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## Lung Injury Pathways: Adenosine Receptor 2B Signaling Limits Development of Ischemic Bronchiolitis Obliterans Organizing Pneumonia

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# Lung Injury Pathways: Adenosine Receptor 2B Signaling Limits Development of Ischemic Bronchiolitis Obliterans Organizing Pneumonia

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## ABSTRACT

**Purpose/Aim of the Study:** Adenosine signaling was studied in bronchiolitis obliterans organizing pneumonia (BOOP) resulting from unilateral lung ischemia. **Materials and Methods:** Ischemia was achieved by either left main pulmonary artery or complete hilar ligation. Sprague–Dawley (SD) rats, Dahl salt sensitive (SS) rats and SS mutant rat strains containing a mutation in the A2B adenosine receptor gene (*Adora2b*) were studied. Adenosine concentrations were measured in bronchoalveolar lavage (BAL) by HPLC. A2A (A2AAR) and A2B adenosine receptor (A2BAR) mRNA and protein were quantified. **Results:** Twenty-four hours after unilateral PA ligation, BAL adenosine concentrations from ischemic lungs were increased relative to contralateral lungs in SD rats. A2BAR mRNA and protein concentrations were increased after PA ligation while miR27a, a negatively regulating microRNA, was decreased in ischemic lungs. A2AAR mRNA and protein concentrations remained unchanged following ischemia. A2BAR protein was increased in PA ligated lungs of SS rats after 7 days, and 4 h after complete hilar ligation in SD rats. SS-*Adora2b* mutants showed a greater extent of BOOP relative to SS rats, and greater inflammatory changes. **Conclusion:** Increased A2BAR and adenosine following unilateral lung ischemia as well as more BOOP in A2BAR mutant rats implicate a protective role for A2BAR signaling in coun

## Keywords:

A2B adenosine receptor; bronchiolitis obliterans organizing pneumonia; lung ischemia; lung transplanting ischemic lung injury.

## Introduction

Lung transplantation is the final option for patients suffering from end-stage lung disease. The efficacy of 2000 yearly transplants in the United States is severely limited by graft availability, preoperative function, and a 50% postoperative failure rate within 5 years.[ 1]Bronchiolitis obliterans (BO), a pathologic condition in which small airways become irreversibly obstructed by granulation tissue and fibrosis, is *the major cause of morbidity and mortality* resulting from lung transplantation.[ 1], [ 2] As a harbinger of BO, bronchiolitis obliterans organizing pneumonia (BOOP) occurs early postoperatively (weeks to months) and is associated with a doubled risk of subsequent BO and graft failure.[ 3], [ 4] Ischemia/reperfusion (I/R) is a major risk factor for all post-transplant problems, including BOOP.[ 5] In order to detect and understand the early ischemic mechanisms inducing BOOP, we developed a rat model of unilateral lung ischemia whereby the left pulmonary artery is ligated *in situ*. This

provides a contralateral control lung for comparison. This model completely avoids immunomodulation and maintains ventilation to both lungs.[ 6] In the present work, we explore the role of adenosine signaling in this injury response.

Pulmonary I/R increases adenosine production, with some studies reporting a proinflammatory role for A<sub>2B</sub> adenosine receptor (A<sub>2B</sub>AR) engagement in the transplanted lung.[ 7], [ 8], [ 9] However, others describe A<sub>2B</sub>AR conferring protection against lipopolysaccharide- or mechanical ventilation-induced direct lung injury.[10], [11], [12] The goal of this study was to examine the role of A<sub>2B</sub>AR signaling promoting postischemic BOOP. The studies presented utilize a novel rat A<sub>2B</sub>AR mutant, A<sub>2B</sub>AR mRNA lung levels, A<sub>2B</sub>AR lung protein expression, and translation-regulating miRNA lung levels to correlate A<sub>2B</sub>AR function with histopathologic changes seen in ischemic BOOP. We report data from three strains of rats, including Sprague Dawley rats, an A<sub>2B</sub>AR mutant, and the background strain for the A<sub>2B</sub> mutant, SS rats. These three strains permit conclusions regarding the role of A<sub>2B</sub> receptors in rodents with two significantly different genetic backgrounds, thus enhancing the translational implications to humans with widely variable genetic makeups.

There are currently three known miRNA sequences that regulate A<sub>2B</sub>AR expression: miR27b, miR128a, and miR128b. [13], [14], [15] All three have been implicated in A<sub>2B</sub>AR *in vitro* signaling (e.g., B-lymphocyte differentiation and 3T3-PKD-1 cell differentiation).[13], [15] Given the negative regulation of A<sub>2B</sub>AR in murine colitis, we focused upon the role of miR27b and miR128a in this model.[14]

Our data provide evidence that adenosine release and activation of A<sub>2B</sub>ARs are protective responses that limit the development of BOOP, and hence BO.

## Materials and methods

antibodies for A<sub>2A</sub>AR and A<sub>2B</sub>AR were purchased from Alomone labs (A<sub>2B</sub>AR Cat# AAR-003, A<sub>2A</sub>AR Cat# AAR-002). Adenosine for standards was obtained from Sigma (Cat#A9251).  $\beta$ -actin antibody was also obtained from Sigma (Cat#A2228).

