how A2BR affects DC maturation, we isolated bone marrow from *A2BR* wild type (*A2BR* WT) mice and *A2BR* $^{-/-}$ knockout (*A2BR* KO) mice and matured the DCs as above in the presence of NECA, A2AR agonist CGS 21680 and A2BR agonist BAY 60-6583. The percentage of wild type $CD11b^{-}CD11c^{+}$ DCs was reduced by NECA compared to control and A2AR and A2BR agonists reduced the effect of the NECA to a small extent (Figure 5D). However, these changes never reached significance. Most interestingly, the percentage of $CD11b^{-}CD11c^{+}$ DCs from *A2BR* KO cells was significantly elevated in the respective control and treatment samples compared to wild type cells. Examination of MHC II expression in $CD11b^{-}CD11c^{+}$ DCs demonstrated that there was no significant difference between the groups (data not shown). OXPHOS and glycolysis levels were evaluated in mature DCs derived from wild type and *A2BR* KO bone marrow cells **(Figure S4A)**. Consistently, *A2BR* KO DCs had a higher glycolysis rate with or without NECA compared to wild type cells (Figure 6**A)**. OXPHOS rates of WT and KO DCs were equivalent without NECA treatment, but was impaired in the KO cells treated with NECA suggesting that A2BR is at least partially necessary for the metabolic changes induced by NECA from a low OXPHOS to a high OXPHOS state (Figure 6B). It is conceivable that inhibition of A2AR is liable to promote immature DCs from tolerance state to immunogenicity accompanied by decreased OXPHOS while generation of functional mature DCs would require increased glycolysis that could be achieved by blockade of A2BR.

Discussion

This study is the first to describe the metabolic effects of targeting adenosine receptors, A2AR and A2BR, in NSCLC and immune cells. Previous studies have addressed many aspects of adenosine receptor signaling in TME using genetic and pharmacological modulation of adenosine receptors [5,6,15,26–31]. However, none of these approaches addressed the question of metabolic homeostasis and nutrient competition among different cells in the TME. The current study revealed an important function of these receptors as regulators of the cellular metabolism in tumor and immune cells at the metabolic level via modulation of A2 adenosine receptor signaling.

Previous observations in cells with *LKB1* mutations show that these cells have greater levels of ROS due to higher levels of metabolic activity and dysfunctional mitochondria [32]. Cells like *LKB1* and *KRAS* co-mutant cells with high metabolic addiction have adapted to counter high oxidative stress [33]. Our analysis of the genes that are involved in detoxifying cells showed that they are up regulated in A549 and H2122, when compared to the *EGFR* mutant cell lines that we used in our study (Figure S5A). This may account for the fact that the A549 and H2122 cell lines, each containing *LKB1* and *KRAS* co-mutation, are resistant to A2BR inhibition despite increased OXPHOS. *EGFR* mutant cell lines PC9, HCC4006, H1650 with lower level of antioxidant gene expression were obviously inhibited in the presence of PSB 603 (Figure S5B-D). The *EGFR* mutant cell line HCC827, which showed the smallest change in OCR with PSB 603 treatment (Figure 3B), was the least sensitive to A2BR inhibition when compared to other *EGFR* mutant cell lines (Figure S5C). Our data suggests that in tumor cells with the characteristics of low metabolic addiction and low sensitivity to nutrient deprivation, such as *EGFR* mutant lung cancer cells, A2BR antagonist might exert anti-tumor activity by induction of oxidative stress. It is thus conceivable that a subset of NSCLC patients with *EGFR* mutation whose tumors increase OXPHOS in response to A2BR antagonism could benefit from an A2BR inhibitor in combination with chemotherapy, since this would enhance the chemotherapy-induced oxidative stress and lead to robust ROS-mediated cell death [34]. In contrast, results of our study suggest that patients with *LKB1* and *KRAS* co-mutation pattern and high level of oxidative phosphorylation may not benefit from A2BR antagonist therapy, as the mechanisms they have developed can ameliorate oxidative stress in these cells.

Despite the impressive outcomes achieved by immunotherapies in the past few years, there is a significant proportion of NSCLC patients who are refractory to ICIs or who develop adaptive resistance after achieving initial clinical responses. In our study, we showed that inhibition of A2BR led to the enhancement of oxidative phosphorylation in T cells, which is needed to meet the basic needs of cell survival and to retain the ability to robustly proliferate upon another encounter with the cognate antigen [35,36]. These findings imply that cellular metabolism can be regulated therapeutically and may represent a strategy to support immune responses. Our results also suggest that appropriate metabolic rewiring of T cells by modulation of A2 adenosine receptor signaling has the potential to improve the design of T-cell mediated immunotherapies [35,37].

