NOX2 Activation of Natural Killer T Cells Is Blocked by the Adenosine A_{2A} Receptor to Inhibit Lung Ischemia–Reperfusion Injury

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Abstract

Rationale: Ischemia–reperfusion (IR) injury after lung transplantation, which affects both short- and long-term allograft survival, involves activation of NADPH oxidase 2 (NOX2) and activation of invariant natural killer T (iNKT) cells to produce IL-17. Adenosine A_{2A} receptor (A_{2A}R) agonists are known to potentially attenuate lung IR injury and IL-17 production. However, mechanisms for iNKT cell activation after IR and A_{2A}R agonist–mediated protection remain unclear.

Objectives: We tested the hypothesis that NOX2 mediates IL-17 production by iNKT cells after IR and that A_{2A}R agonism prevents IR injury by blocking NOX2 activation in iNKT cells.

Methods: An *in vivo* murine hilar ligation model of IR injury was used, in which left lungs underwent 1 hour of ischemia and 2 hours of reperfusion.

Measurements and Main Results: Adoptive transfer of iNKT cells from p47^{phox}^{−/−} or NOX2^{−/−} mice to Jα18^{−/−} (iNKT cell-deficient) mice significantly attenuated lung IR injury and IL-17 production. Treatment with an A_{2A}R agonist attenuated IR injury and IL-17 production in wild-type (WT) mice and in Jα18^{−/−} mice reconstituted with WT, but not A_{2A}R^{−/−}, iNKT cells. Furthermore, the A_{2A}R agonist prevented IL-17 production by murine and human iNKT cells after acute hypoxia–reoxygenation by blocking p47^{phox} phosphorylation, a critical step for NOX2 activation.

Conclusions: NOX2 plays a key role in inducing iNKT cell–mediated IL-17 production and subsequent lung injury after IR. A primary mechanism for A_{2A}R agonist–mediated protection entails inhibition of NOX2 in iNKT cells. Therefore, agonism of A_{2A}Rs on iNKT cells may be a novel therapeutic strategy to prevent primary graft dysfunction after lung transplantation.

Keywords: adenosine A_{2A} receptor; NADPH oxidase; NOX2; IL-17; invariant natural killer T cells

Ischemia–reperfusion (IR) injury after lung transplantation imposes a significant threat to graft and recipient survival (1). IR injury involves a complex cascade of events, including oxidative stress, inflammation, and cellular interactions that can lead to primary graft dysfunction, which significantly increases the risk for rejection and long-term graft dysfunction (2). We

have previously described a pivotal role for invariant natural killer T (iNKT) cells in the initiation of lung IR injury via an IL-17A–dependent mechanism (3). Recent literature has described a prominent role for reactive oxygen species (ROS) in the pathogenesis of IR injury (4–8). Oxidant stresses during reoxygenation mediated by enzymes such as NADPH oxidase, xanthine

oxidase, lipoxygenase, cyclooxygenase, nitric oxide synthase, and cytochrome p450 have been proposed as a source of ROS after IR.

NADPH oxidase represents a family of enzymes that assembles at the cell membrane and reduces molecular oxygen to superoxide by transferring electrons from NADPH (9, 10). Several NADPH oxidase isoforms (NOX1–5, Duox-1, and Duox-2)

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At a Glance Commentary

Scientific Knowledge on the

Subject: Ischemia–reperfusion (IR) injury, which entails NADPH oxidase 2 (NOX2) activation, invariant natural killer T (iNKT) cell activation, and IL-17 production, remains a significant problem after lung transplantation. Although adenosine A_{2A} receptor ($A_{2A}R$) agonism can attenuate IR injury, the mechanisms for this protection and for IL-17 production by iNKT cells after IR remain to be elucidated.