Sequence specific primers for miRNA were obtained from Exiqon. Target sequences follow:

- miR-128-3p: UCACAGUGAACCGUCUCUUU Product# 205677
- miR-27a-3p: UUCACAGUGGCUAAGUCCGC Product#204764
- miR-27b-3p: UUCACAGUGGCUAAGUUCUGC Product# 204782
- miR-191a-5p: CAACGGAAUCCCAAAGCAGCUG Product #204306).
- mRNA primers were obtained from Integrated DNA Technology with following sequences:
- A<sub>2B</sub>AR forward: GCGGCACCTTAGCGGCTGTC (Product#117239370)
- A<sub>2B</sub>AR reverse: TACCTGAGCGGGACGCGAAT (Product#117239371)
- A<sub>2A</sub>AR forward: CCATGCTGGGCTGGAACA (Product# 117239368)
- A<sub>2A</sub>AR reverse: GAAGGGGCAGTAACACGA (Product #117239369)

*Animal models:* AdorA2b mutants. All animal procedures including the generation of the A<sub>2B</sub>AR mutant model, breeding, and phenotypic characterization were performed at the Medical College of Wisconsin using protocols approved by the Institutional Animal Care and Use Committee and in compliance with the National Research

Council's Guide for the Care and Use of Laboratory Animals. Zinc-Finger Nuclease (ZFN) constructs specific for rat *Adora2BA* were designed, assembled, and validated by Sigma-Aldrich, to target the sequence AACTACTTTCTGGTGTccttgGCGACGGCGACGTGGCT within the first exon of *Adora2BA*, where each member of heterodimeric ZFN pair binds to the underlined sequence on opposite strands. mRNA encoding the *Adora2BA* ZFN was injected into SS/JrHsdMcowi (SS) rat embryos, as described previously.[16], [17] Mutant founders were identified using the primers A<sub>2B</sub>AR\_F: ACACAACCCCGGTAGAGGA and A<sub>2B</sub>AR\_R: GATGGAGCTCTGTGTGAGCA.

A founder harboring genomic deletion was identified and validated by Sanger sequencing (Figure 1). A mutant founder was backcrossed to the parental SS strain and heterozygous animals from subsequent generations were intercrossed in order to generate homozygous mutant animals and were used for all reported studies. This strain is designated as SS-Adora2b. Parental SS rats were used as genetic background controls.

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GGCACGAGCG GTCTCGGCGC TGTGGCCATG CCTGCGGCA CCTGCGGCG 201 CTGAGC CCGACACAAC CCGGTAGAG GACTCCCGG GCCCGGCTG
CCGTGCTCGC CAGACCGCG ACACCGGTAC GGACCCCGGT GGAATCGCCG ACAGGACTCG GGCTGTGTTG GGGCCATCTC CTGAGGGGCC CGGCCGAC
M Q L E T Q D A L Y V A L E L V I A A L A V A G N V L V C A A
101 GCGGCAATG CAGTAAAGA TCAAGAGCG GGTATAGTG GACTGAGAG TGGTATATG GCGCTGACA DTGGAGGGA ACCTGCTGCT GTGGCTGGG
GGCCGCTAC GTCGATCTT GCTTCTGCG CGACATGAC CGGACCTCG ACCAATAGCG GGGGACCTG CAGCCCGCT TGCAGGACA GACCGGCGG
V G A S S A L Q T P T N Y F L V S L A T A D V A V G L F A I P F A I
201 GTGGAGCCT CGATGCTTT ACAGACCCG ACCAACTACT TTCTGGTGT CCTGGGACG GCGGACGTGG CTGTGGACT CTTCGCCATC CCCTTTGCCA
APCCCTGGG GGTACGAAA TGTGTGGGG TGTGTTGATGA AAGACAGAG GAAAGCTTC CGCTGACCC GACACCCTGA GAAGCGTAG GGGAAACGGT
T I S L G F C T D P H S C L F L A C F V L V L T Q S S I F S L L A
301 TCACATCAG CTTGGCTTC TGCAGCACT TTCACAGCTG CCTCTCTCC GCTTCCTTCG TCGTGGTGT CACACAGAGC TCCATCTTTA GCTCTTGGC
AGTGTAGTTC GGACCGAAG ACCTGCCGA AAGTGTGAC GGAAGAGAG CCGACGAAGC ACGACACGA GTGTGTCTCG AGGTAGAAAT CCGAGAACCG
V A V D R Y L A I R V P L R Y K G L V T G T R A R G I I A V L W V
401 GTGGCTGTC GACCGTATC TGGCATTGC CGTCCCGCTC AGGTATAAG GTTGGTAC TGGAACAGA GAAAGAGGA TCATCCGTGT CCTTGGGTC
CCACCGACAG CTGGCCATAG ACCGTAAGC GCAGGGGAG TCCATATTC CAACCACTG ACCTGTGCT CGTTCCTCT AGTAGGACA GGAGACCCAG
L A F G I G L T P F L G M H S K D R A T S H C T E P G D G I T N K S
501 CTGGCCTTGG GCATTGACT GACTCTTTC CTGGGTGGA ACAGTAAAGA CCGTGCCACC AGCACTGCA CAGAACCCTG GGATGGCATC ACGAATAAGA
GAACGGAAC CGTAACCTGA CTGAGGAAG GACCCAACCT TGTCAITTTCT GGCACGGTGG TCGTGAAGT GTCTTGGACC CCTACCGTAG TGCTTATCT
C C P V K C L F E N V V P H S Y M V Y F N F F G C V L P P L L I H
601 GCTGCTGCC TTGAAGTCT CTCTTGAAG ACGTAGTCC CATGAGTAC ATGTTTATT TCAACTCTT TG6GTGTC CTTCCTCAC TGCTCATCAT
CGAGACGGG ACACCTTACA GAGAATCTT TGCATCAGG GAACTGATG TACCAATAA AGTTGAGAA ACCCCACAGS GAAGAGGTTG ACCGATGATA
H V I Y I K I F H V A C K Q L O H M E L M E H S R T T L Q R E I H
701 GATGCTGATC TACATCAAAA TCTTCATGGT GGCCTGCAAG CAGCTCAGC ACATGGAAC GATGGAGCAC TCCAGGACA CCGTGCAGCG GGAGATCCAC
CTACCATAG ATGTAGTITT AGAATACCA CCGGACGTTT GTCGAAGTGC TGTACCTTGA CTACCTGCTG AGTCTGCTGT GCGACGTGCG CCTCTAGTGT
A A K S L A M I V G I F A L C W L P V H A I N C I T L F H P A L A K
801 GCGGCCAAGT CACTGGCTAT GATTGTGGG ATCTTTGCTC TGTGTGGCT CCCCCTGACT GGCATCACT GCATCACCTC CTTCATCCA GCGCTGGCCA
CGCCGCTTCA GTGACCGATA TAAACCCCG TAGAACAGAG ACACAACCGA GGGCCAGTA CGGTAGTTGA CGTAGTGGG GAAGTAGGT CCGGACCCGT
D K F K V W H N V A I L L S H A N S V V N P I V Y A Y R N R D F R
901 AGGACAAGCC CAAATGGGTG ATGAAATGG CCATCTCTCT GTCACACGCC AATTCAAGTG TCAATCCAT TGTCTATGCC TACAGGAACC GGGACTTCCG
TCTGTGCGG GTTATCCAC TACTATACC GGTAGGAGA CAGTGTGGG TTAAGTCAAC AGTTAGGGA AFAGATAGCG ATGTCTTGG CCGTGAAGG

