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Human Recombinant Apyrase Therapy Protects Against Canine Pulmonary Ischemia-Reperfusion Injury

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Abstract

INTRODUCTION—There is accumulating evidence that extracellular adenosine triphosphate (eATP) promotes many of the underlying mechanisms that exacerbate acute lung injury. However, much of this data is from inbred rodent models indicating the need for further investigation in higher vertebrates to better establish clinical relevance. To this end we evaluated a human recombinant apyrase therapy in a canine warm pulmonary ischemia-reperfusion injury (IRI) model and measured eATP levels in human lung recipients with or without primary lung allograft dysfunction (PGD).

METHODS—Warm ischemia was induced for 90 minutes in the left lung of 14 mongrel dogs. Seven minutes after reperfusion, the apyrase APT102 (1 mg/kg, N=7) or saline vehicle (N=7) was

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injected into the pulmonary artery. Arterial blood gases were obtained every 30 minutes up to 180 minutes after reperfusion. Bronchioalveolar lavage fluid (BALF) was analyzed for eATP concentration, cellularity and inflammatory mediator accumulation. Thirty bilateral human lung transplant recipients were graded for immediate early PGD and assessed for BALF eATP levels.

RESULTS—APT102-treated dogs had progressively better lung function and less pulmonary edema over the 3-hour reperfusion period when compared to vehicle-treated controls. Protection from IRI was observed with lower BALF eATP levels, fewer airway leukocytes and blunted inflammatory mediator expression. Additionally, human lung recipients with moderate to severe PGD had significantly higher eATP levels when compared to recipients without this injury.

CONCLUSIONS—Extracellular ATP accumulates in acutely injured canine and human lungs. Strategies that target eATP reduction may help protect lung recipients from IRI.

Ischemia-reperfusion injury is the major driver of primary lung allograft dysfunction, a form of acute lung injury that limits both short and long-term survival of lung transplant recipients[1–4]. Pathological features of lung IRI are most prominent in the alveoli where neutrophil congestion, coagulopathy and hemorrhaging lead to the hypoxemia and impaired fluid clearance characteristic of patients with PGD[5]. Although the underlying mechanisms of IRI have not been completely elucidated, it is well established that the accumulation of danger associated molecular patterns (DAMPs) can induce the inflammatory mediator production and leukocyte extravasation associated with acute lung injury[3, 6, 7]. In an attempt to find DAMPs that play a key role in exacerbating lung transplant-mediated IRI we recently reported that there is a significant rise of the DAMP eATP in the bronchioalveolar lavage fluid from rat lung isografts injured by prolonged cold preservation[8].

In healthy tissues eATP is normally maintained in very low concentrations by ectonucleotidases such as triphosphate diphosphohydrolase 1 (CD39), alkaline phosphatases and 5′-ectonucleotidase (CD73). In particular, CD39 and CD73 work in tandem to hydrolyze ATP and ADP to AMP and ultimately generate adenosine, a purine that enforces tissue homeostasis by negatively regulating immune cell activation[9, 10]. However, in injured tissues ATP is released in high amounts, which seemingly overwhelms the pyrophosphatase activity of the endogenous ectonucleotidase network. This in turn leads to eATP engagement of P2 family purigenic receptor family members that promote inflammatory mediator expression[11]. For example, eATP stimulation of the $P2X_7$ receptor triggers Nod-like receptor, pyrin domain containing 3 (NLRP3) inflammasome activation leading to IL-1β post-translational modification and release[12–14].

Data from experimental models of acute lung injury have been important to decipher the relationship between ATP release and pulmonary function. In mouse airways eATP levels were shown elevated in models of acute respiratory distress syndrome and ventilation-induced airway injury[15, 16]. Also, our group has demonstrated that a modified soluble form of the human ecto-ATP diphosphohydrolase CD39L3 (APT102) is effective at depleting eATP and preventing lung transplant-mediated IRI in rats[8]. However, in higher vertebrates it remains unclear if eATP accumulates in lungs damaged by IRI or whether it exacerbates pulmonary dysfunction. In this study we measured BALF eATP levels in a model of canine pulmonary IRI and in human lung recipients with varying degrees of PGD severity. Our

results show that BALF eATP levels are elevated in both dog and human lungs exposed to IRI. Furthermore, prevention of eATP accumulation with APT102 administration protects dogs from lung IRI.