We also showed here that there is no significant difference in metabolic alteration of activated $CD4^{+}$ T cell with A2BR knockout compared to wild type even in the presence of pan-specific adenosine agonist NECA suggesting that A2BR might be required for the full functioning of A2AR. Recent studies demonstrated the possibility of A2AR/A2BR heterodimerization and regulation of A2AR activity by A2BR [38–41]. It is likely that our data reflect the existence of this interaction in T cells and its importance for the regulation of T cell metabolism.

A2AR antagonism exerts effects not only on T cells but also on dendritic cells [42]. We demonstrate that A2AR and A2BR antagonists are distinct in orchestrating the development of dendritic cells via metabolic reprogramming of glucose metabolism. Analysis of cellular metabolism in DCs suggests that inhibition of A2AR and the accompanying decrease in

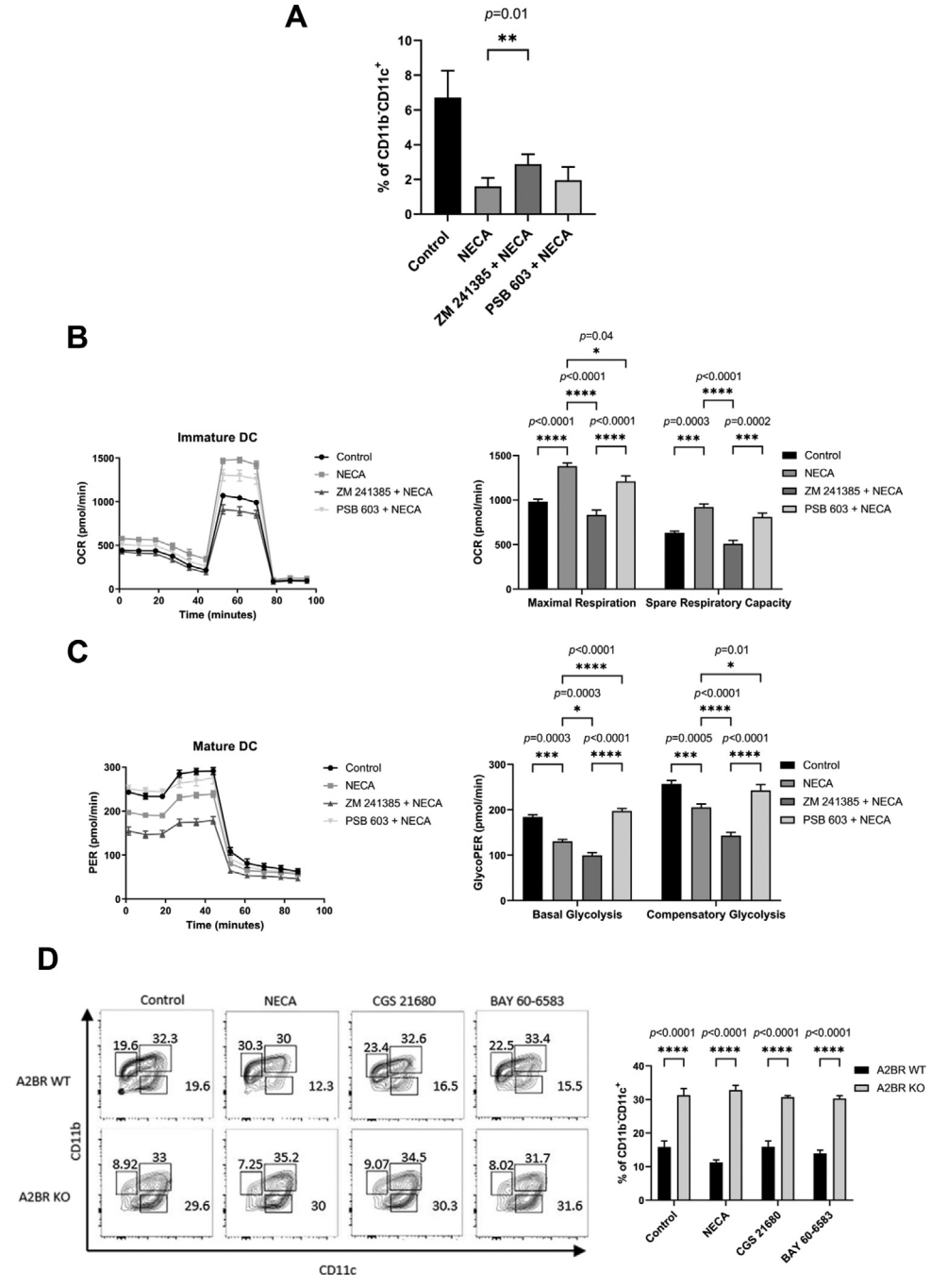

Figure 5. Adenosine receptor antagonists alter the metabolism of immature and mature dendritic cells. (A) Immature DCs were generated from spleenocytes in the presence of vehicle, 30nM NECA alone, or NECA with 500nM ZM 241385 or 30nM PSB 603 to produce CD11b+ CD11c-, CD11b+ CD11c+ and CD11b- CD11c+ cell populations. The population of CD11b- CD11c+ cells are shown and were analyzed by one-way ANOVA. **(B)** Seahorse assay was used to determine OCR profiles (left panel) and calculation of basal respiration, maximal respiration, proton leak, ATP production and SRC (right panel) in immature DCs treated as in A. Significant differences between the indicated groups were determined by a two-way ANOVA. **(C)** PER profiles (left panel) and calculated values of basal glycolysis and compensatory glycolysis (right panel) are shown. Glycolytic rate was determined by seahorse assay in mature DCs from spleenocytes differentiated in presence of vehicle, 3μM NECA alone, or NECA with 100nM ZM 241385 or 300nM PSB 603 with 100ng/ml LPS stimulation for 18h. Significant differences between the indicated groups were determined by a two-way ANOVA. **(D)** Cells were obtained from bone marrow of *A2BR* WT or *A2BR* knockout (*A2BR* KO) mice and matured as described in the presence of vehicle, 3μM NECA, 100nM CGS 21680 or 1μM BAY 60-6583 with 100ng/ml LPS stimulation for 18h. (*$P<0.033$, **$P<0.002$, ***$P<0.0002$, ****$P<0.0001$)