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Graph: Figure 1. cDNA sequence of the A2BAR highlighting the Adora2b mutation, which caused an in-frame deletion. The red region denotes the mutated region, which resulted in deletion of the start codon (blue). The next available start codon is highlighted in blue. This mutated strain is predicted to express a truncated form of the A2BAR lacking the amino terminus, intracellular loops 1 and 2, extracellular loops 1 and 2, and the first 4 transmembrane segments. The green highlights indicate the primer set used for genotyping. Transmembrane regions are highlighted in light grey for easy identification of these areas deleted.

**Surgical procedures:** Male SD (Harlan Labs), SS or A<sub>2B</sub>AR mutant rats (200–400 gm) were used for the experiments. To create unilateral ischemia without compromise of airflow or reperfusion, we used a left anterior thoracotomy to complete an occlusive slip knot tie over the left main pulmonary artery under general anesthesia using isoflurane (2%), as we previously reported.[ 6] In brief, the left main pulmonary artery was identified on the superior aspect of the left mainstem bronchus and then dissected free from the bronchus. A 6-0 suture was passed around the left main pulmonary artery and tied.[ 6] Rats were allowed to recover from surgery, administered carprofen (5 mg/kg b.i.d. for 3 days) for analgesia and studied 1–7 days later.

In selected rats where indicated in the text, a second model of unilateral lung ischemia characterized by increased myeloperoxidase as we previously reported with PA and airway ischemia and reperfusion was employed.[18] A midline incision was made and the chest opened to access the left hilum. The left pulmonary artery, vein, and bronchus were stripped of connective tissue and then occluded with a microvascular clamp to induce ischemia. After 60 minutes, the clamp was removed to allow spontaneous reperfusion and reinitiation of lung ventilation. Lung tissue was harvested three hours after reperfusion.

**Adenosine BAL measurements:** Adenosine was measured by adaptation of methods described by Driver et al.[19] Twenty four hours after left pulmonary artery ligation (see earlier text), Sprague–Dawley rats were anesthetized with 2% isoflurane, intubated, and subjected to thoracotomy via a midline incision. The heart and

lungs were removed from the chest *en block*, after which the left and right lungs were lavaged separately two times with 3.0 mL of PBS (4°C) containing the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 2.5 μM) and the nucleotide transport inhibitor dipyridamole (250 μM) (stopping solutions). In the case of control rats, lavage fluid (5.0 mL) was collected from both lungs. BAL fluid was centrifuged (1000 g for 10 min at 4°C) and then passed through a 0.2 μ filter to remove cellular debris.

To determine the range and sensitivity of our assay as well as potential sources of loss with filtration, centrifugation or freezing, adenosine (cat# A9251 Sigma) was added to lavage fluid from an independent group of control rats to final concentrations of 2, 10, and 50 μM above endogenous adenosine levels either before or after centrifugation and filtration.

Briefly, an Agilent 1100 HPLC system was used and the samples (100 μL) were injected into Kinetex C<sub>18</sub> columns (Phenomenex, 100 mm × 4.6 mm, 2.6 μm) equipped with a guard column and equilibrated with an aqueous solution of KH<sub>2</sub>PO<sub>4</sub> (12.5 mM) containing 7.5% methanol. Isocratic elution of adenosine was achieved using the equilibration buffer at a flow rate of 1.4 ml/min. The retention time of adenosine was 4.2 min by UV detection at 260 nm. Peak area at 260 nm was used for quantification.

*RNA studies:* Total RNA from ~100 mg of right and left lung tissues were extracted with Trizol reagent (Cat# 15596-026, Invitrogen, USA), according to the manufacturer's instructions. The concentration and purity of RNA were determined by a Nanodrop spectrophotometer.

Quantitative<sup>Table 1</sup> real time PCR. Synthesis of cDNA was performed using the miRCURY LNA Universal RT microRNA PCR kit (Cat# 203301, Exiqon). A total of 100 ng of total RNA was used for reverse transcription with polyT-tagged universal primer. Briefly, the tubes were incubated for 60 min at 42°C, followed by heat-inactivation of the reverse transcriptase enzyme for 5 min at 95°C. Real-time PCR was performed with A<sub>2A</sub>AR, A<sub>2B</sub>AR and GAPDH (internal control) primers using recommended annealing temperatures for each primer set (IDT). For miRNA, real-time PCR was performed with miR-27a-3p (cat# 204764), miR-27b-3p (cat# 204782), and miR-128-3p (cat# 295677) LNA primers (Exiqon), as well as SYBR Green master mix (Cat# 203403 Exiqon). MiR-191a-5p (cat # 204306) was used as an internal control along with UniSP6 provided with the cDNA kit to account for efficiency of reverse transcription for miRNA analyses. PCR was carried out according to the manufacturer's instructions (Cat# 203301, Exiqon) using a Bio-Rad icycler (Model CFX 96 Real time system). The following cycling conditions were employed for miRNA: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 1 min. The expression level of mRNA in each sample was normalized to GAPDH and miRNA to either miR-191a-5p or to UniSP6. Then, relative expression levels in right and left lung samples were calculated using the CT values for the RNA of interest by using 2<sup>-ΔΔCT</sup> method.