Methods

Animals and Reagents

14 mongrel dogs (Oak Hill Genetics, Ewing, IL), weighing 22 to 30 kg, were used in all experiments. All surgical and experimental procedures were approved by the Animal Studies Committee at the Washington University School of Medicine. Animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. APT Therapeutics of St. Louis, MO supplied APT102, a recombinant soluble form of the modified human ecto-ATP diphosphohydrolase CD39L3.

Operative procedure and APT102 treatment

For surgery, dogs were sedated with propofol (5–7 mg/kg), intubated and mechanically ventilated at a tidal volume average of 20 ml/kg and a rate of 12 breaths/min to maintain a physiological P_{CO2}. A positive end-expiratory pressure of 5.0 cm H_2O, and an inspired O_2 fraction (FiO_2) of 1.0 was maintained and anesthesia during surgery was administered with inhalation of between 1 to 3% isoflurane and intravenous 7–15 μg/kg/hr of hydromorphone hydrochloride. The left carotid artery was dissected and catheterized for continuous pressure monitoring and arterial blood gas sampling. The right jugular vein was dissected and catheterized for continuous monitoring of right heart pressure and serum sampling. Periodic palpebral reflex and spontaneous movements were observed to monitor the depth of anesthesia. A left thoracotomy was performed in both groups. The chest incision was made between the 4th and 5th intercostal space to get access to the left main bronchus, pulmonary veins and artery. Hilar stripping of the left lung was performed along with complete transection of the nerves, bronchial arteries and lymphatics to prevent possible collateral circulation. Stripping of the right pulmonary artery was performed and warm ischemia was induced for 90 minutes by clamping the left pulmonary artery (LPA) and veins, and by excluding the left lung from ventilation via insertion of a bronchial blocker (Phycon, Hamberg Germany). After unclamping the LPA and removal of the bronchial blocker, the right pulmonary artery was clamped for 15 minutes, to normalize blood flow into the left lung due to transient pulmonary resistance following ischemia. After 7-minutes of reperfusion to the left lung, APT102 (1 mg/kg) (N=7) or saline (N=7) along with 250 mg of SoluMedrol (methylprednisolone) was administered into the LPA. For single lung ventilation peak airway pressures (PAP) were initiated at an average of 20 cm of H_2O for a period of no longer than 20 minutes at the start of single lung ventilation and then kept near 10 cm of H_2O for the duration of the procedure. Minute ventilation adjustments were also made to maintain CO_2 levels at 35mm of Hg. The average tidal volume ultimately used for the study was 15 ± 5 ml/kg. Sodium Bicarbonate was administered as needed at 30 minutes after reperfusion to counter hyperkalemia. Sodium heparin was also administered to keep the activated clotting time above 350 seconds. Left lung bronchial lavage fluid (BALF) and

biopsies were obtained at 90 and 180 minutes after reperfusion. At 180 minutes after reperfusion saturated KCl was administered for euthanization while under deep anesthesia.

Measurement of blood gas

Arterial blood samples were taken for blood gas analysis before ischemia and every 30 minutes after reperfusion. The arterial partial pressure of oxygen (P_aO_2) was determined with a blood-gas analyzer (Nova Biomedical, Stat Profile, PhoxPlusC).

Bronchoalveolar lavage analysis

The left lung was lavaged twice with saline (40 mL per lavage). BAL was centrifuged at 1500 rpm for 8 minutes within an approximately thirty-minute period after isolation from the patient. The pellet was resuspended in PBS, and total cell numbers were counted with a hemocytometer using Trypan blue to exclude dead cells. A portion of BALF was further clarified by centrifugation ($10^4 \times g$, 5 min) and then stabilized with heat (95 °C for 20 minutes) for measurement of eATP concentration using Enzilight ATP assay kits (BioAssay Systems, Hayward CA) in accordance with manufacturers recommendations. Remaining BALF portions were analyzed for Tumor necrosis factor-α (TNF-α), inteleukin-8 (IL-8) and interleukin-1β (IL-1β) levels by ELISA (BioSource International, Inc, Camarillo, CA & Genway Biotech San Diego, CA) in accordance to manufacturer's instructions.

Wet-to-Dry Lung Weight Ratio

Left lung tissues of 100 to 400 mg were used for wet-to-dry weight ratio measurements before ischemia, at 90 and, 180 minutes after reperfusion. The tissues were excised from the left lung using a TA 60mm Stapler (Auto suture, Covidien, USA). Tissues were biopsied from non-dependent areas of the lung. The wet weight was measured first. The dry weight was measured after drying the tissue at 80°C for 72 hours. The wet to dry ratio was calculated using the following formula: wet weight (mg) − dry weight (mg)]/dry weight (mg).