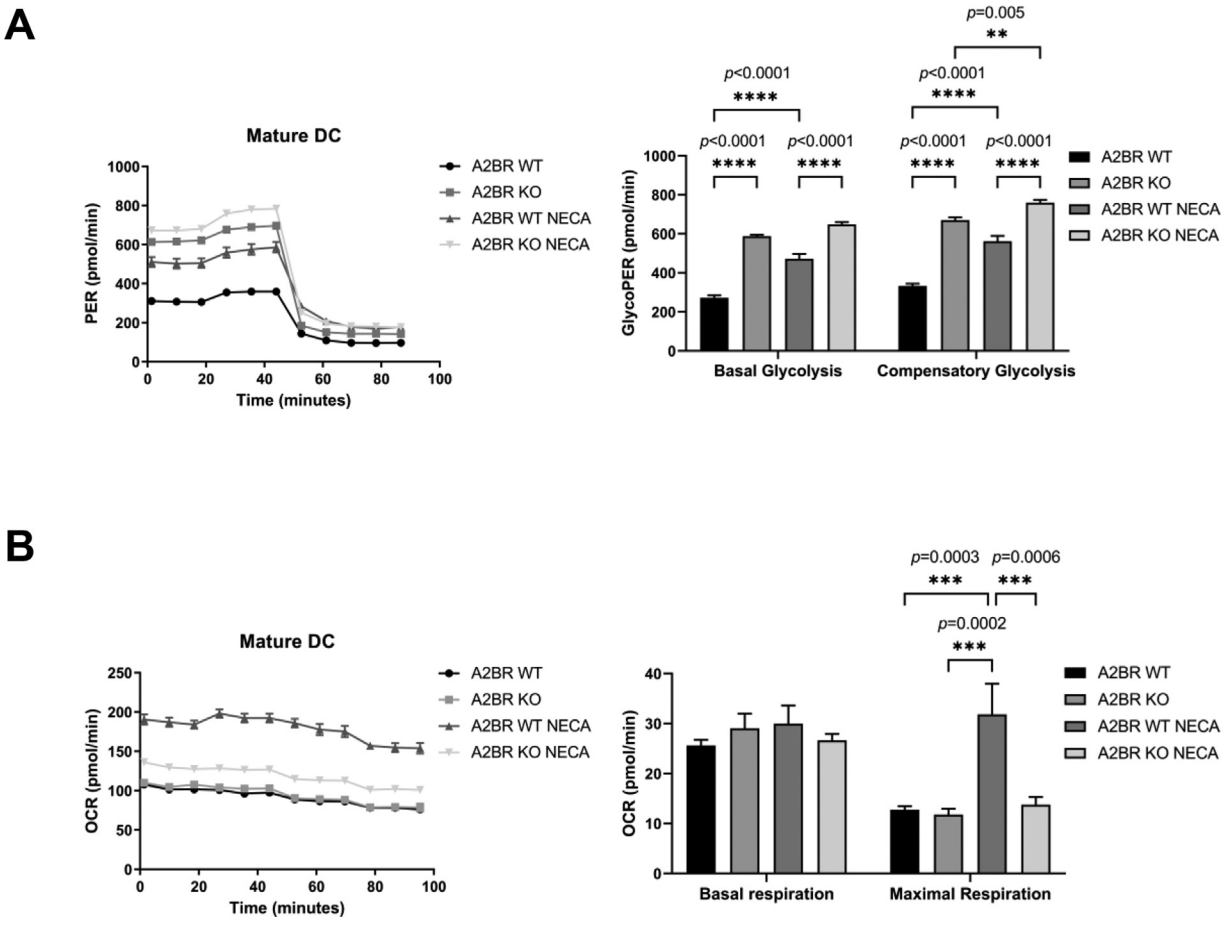

Figure 6. Adenosine receptor antagonists alter the metabolic regulation of immature and mature dendritic cells. (A-B) Seahorse assays were used to determine PER and OCR in matured bone marrow derived DCs from *A2BR* WT or *A2BR* KO mice in the presence of 3μM NECA. All data are representative of at least 3 independent experiments. ($^{*}P<0.033$, $^{**}P<0.002$, $^{***}P<0.0002$, $^{****}P<0.0001$)

OXPHOS promotes immature DCs from a tolerance state to immunogenic state, while the functional DCs after maturation are programmed by blockade of A2BR via increased glycolysis. We found that A2AR in mature DCs can alter cell metabolism even when A2BR is deleted, which is different from what we observed in $CD4^+$ T cells. This suggests that A2AR signaling is different in the metabolic regulation in T cells and DCs. We found that A2AR and A2BR reprogram opposite metabolic alterations in splenocyte derived mature DCs for unclear reasons.

We also noted a remarkable increase in the proportion of $CD11b^-$ $CD11c^+$ mature DCs generated from *A2BR*$^{-/-}$ knockout cells correlates with the enhanced glycolysis and indicates the importance of A2BR on the generation of mature DCs. This is supported by the fact that the effect of NECA on glycolysis is weakened when A2BR was deleted compared to A2BR wild type. This again may be indicative of the interaction between A2AR and A2BR and the existence of A2BR control over A2AR activity, as was found previously [38,39]. In the current study, we also revealed that mature DCs from *A2BR*$^{-/-}$ knockout cells had decreased A2AR expression compared with A2BR wild type cells **(Figure S6A)**. The studies discussed above could lead to the development of various therapeutic strategies to improve anti-cancer immunity by modulating DC metabolism and function in vivo. *Ex vivo* manipulation of DC metabolism for therapeutic vaccination purposes also might be an attractive strategy, targeting metabolic pathways associated with DC tolerogenicity [43–45]. Thus, we posit that therapeutic transformation of DCs from an immunosuppressive to an immunostimulatory state by modulating cellular metabolism through the adenosine receptors might represent an effective therapeutic strategy.