What This Study Adds to the

Field: This study defines a novel signaling mechanism of IL-17 production by iNKT cells that is mediated by NOX2 activation after lung IR. Furthermore, this study demonstrates that a primary mechanism for $A_{2A}R$ agonist-mediated prevention of IR injury involves the inhibition of NOX2 in iNKT cells. Thus, agonism of $A_{2A}Rs$ on iNKT cells may be a novel therapeutic strategy to prevent primary graft dysfunction after lung transplantation.

have been characterized that differ in their tissue distribution, subunit composition, and mode of activation (11). The NOX2 isoform, which was first discovered as a component of the oxidative burst in neutrophils, is a complex consisting of several cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac1 or Rac2) and a membrane-associated cytochrome b558 reductase made up of $gp91^{phox}$ and $p22^{phox}$ subunits. The assembly, translocation, and binding of the cytosolic subunits to the $gp91^{phox}/p22^{phox}$ catalytic complex is facilitated by $p47^{phox}$. The sequential activation of $p47^{phox}$ involves serine phosphorylation, membrane translocation, and binding with $p22^{phox}$ (12, 13). A multitude of protein kinases, including various isoforms of protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) can regulate $p47^{phox}$ phosphorylation in cells, such as neutrophils, lung endothelial cells, and alveolar epithelial cells (14–16). However, a role for NADPH oxidase in the activation of iNKT cells, especially after IR, and their production of IL-17 has not yet been explored.

Adenosine is a nucleoside released in response to cellular stress (such as IR) and binds to a G-protein coupled family of four adenosine receptors (A_1R , $A_{2A}R$, $A_{2B}R$, and A_3R) (17–19). $A_{2A}R$ activation leads to increased cAMP production that culminates in the attenuation of transcriptional signaling that leads to inflammation. In response to inflammatory stimuli such as IR, activation of innate immune cells leads to robust production of proinflammatory cytokines (e.g., tumor necrosis factor- α [TNF- α], IL-17, and IFN- γ), which can be blocked by treatment with $A_{2A}R$ agonists (3, 20–23). However, the mechanism of $A_{2A}R$ agonist-mediated prevention of lung IR injury, especially in the context of iNKT cell-mediated IL-17 production, remains to be elucidated.

The present study tests the hypothesis that iNKT cell-produced IL-17 after IR is mediated by NADPH oxidase and that $A_{2A}R$ agonist attenuates IR injury largely by blocking NOX2 activation in iNKT cells. Results demonstrate that NOX2 mediates IL-17 production by iNKT cells after IR. Furthermore, treatment with an $A_{2A}R$ agonist attenuates NOX2 activation and IL-17 production by iNKT cells, leading to attenuation of lung IR injury. Finally, we show that the mechanism of $A_{2A}R$ -mediated protection involves inhibition of $p47^{phox}$ phosphorylation (and thus, NOX2) in iNKT cells, thereby identifying a potentially effective therapeutic target for the prevention of lung IR injury.

Methods

Detailed methods are described in the online supplement.

Animals and Reagents

This study used 8- to 12-week-old male C57BL/6 wild-type (WT), $p47^{phox-/-}$ and $NOX2^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME). $J\alpha 18^{-/-}$ mice (24) were provided by Masaru Taniguchi, RIKEN Research Center for Allergy and Immunology (Yokohama City, Japan). $A_{2A}R^{-/-}$ mice (25) were provided by Joel Linden, La Jolla Institute for Allergy and Immunology (La Jolla, CA). This study conformed to the National Institutes of Health guidelines and was conducted under animal protocols

approved by the University of Virginia's Institutional Animal Care and Use Committee.

Murine Lung IR Model

An *in vivo* hilar ligation model of lung IR was used in which mice underwent 1 hour left lung ischemia and 2 hours of reperfusion as previously described (26). Mice were extubated and returned to their cage during both the ischemic and reperfusion periods to minimize potential ventilator-induced injury. ATL313 (3 μ g/kg), with or without ZM241385 (1 mg/kg), was administered intravenously 5 min before ischemia. *In vivo* blockade of CD1d was performed by treating the WT mice with anti-CD1d antibody (50 μ g; eBioscience, San Diego, CA) or isotype control antibody intraperitoneally 24 hours before ischemia.

Pulmonary Function

At the end of reperfusion, pulmonary function was evaluated using an isolated, buffer-perfused lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany) as described (27).