Histologic Studies

The left lung specimens were used for histologic study were obtained at 90 and 180 minutes after reperfusion. Specimens were fixed in 10% buffer formalin, dehydrated, and embedded in paraffin. Sections (3 to 5 μm) were cut, mounted and stained with hematoxylin–eosin. PMNs were counted under oil immersion light microscopy (BX-51 microscope, Olympus, Japan) at 1000x magnification in 10 fields randomly selected for each specimen. The total number of PMNs was calculated only within whole alveoli. To measure alveolar thickness, we evaluated six randomly selected viewing fields (with size of 65.7 μm^2 at 400x magnification). Then, ten randomly selected alveolar septa were measured within each field using computer image analysis software (ImageJ 1.47V, NIH, USA).

Study Subjects

Thirty consecutive consenting adults undergoing bilateral lung transplantation at the Barnes-Jewish Hospital were prospectively enrolled in this study from 2011 to 2013. PGD was graded in accordance with ISHLT guidelines[3] immediately after arriving to the intensive

care unit where BAL was isolated between 6 to 12 hours later as part of our standard of care. Patients were excluded from the study for other causes of acute lung allograft injury consistent with the definition of PGD, which include cardiogenic pulmonary edema, hyperacute rejection, pneumonia and anastomosis complications.

Statistical Analyses

All values are described as means ± standard deviation (SD). Statistical analysis to assess significant differences between experimental groups was performed by using non-parametric Mann-Whitney test. For mean comparisons for greater than two groups non-parametric Kruskal-Wallis test was performed. In all cases, a probability value of less than 0.05 was considered significant. All statistical analyses were performed using GraphPad prism 5.0 (GraphPad Software Inc, La Jolla, CA).

Results

Apyrase treatment preserves the function of acutely injured dog lungs

In order to study the effects of eATP on acute pulmonary injury in higher vertebrates, canine left lungs were subjected to warm ischemia for 90 minutes and 7 minutes after reperfusion the recombinant apyrase APT102 or saline vehicle was administered directly into the left pulmonary artery. Importantly, immediately after APT102 or vehicle treatment methylprednisolone was also injected into the left pulmonary artery to model steroid immunosuppression that is commonly intra-operatively administered to human lung transplant recipients. As refractory hypoxemia is a defining characteristic of acute lung injury we assessed arterial oxygen saturation from the carotid artery over 30-minute intervals up to 3 hours post-reperfusion at a FiO_2 of 1.0 (Fig. 1A). APT102-treated dogs trended with moderately better P_aO_2 levels at 1-hour post-reperfusion and then became sharply less hypoxic at time intervals greater than 2 hours after reperfusion when compared to vehicle-treated recipients. Of note, P_aO_2 measurements from the pulmonary vein were not significantly different from the carotid artery at the conclusion of the study (Fig. 1B). Consistent with these observations we also noted significantly less edema in pulmonary biopsies obtained at 90 and 180 minutes post-reperfusion in APT102-treated dogs (Fig. 1C).

Elevated extracellular ATP levels in acutely injured dog and human lungs

To measure ATP release from pulmonary tissues BALF was obtained from vehicle and APT102-treated dogs at 90 and 180 minutes following reperfusion and assayed for eATP concentration. In vehicle-treated dogs eATP levels remained in a range above 100 nM at both time points. By contrast, in dogs that received APT102 eATP levels remained in a tight distribution around 16 nM over the same time period (Fig. 2A). Notably, we also observed a significant decline in circulating K^+ in APT102-treated dogs suggesting protection against hyperkalemia (Fig. 2B).

We next analyzed BALF eATP concentrations from thirty human bilateral lung transplant recipients (Fig. 3 and Table 1). Lung recipients were scored in accordance with ISHLT criteria[3] for immediate early PGD where 0 (none), 1 (mild), 2 (moderate) and 3 (severe) represent increasing grades of acute lung injury. As compared to lung recipients without

PGD eATP levels were significantly higher in patients with either moderate or severe PGD. Furthermore, transplant recipients with mild PGD also had less eATP accumulation when compared to recipients of lungs with more severe grades of PGD. Importantly, there was no correlation between some common indications for lung transplantation such as interstitial lung disease, chronic obstructive pulmonary disease or cystic fibrosis and ATP release, suggesting that accumulation of this DAMP is regulated directly by the allograft.