*Western immunoblotting of A<sub>2A</sub>AR and A<sub>2B</sub>AR:* Western blot analysis was performed as described previously.[18] Briefly, whole lung homogenates in a buffer supplemented with protease inhibitor cocktail were centrifuged for 10 min at 20,000 g. Blots were developed with a primary antibody to A<sub>2A</sub>AR, A<sub>2B</sub>AR or β-actin (Sigma #A2228) and matched secondary antibodies conjugated to horseradish peroxidase. ECL detection reagent (Pierce # 32106) was used for visualization. The relative densities of protein bands were compared in images scanned with an Alpha Image 220 Analysis System, with β-actin used to correct for protein loading. Bands at ~48 and 37 kDa were observed in our immunoblots. The band just smaller than 50 kDa is consistent with immunospecific band identified by this Alomone antibody in rodent tissue.[20] Both bands were competed away by blocking peptide (Alomone Labs: [www.alomone.com/p/anti-A2BA%5fadenosine%5freceptor%5f\(extracellular\)/aar-003/114](http://www.alomone.com/p/anti-A2BA%5fadenosine%5freceptor%5f(extracellular)/aar-003/114)). The band just smaller than 50 kDa band was used for quantification as it was the most reproducible in our hands and was consistent with results by Vindeirinho et al.[20]

**Histology:** The heart and lungs were removed *en bloc* under isoflurane anesthesia. Lungs were fixed with paraformaldehyde in the inflated state. Four-micron thick slices of paraffin-embedded, fixed whole-mount coronal sections of left and right lung were stained for hematoxylin and eosin. Extent of BOOP was quantified by assessing percentage of the cross sectional area of the lung involved at low power, morphology confirmed with high-resolution JPEG images, as we previously reported.[ 6] Histology consistent with BOOP (Masson bodies, inflammation, and young fibroblasts filling the distal airways and alveoli) was assessed with high power images. Using a scale developed for quantification of BOOP,[ 6], [21] we also performed grading of epithelial injury, fibrosis, and inflammatory changes (EFI) in the most severely affected areas of cross sectional lung images, each on a zero to three scale. The presence or absence of pulmonary vascular changes (thickening of intima or media or vascular drop out) and necrosis were noted in each image (Table 1). Two blinded reviewers graded all images. Average values were used for analysis.

Table 1. To quantify BOOP, we developed a grading scale for our studies[ 6] based on methods to quantify BOOP in human transplant patients.[21] This scale includes grading epithelial injury, fibrosis, and inflammatory changes, each on a scale from zero to three, and estimation of the percentage area of the lung exhibiting BOOP. Images were graded by two investigators blinded to the treatment group.

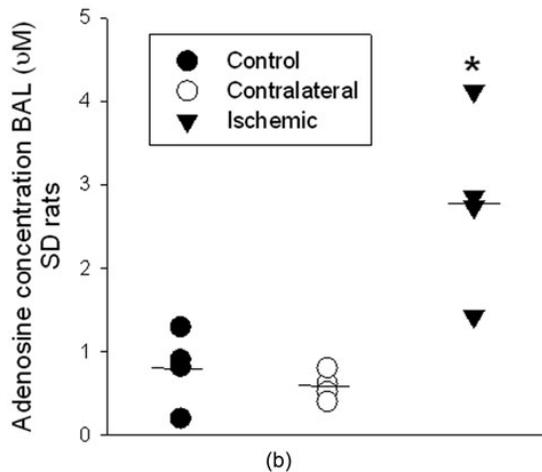
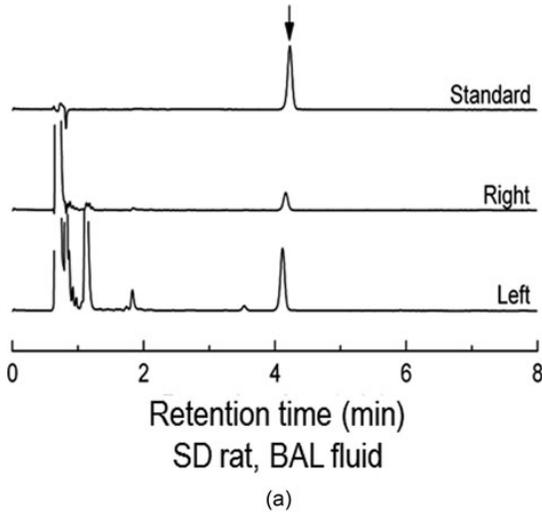
Histological grading of BOOP				
Grade	0	1 (mild)	2 (moderate)	3 (severe)
E (epithelial)	Normal	Reactive epithelium ±intermittent disruption of epithelial layer	Moderately prevalent disruption and loss of epithelium	Epithelium largely denuded
F (fibrotic)	Normal	Young fibroblasts	Intraluminal fibromyxoid filling	Dense fibrosis
I (inflammatory)	Normal	Perivascular or peribronchial cuffing with mixed inflammatory cells	Moderate mixed inflammatory infiltration throughout the lungs	Severe infiltration of mixed inflammatory cells
Percent involvement	(from 0 to 100% in 10% increments)			

**Statistical analysis:** All analyses were not performed on all rats, as some (e.g., histological analysis and RNA extraction) were mutually exclusive. For each assay, a minimum of four, and typically five to eight, separate rats were studied per group. The group size is shown in the figure legends, figures, text, or table. Comparisons between ischemic and nonischemic lungs were performed using *t* tests or rank sum tests followed by Mann–Whitney Rank Sum test when data were not normally distributed. Grouped data for three or more conditions were compared using one-way analysis of variance. When differences were found, a Holm–Sidak's post hoc test was employed. Statistical significance was assumed for  $p < 0.05$ . All grouped data shown in figures are presented as means ± standard error of the mean (SEM), means ± standard deviation (SD), or medians and confidence intervals in the case of nonparametric data.