Purification and Adoptive Transfer of iNKT Cells

Reconstitution of $J\alpha 18^{-/-}$ mice was performed by adoptive transfer of 2.5×10^5 primary murine iNKT cells via tail vein injection 4 days before surgery as previously described (3).

iNKT Cell Culture and Exposure to Hypoxia–Reoxygenation

Primary iNKT cells were cultured overnight in RPMI media containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). For exposure to hypoxia–reoxygenation (HR), 24-well culture dishes or chamber slides were placed in a humidified, sealed hypoxic chamber (Billups-Rothenberg, Del Mar, CA) that was purged with 95% N_2 and 5% CO_2 for 25 min to establish hypoxia as described previously (28). Reoxygenation was achieved by removing the plates from the hypoxic chamber and placing them in a normoxic, humidified incubator (37°C, 5% CO_2 , and 95% O_2) for 1 hour.

$p47^{phox}$ Phosphorylation

A colorimetric cell-based ELISA was used to measure $p47^{phox}$ protein phosphorylation per the manufacturer's instructions (Assay Biotechnology, Sunnyvale, CA).

p47^{phox} Immunofluorescence

Primary iNKT cells were incubated with anti-p47^{phox} primary antibody (Santa Cruz Biotechnology, Dallas, TX; 1:200 dilution in blocking buffer for 1 h), rinsed with Tris-buffered saline with Tween 20, and incubated with Alexa Fluor 488 secondary antibody (1:200 dilution in blocking buffer for 1 h). Slides were viewed using a Zeiss LSM510 confocal microscope using a 63 $\times$ oil immersion objective and Zen 2012 imaging software (Carl Zeiss Microscopy, Thornwood, NY).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA), and data are presented

as the mean $\pm$ SEM. One-way analysis of variance with *post hoc* Bonferroni's multiple comparisons, Mann-Whitney *U* test, or Student's *t* test were used as appropriate to compare experimental groups. A *P* value of <0.05 was considered significant.

Results

Pulmonary Dysfunction and Inflammation after IR Is Mediated by NADPH Oxidase-Dependent IL-17 Production by iNKT Cells

Significant pulmonary dysfunction occurred after IR in WT mice compared with sham as indicated by increased pulmonary artery pressure (12.23 ± 0.57 cm H₂O vs.

5.96 ± 0.11 cm H₂O), increased airway resistance (2.61 ± 0.10 cm H₂O/ μ l/s vs. 0.80 ± 0.05 cm H₂O/ μ l/s), and decreased pulmonary compliance (2.28 ± 0.15 μ l/cm H₂O vs. 5.82 ± 0.16 μ l/cm H₂O) (Figure 1A). Lungs of J α 18^{-/-} mice (deficient in iNKT cells) were protected after IR compared with WT mice, as shown by significantly reduced pulmonary artery pressure (6.10 ± 0.18 cm H₂O vs. 12.23 ± 0.57 cm H₂O), reduced airway resistance (1.01 ± 0.03 cm H₂O/ μ l/s vs. 2.61 ± 0.10 cm H₂O/ μ l/s), and increased pulmonary compliance (5.65 ± 0.16 μ l/cm H₂O vs. 2.28 ± 0.15 μ l/cm H₂O). Reconstitution of J α 18^{-/-} mice with iNKT cells from p47^{phox}^{-/-} mice (deficient in p47^{phox}, an essential subunit for NOX2 activation), but not WT mice, resulted

Figure 1. Pulmonary dysfunction and cytokine production after ischemia-reperfusion (IR) is mediated by NADPH oxidase-dependent activation of invariant natural killer T (iNKT) cells. (A) Significant improvement in lung function (decreased pulmonary artery pressure, decreased airway resistance, and increased pulmonary compliance) occurred after IR in J α 18^{-/-} mice compared with wild-type (WT) mice. Adoptive transfer of iNKT cells from p47^{phox}^{-/-} (p47^{-/-}), but not WT mice, to J α 18^{-/-} mice resulted in significantly reduced lung dysfunction after IR. (B) Proinflammatory cytokine levels, especially IL-17, were significantly attenuated after IR in lungs of J α 18^{-/-} mice compared with WT mice. Adoptive transfer of iNKT cells from p47^{phox}^{-/-} mice, but not WT mice, to J α 18^{-/-} mice significantly attenuated cytokine production after IR. Data are presented as mean $\pm$ SEM; *n* = 5–7/group. **P* < 0.05 versus sham; #*P* < 0.05 versus WT IR; δ *P* < 0.05 versus J α 18^{-/-} IR; ##*P* < 0.05 versus J α 18^{-/-} IR (WT). CXCL1 = chemokine (C-X-C) motif ligand 1; MCP-1 = monocyte chemoattractant protein 1; RANTES = regulated upon activation, normal T-cell expressed and secreted; TNF- α = tumor necrosis factor- α .