Attenuation of pulmonary tissue inflammation in APT102 treated dogs

We next wanted to determine if apyrase treatment inhibited cytokine production known to exacerbate acute lung injury. In vehicle-treated dogs we observed high levels of IL-8, TNF-α and IL-1β in the BALF with a marked downward expression trajectory between 90 and 180 minutes after reperfusion (Fig. 4). However, APT102-treated dogs had significantly lower concentrations of cytokines at both of these time points. Along with less inflammatory mediator expression there were also fewer BAL leukocytes in these dogs (Fig. 5A). Moreover, histological analysis of lung biopsies showed less evidence of tissue inflammation as APT102-treated dogs had lower numbers of alveolar neutrophils (Fig. 5B and C) and reduced alveolar septal thickness at both 90 (5.44 ± 0.29 vs. 11.4 ± 0.94; $p<0.001$) and 180 (3.52 ± 0.21 vs. 9.29 ± 0.99; $p<0.001$) minutes post-reperfusion as compared to canines treated with vehicle.

Discussion

Over the last decade data from rodent models have led to considerable progress in uncovering the mechanisms that promote eATP-mediated acute inflammation[17–19]. Studies of mice deficient in CD39 and CD73 ectonucleotidases have shown an increased susceptibility to ventilator-induced lung injury[9], LPS-mediated acute lung injury[20], and hyperoxia mediated-pulmonary injury[21]. Additionally, the lack of the $P2X_7$ receptor in mice results in resistance to LPS-mediated acute lung injury[11, 22]. Lastly, we have reported that apyrase treatment of rat lung isografts injured by prolonged cold preservation enhances graft function[8]. Although these accounts clearly point to the benefits of inhibiting eATP-mediated pulmonary inflammation in rodents, it still remains controversial if data derived from murine models of acute inflammation is relevant to the clinic[23]. To this end we sought to further analyze the role of eATP in dog and human lung injury. Importantly, and in contrast to previous studies with rodents, we assessed the effectiveness of mitigating eATP-mediated inflammation in the presence of steroids, which are routinely administered during human lung transplantation[24]. Also, given the size of dogs, we could more precisely monitor how APT102 controls pulmonary inflammation and function as multiple blood, pulmonary tissue and BAL samples could be obtained from the same dog.

To the best of our knowledge eATP levels have not been reported in humans with acute lung injury. However, elevated ATP levels have been documented in the BALF of patients with asthma[25] and idiopathic pulmonary fibrosis (IPF)[11]. In these studies healthy volunteers had levels of eATP comparable to patients with mild to no PGD. By contrast, in patients with severe exacerbations of IPF, eATP concentrations were similar to what we observed in lung recipients with severe PGD suggesting that elevated ATP release is characteristic of both

acute and chronic human pulmonary inflammation. Interestingly, lung recipients with PGD are more likely to develop Bronchiolitis Obliterans Syndrome (BOS), a chronic fibrotic disease of the airways[26]. The risk of BOS development increases with the degree of PGD severity independent of other common risk factors associated with transplant rejection. We observed that PGD severity trended with eATP levels raising the possibility that ATP release indirectly contributes to BOS through promoting acute pulmonary injury.

The burst of inflammatory mediator production following lung IRI is thought promoted by the release of DAMPs from injured cells and macrophages[27, 28]. Notably, we detected evidence of this relationship in injured dog lungs, as IL-1β, TNF-α and IL-8 levels were all significantly lower in APT102-treated dogs. In particular our observation of blunted IL-1β production is in accordance with previous reports that show eATP engagement of the $P2X_7$ receptor promotes NLRP3 inflammasome activation[12, 13]. IL-1β production in this manner is also critically dependent on $P2X_7$ receptor-mediated oligomerization of non-selective cation channels leading to a large outward K^+ current[29]. Interestingly, we may have been able to detect the eATP-mediated opening of these channels as there was significantly higher mean circulating K^+ levels in vehicle-treated dogs as compared to dogs that received APT102. However, it cannot be ruled out that K^+ release could at least in part be due to IRI-mediated cellular necrosis. Furthermore, we were unable to measure IRI-mediated K^+ release beyond 30 minutes post-reperfusion because at later time points the administration of bicarbonate was necessary to prevent hyperkalemia in vehicle-treated dogs. Finally, apyrase treatment may also be inhibiting inflammatory cell trafficking as eATP triggered inflammasome activation drives neutrophil extravasation through inducing IL-1β-dependent ICAM-1 upregulation on vascular endothelium[12, 13, 18, 30]. Consistent with these reports we observed less evidence of BAL leukocytosis and alveolar neutrophilia in dogs treated with APT102. Nevertheless, some of the deleterious effects of eATP may not involve the direct activity of cytokines. For example, eATP drives the production of stromal factors such as vascular endothelial growth factor[31], which could have possibly contributed to the thickening of alveolar septa in vehicle-treated dogs.