## Results

### Adenosine concentrations in BAL

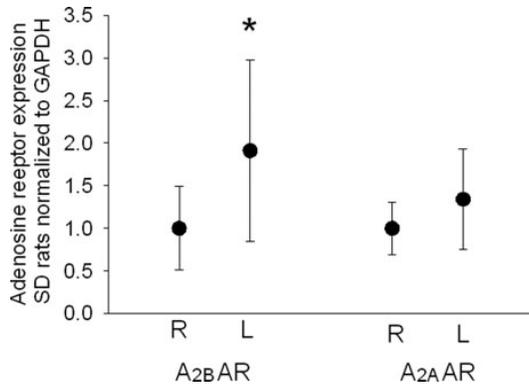
Representative HPLCs for quantification of adenosine concentrations appear in Figure 2a. Adenosine concentration values for control lungs of SD rats were not different than those from contralateral, nonischemic lungs from SD rats ( $0.89 \pm 0.23 \mu\text{M}$  vs.  $0.59 \pm 0.09$ ;  $n = 4$  each). BAL from ischemic relative to contralateral lungs exhibited adenosine concentrations, which were increased approximately fourfold over those of contralateral lungs 24 h after PA ligation (Figure 2a and 2b).



Graph: Figure 2. Adenosine concentrations were measured in BAL from four SD control rats or from isolated left and right lungs from four SD rats collected 24 h after left pulmonary artery ligation. Figure 2a shows representative chromatograms from an adenosine standard ( $3 \mu\text{M}$ ) and from BAL collected from the right and left lungs of ischemic/reperfused rats. The graph in 2b shows individual values for samples. The concentration of adenosine was increased more than fourfold in BAL from ischemic lungs versus contralateral non-ischemic lungs ( $*p < 0.008$ ). Horizontal lines represent median values for each of the three groups.

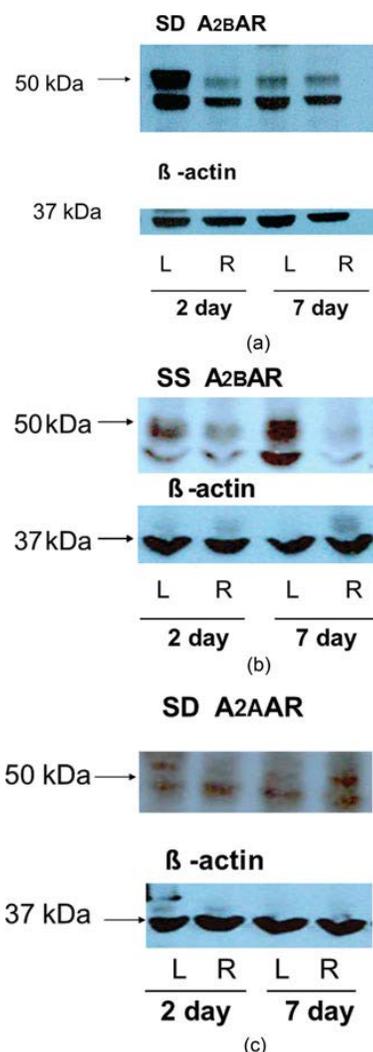
### Adenosine receptor mRNA and protein expression

Next we evaluated  $A_{2A}$ AR and  $A_{2B}$ AR mRNA expression in ischemic versus nonischemic lungs. Message for  $A_{2B}$ AR ( $n = 17$  pairs, Mann–Whitney Rank Sum Test  $p = 0.03$ ) but not  $A_{2A}$ AR ( $n = 13$  pairs, Mann–Whitney Rank Sum Test  $p = 0.20$ ) was increased in ischemic lungs of SD rats 7 days after tie off of the left pulmonary artery (Figure 3).

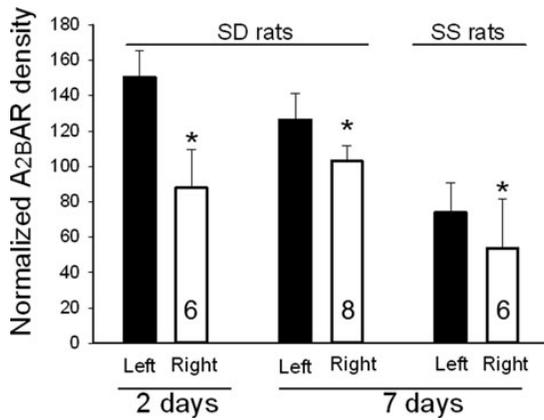


Graph: Figure 3. Graphic representation showing GAPDH normalized expression of A<sub>2B</sub>AR and A<sub>2A</sub>AR mRNA from ischemic (L = left) and nonischemic (R = right) in 13 SD rats 7 days after left PA tie. The bars represent medians and bars show confidence intervals. A<sub>2B</sub>AR expression was greater in ischemic than nonischemic lungs ( $p = 0.03$ ) while A<sub>2A</sub>AR was not different ( $p = 0.20$ ).

Western blots of lung homogenates from ischemic left lungs of SD rats exhibited increased anti-A<sub>2B</sub>AR immunoreactivity at two and seven days after unilateral ligation of the pulmonary arteries (Figures 4a and 5). Similarly, SS rats had increased anti-A<sub>2B</sub>AR immunoreactivity in ischemic versus contralateral non-ischemic lungs (Figure 4b and 5). In contrast, anti-A<sub>2A</sub>AR immunoreactivity from the same rats was not different between ischemic and nonischemic lungs (Figure 4c;  $n = 9$  pairs,  $p = 0.8$ ). In a second lung injury model involving unilateral warm ischemia of the lung for one hour followed by *in vivo* reperfusion for three additional hours, A<sub>2B</sub>AR protein density normalized to beta actin was also increased in ischemic relative to contralateral lungs ( $n = 8$  pairs;  $125 \pm 10$  vs.  $90 \pm 12$ ,  $p < 0.05$ ; data not shown).