It should be stressed, however, that our data only provides a preliminary glimpse into the metabolic regulation of tumor and immune cells by A2R modulation. Much more work needs to be done to improve our understanding of the involvement of A2R signaling in cancer and, more specifically, in the TME. Additional experimentation will determine the potential benefits A2R antagonism/agonism and the ability to modulate cellular metabolism to increase the efficacy immunotherapy and chemotherapy in treating cancer.

Conclusions

To gain an understanding of how tumor cells and immune cells respond metabolically to adenosine receptor antagonism, we have investigated the metabolic changes that take place in multiple cell types by targeting of the A2AR and A2BR. We have discovered that tumor cells, CD4 T cells, and DCs are all affected by A2 receptor targeting. Specifically, a subset of *EGFR* mutant tumors may be sensitive to A2BR inhibition. In addition, targeting of A2AR and A2BR in immune cells has the potential to improve their anti-tumor capabilities by favorably modifying the metabolism of T cells and dendritic cells to a more immunogenic phenotype, although timing of intervention and duration need further exploration. Our findings present compelling evidence for the impact of A2AR and A2BR modulation on metabolic reprogramming

in tumor and immune cells supporting their use in combination with other immunotherapies in NSCLC.

Authors' contributions

DPC, MMD, JW, SG - Conceptualization; DPC, MMD, JW - Funding acquisition; DPC, MMD, JW, JMA - Supervision; SG, SS - Data collection; SG - Writing original draft; SG, JW, DPC, MMD, JMA, RW - Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2022.100824.

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