There are several limitations to this study. We clamped the pulmonary artery for 15 minutes to redirect blood flow to left lung, which may have of further exacerbated IRI. Also, the use of steroids in our model makes it more difficult to discern the direct effects of apyrase on lung inflammation. For example, its well-accepted that steroids inhibit inflammatory cytokine production by macrophages[32, 33] but they may also worsen pulmonary neutrophilia as dexamethasone is reported to enhance eATP-mediated endothelial cell expression of adhesion molecules and IL-8[34]. Additionally, we did not obtain pre-transplant BALF samples from donors or recipients where data on eATP levels may have been informative to better assess the impact of pre-existing pathologies on the release of this DAMP following engraftment. This may be evident in diseases such as pulmonary hypertension, which is an independent risk factor for PGD and therefore could contribute to eATP levels[35]. In conclusion we have demonstrated that following lung IRI eATP is significantly elevated in both dog and human BALF. Our data provide a new basis for the use of intraoperative apyrase treatment for the prevention of lung transplant-mediated IRI.

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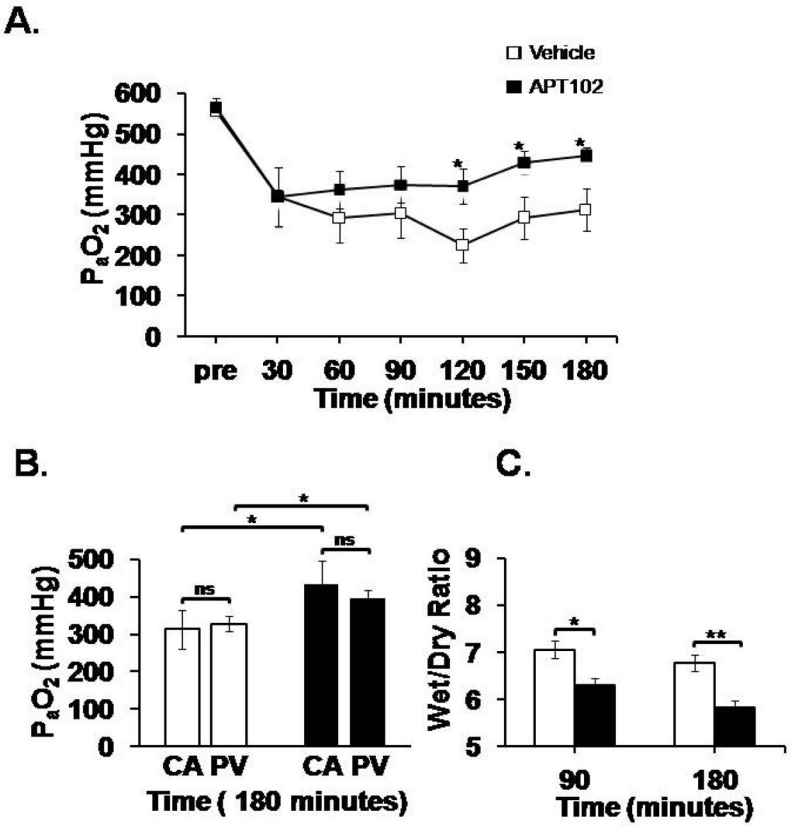

Figure 1. APT102 treatment improves lung function and attenuates vascular permeability

Dogs treated with vehicle or APT102 were assayed for (A) arterial blood oxygenation at 30-minutes intervals up to 3-hours after reperfusion at a FiO_2 of 1.0, (B) arterial blood oxygenation from carotid artery (CA) and pulmonary vein (PV) at 180 minutes after reperfusion at a FiO_2 of 1.0 or (C) pulmonary edema via left lung biopsy wet and dry weight obtained at 90 and 180 minutes post-reperfusion. Results in (A) are represented as a mean ± S.D. In (B) data is represented as mean wet to dry ratios. In (A), (B) and (C) indicated significant differences between means are represented as $*P< 0.05$ or $**P< 0.01$.