in significantly improved function as shown by decreased pulmonary artery pressure (5.80 ± 0.13 cm H₂O vs. 11.67 ± 0.37 cm H₂O), decreased airway resistance (0.82 ± 0.06 cm H₂O/ μ l/s vs. 2.39 ± 0.12 cm H₂O/ μ l/s), and increased pulmonary compliance (5.59 ± 0.15 μ l/cm H₂O vs. 2.84 ± 0.17 μ l/cm H₂O) (Figure 1A). Furthermore, expression of IL-17 and other proinflammatory cytokines (TNF- α , IL-6, monocyte chemoattractant protein 1 [MCP-1], regulated upon activation, normal T-cell expressed and secreted [RANTES], chemokine [C-X-C motif] ligand 1 [CXCL1]) was attenuated in lungs from $J\alpha 18^{-/-}$ mice reconstituted with $p47^{\text{phox-/-}}$, but not WT, iNKT cells (Figure 1B). These results confirm a critical role for NADPH oxidase in iNKT cell-mediated lung IR injury.

To determine the role of NADPH oxidase in iNKT cells on lung IR injury, pulmonary

edema (wet/dry weight), neutrophil infiltration, and myeloperoxidase (MPO) levels in bronchoalveolar lavage fluid were measured (Figure 2). A marked increase in neutrophil infiltration, MPO levels, and edema occurred in WT mice after IR, which was completely blocked in $J\alpha 18^{-/-}$ mice. Reconstitution of $J\alpha 18^{-/-}$ mice with WT, but not $p47^{\text{phox-/-}}$, iNKT cells restored pulmonary edema, MPO levels, and neutrophil infiltration after IR. These results confirm that NADPH oxidase in iNKT cells mediates vascular and epithelial cell permeability after IR, which leads to edema and neutrophil infiltration.

Pulmonary Dysfunction, Inflammation, and Injury after IR Is Attenuated by Selective Activation of A_{2A}Rs on iNKT Cells

To determine if iNKT cells are the predominant A_{2A}R-expressing cell

population responsible for A_{2A}R agonist-mediated protection after IR, pulmonary function was assessed in WT and $J\alpha 18^{-/-}$ mice with or without treatment with ATL313, a potent and selective A_{2A}R agonist that attenuates IR injury in the lung, heart, and liver (23, 29, 30). WT mice treated with ATL313 demonstrated marked attenuation of pulmonary dysfunction versus untreated mice as shown by significantly decreased pulmonary artery pressure (6.35 ± 0.26 cm H₂O vs. 12.23 ± 0.57 cm H₂O), decreased airway resistance (1.02 ± 0.08 cm H₂O/ μ l/s vs. 2.61 ± 0.10 cm H₂O/ μ l/s), and increased pulmonary compliance (5.16 ± 0.24 μ l/cm H₂O vs. 2.20 ± 0.15 μ l/cm H₂O) (Figure 3A). Coadministration of ZM241385, an A_{2A}R antagonist, blocked ATL313-mediated attenuation of dysfunction and cytokine production

Figure 2. NADPH oxidase activation in invariant natural killer T (iNKT) cells mediates lung ischemia-reperfusion (IR) injury. (A) Representative images (top) showing neutrophil immunostaining in lungs of wild-type (WT) and $J\alpha 18^{-/-}$ mice. Neutrophils are stained red, sections are counterstained with hematoxylin, and images are at $\times 40$ magnification. The number of neutrophils per high power field (HPF) was quantified from immunostained sections (bottom). Neutrophil infiltration was significantly attenuated after IR in $J\alpha 18^{-/-}$ mice compared with WT mice. Adoptive transfer of iNKT cells from $p47^{\text{phox-/-}}$ mice ($p47^{-/-}$), but not WT mice, to $J\alpha 18^{-/-}$ mice resulted in significant attenuation of neutrophil infiltration after IR. Scale bars = 50 μ m. (B) Myeloperoxidase (MPO) levels in bronchoalveolar lavage fluid (top) and lung wet/dry weight (bottom) were significantly increased after IR in WT mice but attenuated in $J\alpha 18^{-/-}$ mice. Reconstitution of $J\alpha 18^{-/-}$ mice with iNKT cells from WT mice, but not $p47^{\text{phox-/-}}$ mice, resulted in significantly elevated MPO and wet/dry weight after IR. Data are presented as mean $\pm$ SEM; $n = 5-8$ /group. * $P < 0.05$ versus sham; # $P < 0.05$ versus WT IR; $\delta P < 0.05$ versus $J\alpha 18^{-/-}$ IR; ## $P < 0.05$ versus $J\alpha 18^{-/-}$ IR (WT).