Graph: Figure 4. 4a shows representative Western blots of homogenates from ischemic (L = left) and contralateral (R = right) lungs from SD rats harvested either 2 or 7 days after pulmonary artery ligation probed with the A<sub>2</sub>BAR antibody. A prominent band under 50 kDa was observed in all of the studies along with a secondary band near 37 kDa in some instances. The larger band just under 50 kDa was used for quantification. After quantification of A<sub>2</sub>BAR protein, membranes were probed for beta actin, which was not different in left and right lungs. Beta actin was used to normalize A<sub>2</sub>BAR immunoreactivity for protein loading. Ischemic left lungs exhibited increased A<sub>2</sub>BAR immunoreactivity compared to their control right lung counterparts. 4b. Representative images from studies similar to those in Figure 4a except that homogenates were obtained from lungs of SS rather than SD rats. Again, increased A<sub>2</sub>BAR immunoreactivity in ischemic left compared to contralateral right lungs was observed seven days after ischemia. 4c. Representative Western blots from the same samples as Figure 4a (SD rats) probed with primary A<sub>2</sub>AAR antibodies. The band between 37 and 50 kDa was quantified.[20] Bands were less consistent than those probed A<sub>2</sub>BAR antibody and no differences in A<sub>2</sub>AAR immunoreactivity between ischemic and contralateral lungs were observed.



Graph: Figure 5. Graph shows averaged density of A<sub>2B</sub>AR immunoreactivity normalized to beta actin in lung homogenates from left and right lungs of SD rats at 2 (n = 6) and 7 days (n = 8) after ischemia, and in SS rats seven days (n = 6) after the same ischemic injury. These are aggregate data including those examples shown in Figure 4. "N's in the bars represent the number of paired samples on each group. In each case, the density of immunoreactivity is increased in ischemic left over contralateral right lungs with p < 0.005 by paired t test. Bars represent standard deviation.

Expression of the A<sub>2B</sub>AR is reported to be modulated by miRNAs 27a-3p, 27b-3p, and 128-3p.[14] RT-qPCR data normalized for an internal miRNA, miR191-5p demonstrated decreased 27a and 27b miRNA in ischemic versus nonischemic lung tissue at 7 days (Table 2). Normalization for the spiked sequence UniSP6 also showed decreased 27a-3p miRNA in ischemic lungs compared to contralateral controls. There was no difference in miR191-5p expression when normalized to UniSP6. These results confirm a decrease in miR27a-3p after ischemia.

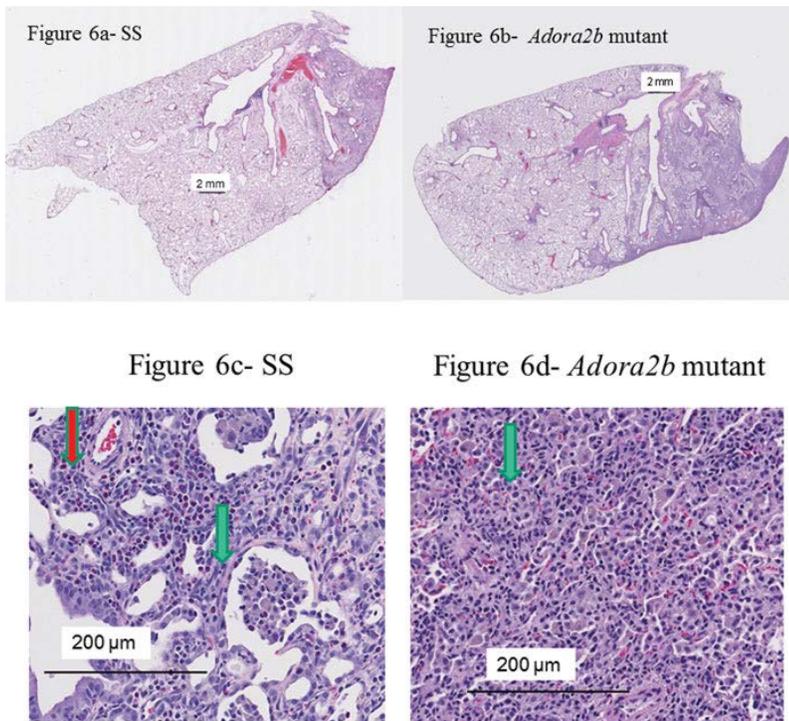
Table 2. qPCR and p-values for miR27b-3p, miR128-3p, miR27a-3p, and miR191-5p normalized to either 191-5p or UniSP6 for left and right lungs harvested 7 days after ischemia. Once normalized for 191-5p or UniSP6, qPCR values for right lung (nonischemic) were set at 1, the left lung values expressed as a fraction of the right. P-values are different in left and right lungs for miR27b-3p and miR27a-3p when qPCR values are normalized to miR191, and for miR27a when normalized for UniSP6.