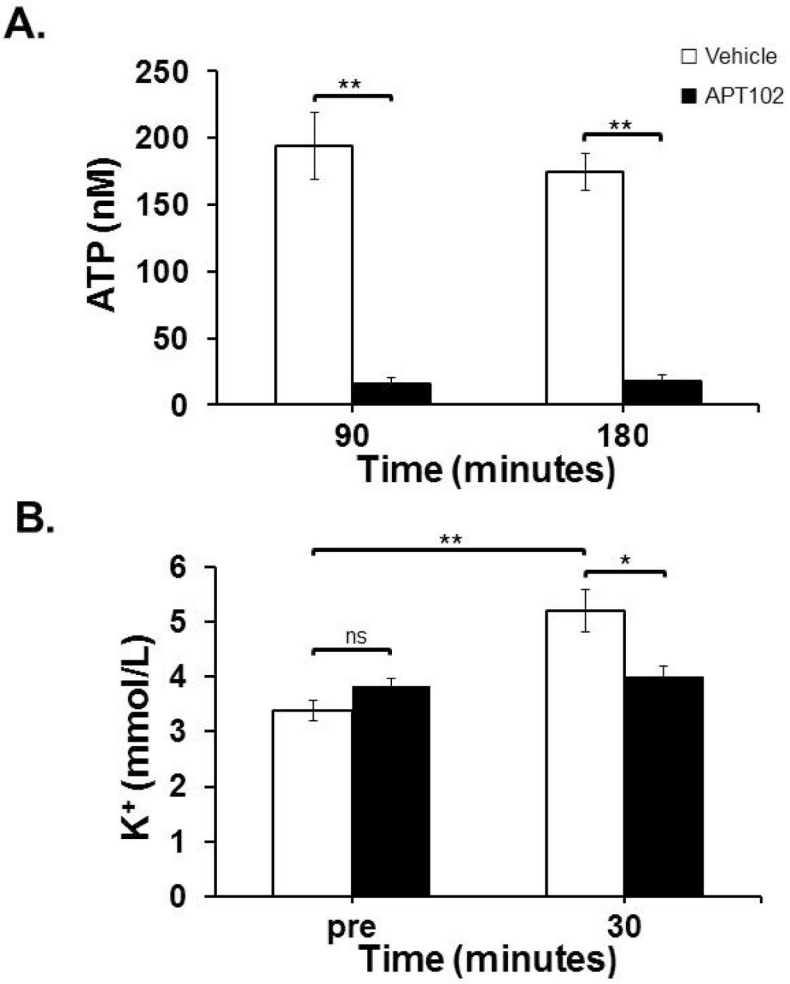

Figure 2. Airway eATP and circulating K^+ levels are reduced by APT102 treatment

Dogs treated with vehicle or APT102 assayed for (A) BALF eATP concentration at 90 and 180 minutes after reperfusion or (B) for circulating plasma potassium ion concentration before clamping (pre) or 30 minutes post-reperfusion. Results in (A) are shown as a mean ± S.D. In (A) and (B) indicated significant differences between means are represented as $*P< 0.05$ or $**P< 0.01$.