Figure 3. Activation of A_{2A} receptors (A_{2A}Rs) on invariant natural killer T (iNKT) cells attenuates lung dysfunction and cytokine production after lung ischemia–reperfusion (IR) injury. (A) Pulmonary dysfunction in wild-type (WT) mice after IR (increased pulmonary artery pressure, increased airway resistance, and decreased pulmonary compliance) was significantly attenuated by treatment with ATL313 (ATL), an A_{2A}R agonist. **P* < 0.05 versus sham; ***P* < 0.05 versus IR; *n* = 6/group. (B) Reconstitution of Jα18^{−/−} mice with iNKT cells from WT mice or A_{2A}R^{−/−} mice restored lung dysfunction after IR compared with Jα18^{−/−} mice alone. ATL313 treatment significantly attenuated lung dysfunction after IR in Jα18^{−/−} mice reconstituted with WT, but not A_{2A}R^{−/−} iNKT cells. #*P* < 0.05 versus Jα18^{−/−} IR; δ*P* < 0.05 versus Jα18^{−/−} IR (WT), *n* = 5–7/group. (C) Proinflammatory cytokine expression after IR in Jα18^{−/−} mice was restored by reconstitution with WT or A_{2A}R^{−/−} iNKT cells, and ATL313 treatment significantly attenuated cytokine production after reconstitution with WT, but not A_{2A}R^{−/−} iNKT cells. Data are presented as mean ± SEM; *n* = 5–7/group. #*P* < 0.05 versus Jα18^{−/−} IR; δ*P* < 0.05 versus Jα18^{−/−} IR (WT). CXCL1 = chemokine (C-X-C) motif ligand 1; MCP-1 = monocyte chemoattractant protein 1; RANTES = regulated upon activation, normal T-cell expressed and secreted; TNF-α = tumor necrosis factor-α.

(see Figure E1 in the online supplement). Moreover, lung dysfunction and production of proinflammatory cytokines (including IL-17) after IR were restored in Jα18^{−/−} mice reconstituted with WT or A_{2A}R^{−/−} iNKT cells (Figures 3B and 3C). However, ATL313 had no significant protective effect in Jα18^{−/−} mice reconstituted with A_{2A}R^{−/−} iNKT cells (Figures 3B and 3C).

Similar to dysfunction, pulmonary edema, neutrophil infiltration, and MPO levels after IR were attenuated by ATL313 treatment of WT mice and Jα18^{−/−} mice reconstituted with WT, but not A_{2A}R^{−/−} iNKT cells (Figure 4). These results suggest that the primary mechanism of A_{2A}R agonist-mediated protection involves its antiinflammatory actions specifically on

iNKT cells, resulting in preservation of lung function and mitigation of proinflammatory cytokines, especially IL-17, after IR.

A_{2A}R Agonist Blocks NADPH Oxidase Activation in iNKT Cells after HR

To investigate the role of NADPH oxidase activation specifically in iNKT cells, primary murine iNKT cells were exposed to acute HR as a surrogate *in vitro* model of lung IR, which we previously showed induced IL-17 production by iNKT cells (31). ROS generation, as assessed using dichlorofluorescein dye, was markedly elevated in WT iNKT cells after HR (3 h hypoxia plus 1 h normoxia) compared with normoxia alone, which was significantly attenuated by ATL313 treatment (Figure 5A).

ROS generation after HR was also significantly attenuated in p47^{phox}−/− iNKT cells, and treatment of WT iNKT cells with apocynin (600 μM; an NADPH oxidase inhibitor) also attenuated ROS production.