	Normalized by	Nonischemic R (n = 8) mean ± SD	Ischemic L (n = 8) mean ± SD	Sig by t test
miR 27b-3p	miR191	1 ± 0.11	0.82 ± 0.17	*P = 0.021
miR 128-3p	miR191	1 ± 0.28	0.68 ± 0.26	P = 0.976
miR 27a	miR191	1 ± 0.17	0.66 ± 0.07	*P = 0.000135
miR191-5p	SP6	1 ± 0.12	1.04 ± 0.17	P = 0.610
miR 27b-3p	SP6	1 ± 0.08	0.86 ± 0.23	P = 0.113
miR 128-3p	SP6	1 ± 0.09	0.97 ± 0.11	P = 0.551
miR 27a	SP6	1 ± 0.1	0.7 ± 0.13	*P = 0.00014

## Histology

We have reported that unilateral ischemia of the lung induced by pulmonary artery tie results in BOOP and inflammatory changes seven days after surgery in SD rats.[ 6] To begin to test the contribution of A<sub>2B</sub>AR expression to the development of BOOP, we quantified percent BOOP in ischemic lungs of a genetically modified rat strain with deletions of A<sub>2B</sub>AR relative to the background strain (Dahl SS).[22] Representative images of cross sections of whole mount ischemic lungs appear in Figure 6(a–c). We observed more lung involvement by BOOP in rats genetically modified by *Adora2b* disruption (Table 3 and Figure 6a–d). Additional studies to assess for injury were completed. Higher epithelial and inflammatory but not fibrotic scores were assigned to images of

lung cross sections from Adora2b disrupted rats (Table 3). Pulmonary vascular changes (thickened media or intima) were not different between Adora2b mutant rats and background controls (Table 3). Scattered pockets of necrosis were observed in one of five and one of four cross sectional areas, respectively, of a SS control rat and an Adora2b rat (also not different and not shown).



Graph: Figure 6. Representative histological images from lung cross sections from SS (a and c) and SS-AdorA2b mutant rats (b and d) seven days after ischemia. Low power (a and b) shows areas of increased cellularity, which were consistent with BOOP under high power magnification (c and d). The area of BOOP is modestly larger in the image from lungs of mutant rats. The green arrows in the magnified images (c and d) show typical epithelial changes, whereas the red arrow shows perivascular inflammation (c). In some images from mutants (not shown), areas of inflammation outside sections showing significant epithelioid changes were observed.

Table 3. Mean and SEM as well as median and 95% confidence interval values for epithelial, inflammation, and fibrosis changes in images from lungs scored by investigators blinded to the treatment group. Epithelial and inflammatory changes were different in ischemic lungs of SS and SS-Adora2b mutant rats while fibrotic changes were not. Confidence intervals: CI and standard error of the mean: SEM.

	Dahl SS (n = 5)	A2bAR knock out (n = 4)	P-value relative to Dahl SS
%BOOP			
Mean/median	18/25	60/67.5	0.02*
SEM/CI	3.4/19	10/32	
Epithelial			
Mean/median	1.08/1.00	1.6/2.0	0.031*
SEM/CI	0.15/0.3	0.18/0.39	
Inflammation			
Mean/median	1.5/2.0	2.43/2.0	0.048*
SEM/CI	0.19/0.43	0.21/0.44	
Fibrosis			
Mean/median	0.79/1.0	1.32/2.0	0.5 ns

SEM/CI	0.19/0.42	0.26/0.55	
Vascular changes			
Mean/median	0.2/0.0	0.5/0.5	0.4 ns
SEM/CI	0.5/0.55	0.29/0.92	

## Discussion

We provide compelling evidence that expression of A<sub>2B</sub>AR mRNA and/or protein is increased in the lungs of two different rat strains (SD and SS) for at least seven days following ischemia by either isolated pulmonary artery ligation or hilar ligation (no ventilation or perfusion) with ischemia/reperfusion. Coincident with increased A<sub>2B</sub>AR expression, a candidate miRNA for at least one of three sequences reported to suppress *Adora2BA* transcription, miR27a, is decreased. Physiologically, we observed increased adenosine levels in bronchoalveolar lavage fluid and increased BOOP in ischemic lungs from rat strains that contain mutations in *Adora2b*. Collectively, these findings indicate that adenosine signaling via the A<sub>2B</sub>AR may mediate a protective role in ischemia-induced BOOP.

Adenosine and its receptors have an established modulatory role during acute organ injury as well as during tissue remodeling that occur during chronic inflammatory states. Extracellular adenosine elicits biological effects through activation of four G-coupled protein adenosine receptors (A<sub>1</sub>AR, A<sub>2A</sub>AR, A<sub>2B</sub>AR, and A<sub>3</sub>AR).[23] In the lung, A<sub>2B</sub>AR expression has been reported in bronchial epithelial cells in both medium and large airways, pulmonary vascular cells, and alveolar macrophages.[24], [25] A major site of A<sub>2B</sub>AR translation is the type II alveolar epithelial cell.[24] Endogenous adenosine released following cell injury has been reported to protect against I/R injury in lungs.[26] Further, protection from I/R in isolated perfused rabbit lungs is achieved with treatment by agonists for A<sub>1</sub>, A<sub>2A</sub>, or A<sub>3</sub> adenosine receptors.[27] In these models, decreased elaboration of the proinflammatory cytokine, TNF- $\alpha$ , and decreased neutrophil sequestration are postulated to contribute to the overall improvement in pulmonary function, a signaling pathway triggered by adenosine receptor binding. Koeppen *et al.* report that A<sub>2B</sub>AR activation requires higher ligand concentrations as compared to other adenosine receptors.[ 7] Given the abundance of adenosine during I/R and induction of A<sub>2B</sub>AR expression, signaling by this pathway likely promotes homeostasis. We propose that this compensatory mechanism serves an important role to protect the lung tissue from remodeling to a state of BOOP.