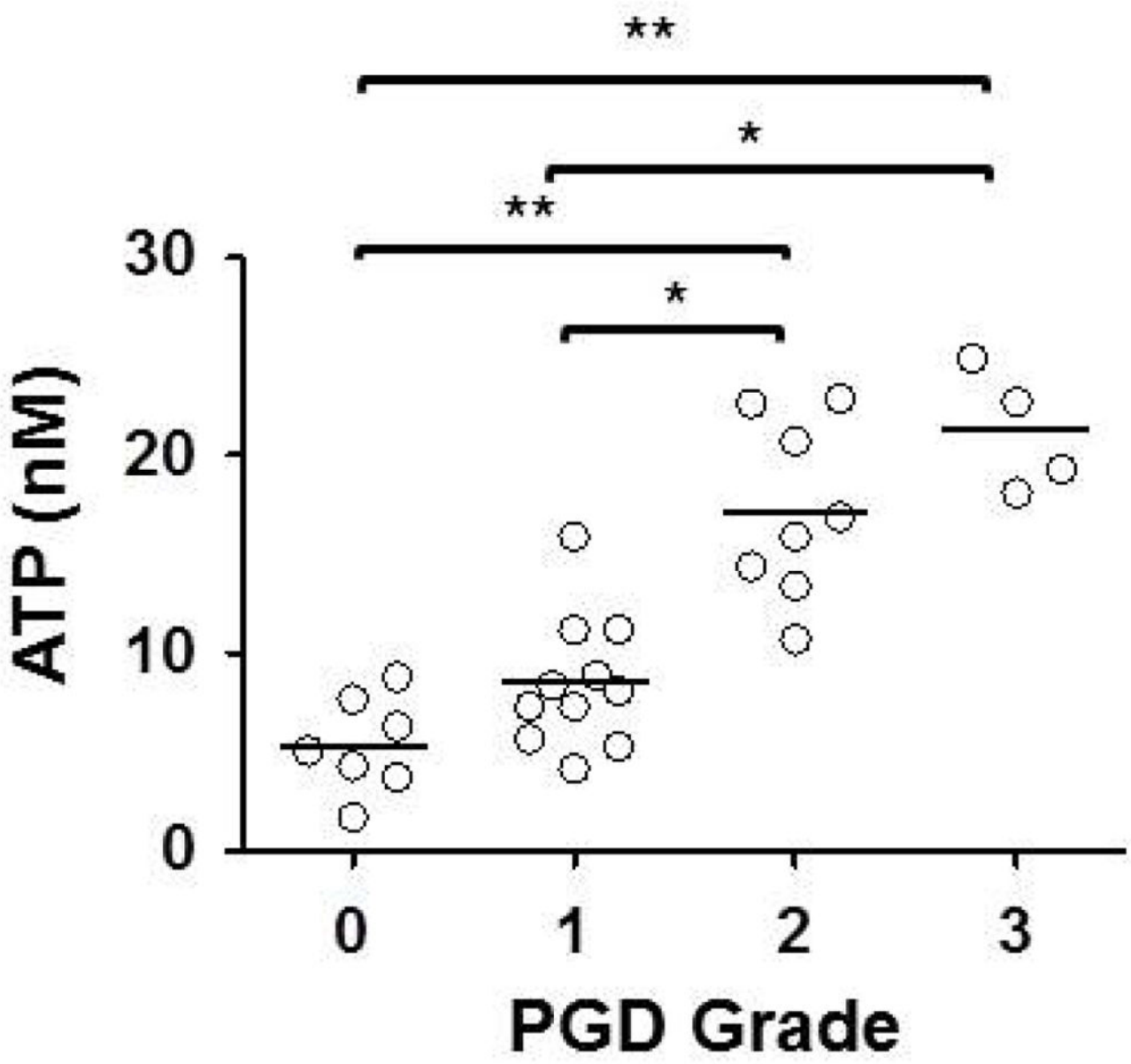

Figure 3. Human lung recipients with moderate or severe PGD have elevated airway eATP levels

Thirty bilateral lung transplant recipients were graded for immediate early PGD and had BAL retrieved for eATP concentration between 8 and 12 hours after ICU arrival. Bars represent mean eATP concentrations where indicated significant differences are represented as *P< 0.05 and **P< 0.01.

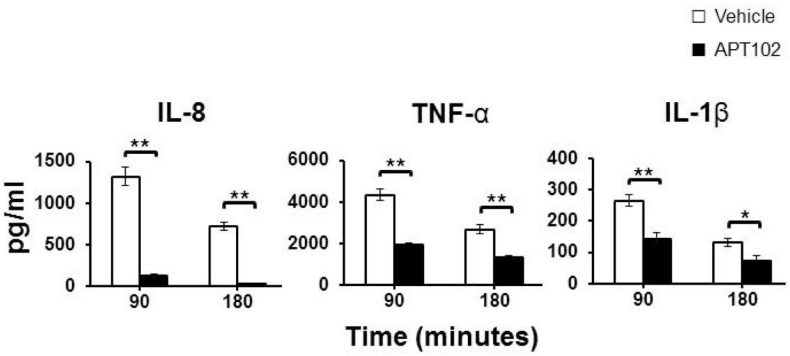

Figure 4. Inflammatory mediator expression is blunted by APT102 administration

BALF was collected from vehicle or APT012-treated dogs 90 and 180 minutes after reperfusion and evaluated for the concentration of IL-8, TNF-α and IL-1β by ELISA. Results shown are indicated differences between means are represented as **P*< 0.05 or ***P*< 0.01.