While some studies demonstrate a protective role by A<sub>2A</sub>AR signaling during lung injury[27], [28], [29], [30] we did not detect changes in expression of this important adenosine receptor subtype in our studies following unilateral lung ischemia. A<sub>2B</sub>AR expression, however, increases over time after ischemia and I/R (Figures 3 and 5). This result implicates A<sub>2B</sub>AR expression as an inducible and adaptive response to ischemia. Notably, a binding site for hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) exists within the A<sub>2B</sub>AR promoter.[31], [32], [33]

The role of A<sub>2B</sub>AR signaling in all cause injury is controversial. On the side of promoting injury, A<sub>2B</sub>AR stimulation has been reported to promote the development of BO in a heterotopic tracheal implantation mouse model by inhibiting CD4+/CD25+/Foxp3+ regulatory T cell infiltration.[ 9] In the presence of hypoxia, A<sub>2B</sub>AR activation synergistically activates human fibroblasts, presumably leading to fibrosis.[32] Activation of A<sub>2B</sub>AR in smooth bronchial muscle cells enhances release of IL-6 and monocyte chemotactic protein (MCP-1).[34] A<sub>2B</sub>AR transcriptionally activates HIF-1 $\alpha$  and hence favors an ischemic response.[ 7] Most germane to the present studies, disruption of the A<sub>2b</sub> adenosine receptor in the rats used for our studies is associated with increased body weight, delayed glucose clearance, modestly elevated systemic blood pressures with age, and reduced cytokine production after challenge with lipopolysaccharide.[22] These data would suggest increased inflammatory responses evoked by ischemia in our genetically modified rats are either mediated by different cytokines than those evoked by LPS, or is driven largely by noncytokine dependent signaling.

Several other models demonstrate *protection* with A<sub>2B</sub>AR activation. Pharmacologic inhibition and/or genetic elimination of A<sub>2B</sub>AR *increases* murine endotoxin-induced lung injury.[12] In a murine model of LPS-induced lung injury, activation of A<sub>2B</sub>AR on pulmonary endothelial cells decreases microvascular permeability and neutrophil migration into alveolar spaces.[35] A<sub>2B</sub>AR-deficient mice enjoy greater survival than their wild-type counterparts in polymicrobial sepsis.[36] A<sub>2B</sub>AR signaling enhances amiloride-sensitive fluid transport and elevation of pulmonary cAMP levels following ventilator associated lung injury in mice, suggesting that A<sub>2B</sub>AR agonist treatment protects by facilitating fluid resorption from the lungs.[10] Perhaps most applicable to our data, ischemia reperfusion injury to the liver in mice is associated with robust induction of A<sub>2B</sub>AR transcript and protein.[37] Mice with *Adora2BA*-deletion exhibited increased sensitivity to injury. Finally, treatment of wild-type mice with an A<sub>2B</sub>AR-selective antagonist resulted in worsened liver injury, whereas A<sub>2B</sub>AR-agonist treatment attenuated hepatic injury in wild type, but not in *Adora2BA*<sup>-/-</sup> mice. Our data demonstrating increased BOOP 7 days after ischemia in *Adora2BA* mutant rats add additional credence to a protective role for the adenosine/A<sub>2B</sub>AR signaling axis after ischemia. To our knowledge, this is the first report that A<sub>2B</sub>AR signaling is protective from BOOP after ischemic injury in the lung.

Micro RNA (miRNA) are short (20–30 nucleotide) translational regulator sequences encoded in the eukaryotic DNA genome. Their usual function is to bind complementary single-stranded mRNA sequences undergoing translation by ribosomal complexes. When this binding occurs, the ribosomal translation is halted, resulting in an ineffective protein product. At least two miRNAs have been reported to modulate posttranscriptional expression of A<sub>2B</sub>AR in a model of mouse colitis: miR27b, miR128a.[14] These miRNAs decrease A<sub>2B</sub>AR translation in colonocytes from this model. Furthermore, miR27b and miR128a transcription was suppressed by TNF- $\alpha$ . [14] Our data demonstrate decreases in miR27a and 27b, consistent with the hypothesis that these miRNA contribute to post-transcriptional increase of A<sub>2B</sub>AR after injury. Further studies will help determine the factors/cytokines that are induced by ischemia to regulate miRNA expression, especially downregulation of miR27a.

There are limitations to our studies. First, the cellular sources of increased A<sub>2B</sub>AR expression in ischemic lungs remain to be determined. Unfortunately, attempts at immunohistochemical localization were unsuccessful due to interference by background staining. Second, the specificity of commercially available antibodies for adenosine receptors has been questioned raising concerns regarding accuracy of assessing changes in adenosine receptor protein expression by immunological approaches. Nevertheless, we followed procedures utilized successfully in several previously published reports.[ 7], [20], [36], [37] With this in mind, it was important that changes in A<sub>2B</sub>AR protein expression in lung tissue observed in our studies by Western immunoblotting were paralleled by changes in mRNA expression. Although we did not obtain evidence for increased A<sub>2A</sub>AR expression, our data do not exclude a role for adenosine signaling through these or other adenosine receptor subtype during ischemia-induced BOOP. They do suggest, however, that increases in A<sub>2A</sub>AR expression and cross-reactivity with A<sub>2B</sub>AR do *not* account for the increases in A<sub>2B</sub>AR protein we observed in ischemic lungs. Lastly, the effects seen may be due to other factors besides tissue oxidative state (e.g., resulting from hemostasis in the pulmonary circulation and resultant proinflammatory changes in the endothelium).

Our work provides strong justification for additional studies. If A<sub>2B</sub>AR signaling protects from ischemic lung injury, then this axis affords a target to diminish the *morbidity and mortality* resulting from lung transplantation.[ 1], [ 2] Given the ~50% 5-year mortality from BO in transplanted lungs and the extensive waiting lists for those with end-stage lung disease,[38], [39], [40] interventions that may extend the survival of transplanted lung recipients by even a few years with good functional capacity are highly desirable. Successful therapies will most likely be based on countering mechanisms of injury or enhancing protective signaling pathways. Our work provides justification for further studies of A<sub>2B</sub>AR-mediated events as a strategy to enhance protection of ischemic lungs.

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## Declaration of interest

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## Footnotes

1 Color versions of one or more of the figures in the article can be found online at [www.tandfonline.com/ielu](http://www.tandfonline.com/ielu).

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