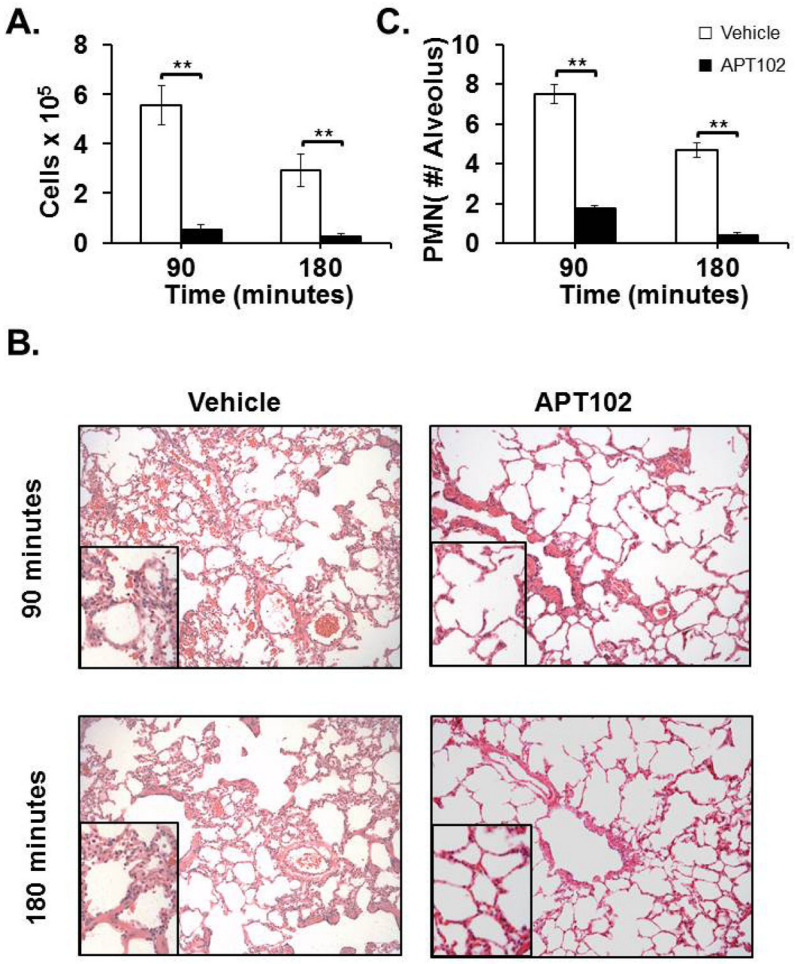

Figure 5. APT102 treatment preserves pulmonary architecture and prevents inflammatory cell sequestration

Vehicle or APT102-treated dogs 90 and 180 minutes after reperfusion were processed for (A) BAL total cell counts, (B) Hematoxylin and Eosin histological analysis and (C) mean PMN counts per alveolus. Results in (A) and (C) are shown as individual cell counts where indicated significant differences between means are represented as $^{**}P < 0.01$ and in (B) are representative per group (N=7) and depicted at 100 X (400 X inset).

Table 1

Indications for Lung Transplant

PRIMARY INDICATION FOR LUNG TRANSPLANT	AGE	SEX	PGD GRADE 0 HRS	eATP (nM)
A1E	49	M	3	24.9
A1E	50	M	2	14.4
BOS	41	F	2	20.7
CF	40	F	1	11.2
CF	32	M	2	10.7
CF	26	M	1	5.3
CF	22	F	3	18.1
CF	42	F	0	5.1
CF	33	M	0	4.3
CF	52	M	0	8.8
COPD	64	F	2	13.4
COPD	66	M	1	4.2
COPD	69	M	1	8.1
COPD	62	M	2	22.9
COPD	60	M	0	1.7
COPD	54	M	0	7.7
COPD	65	F	1	15.9
COPD	58	F	0	3.7
ILD	67	F	3	22.7
ILD	59	M	3	19.3
ILD	64	M	1	8.9
ILD	59	M	1	5.7
ILD	65	F	1	7.3
ILD	59	M	2	22.6
ILD	42	M	2	15.9
ILD	65	M	1	7.3
ILD	65	F	1	11.2
ILD	63	M	1	8.4
ILD	66	M	0	6.3
ILD	60	M	2	16.9

A1E = Alpha-1-antitrypsin deficiency emphysema, BOS = Bronchiolitis obliterans syndrome, CF = Cystic fibrosis, COPD = Chronic obstructive pulmonary disease, ILD = Interstitial lung disease