NADPH oxidase activity, via measurement of superoxide anion generation, in iNKT cells was then measured by a chemiluminescence-based assay (Figure 5B). A significant increase in superoxide anion generation occurred at different time points in iNKT cells after HR, which was blocked by ATL313 or apocynin treatment at all comparative time points. Also, iNKT cells from p47^{phox}−/− mice demonstrated no significant induction of superoxide anion after HR. These results confirm that NADPH oxidase is the primary source of ROS

Figure 4. Neutrophil infiltration and pulmonary edema after ischemia–reperfusion (IR) are attenuated by activation of A_{2A} receptors ($A_{2A}Rs$) on invariant natural killer T (iNKT) cells. (A) Neutrophil immunostaining in lungs of wild-type (WT) and $J\alpha 18^{-/-}$ ($J\alpha 18$) mice. Neutrophils are stained red, sections are counterstained with hematoxylin, and images are at $\times 40$ magnification. Scale bars = 50 μm . (B) The number of neutrophils/high-powered field (HPF) (top), quantification of immunostained sections shown in (A), myeloperoxidase (MPO) levels in bronchoalveolar lavage fluid (center), and lung wet/dry weight (bottom) are all significantly increased in WT mice after IR, which is attenuated by treatment with ATL313 (ATL). * $P < 0.05$ versus sham; ** $P < 0.05$ versus WT IR; $n = 5$ –8/group. (C) The number of neutrophils/HPF (quantification of immunostained sections shown in A), MPO levels, and lung wet/dry weight are significantly increased after IR in $J\alpha 18^{-/-}$ mice reconstituted with iNKT cells from WT mice, which are significantly attenuated by ATL313 treatment. Neutrophil numbers, MPO levels, and wet/dry weight are also elevated after IR in $J\alpha 18^{-/-}$ mice reconstituted with iNKT cells from $A_{2A}R^{-/-}$ mice, which are not attenuated by ATL313 treatment. Data are presented as mean $\pm$ SEM; $n = 5$ –8/group. # $P < 0.05$ versus $J\alpha 18^{-/-}$ IR; $\delta P < 0.05$ versus $J\alpha 18^{-/-}$ IR (WT).

generation in iNKT cells after HR and that $A_{2A}R$ agonism can effectively attenuate superoxide anion production.

$A_{2A}R$ Agonist Blocks NADPH Oxidase Activation by Inhibiting $p47^{phox}$ Phosphorylation

To define a mechanism for $A_{2A}R$ agonist-mediated inhibition of NADPH oxidase activation, phosphorylation of $p47^{phox}$ was evaluated. HR induced significant $p47^{phox}$ phosphorylation in iNKT cells compared with normoxia, which was significantly attenuated by ATL313 (Figure 5C). Also, translocation of $p47^{phox}$ from the cytoplasm to the plasma membrane (signifying activation of the NADPH oxidase complex) was observed in HR-exposed

iNKT cells compared with normoxic iNKT cells, which was attenuated by ATL313 treatment (Figure 5D). Taken together, these results show that $A_{2A}R$ agonism on iNKT cells blocks ROS production by influencing NADPH oxidase-dependent superoxide anion generation via inhibition of $p47^{phox}$ phosphorylation.

IL-17 Production by iNKT Cells Is Mediated by NADPH Oxidase and Attenuated by $A_{2A}R$ Activation

To determine if IL-17 production by iNKT cells is dependent on NADPH oxidase, cytokine secretion was measured *in vitro* after HR. HR induced a significant increase in IL-17 secretion by WT iNKT cells compared with normoxia

(105 ± 7.85 pg/ml vs. 13.01 ± 1.42 pg/ml), which was attenuated by treatment with ATL313 or apocynin (33.29 ± 4.38 and 25.9 ± 1.30 pg/ml, respectively) (Figure 6A). iNKT cells from $p47^{phox-/-}$ mice also demonstrated significantly reduced IL-17 production after HR compared with WT iNKT cells (36.2 ± 3.10 pg/ml vs. 105 ± 7.85 pg/ml). Similar results were also observed for RANTES and macrophage inflammatory protein 1 α (MCP-1 α) (data not shown). Furthermore, pretreatment of HR-exposed iNKT cells with a PKA inhibitor KT 5720 (0.1 μM ; Tocris Bioscience, Bristol, UK) abolished the ATL313-mediated attenuation of IL-17 production (93.19 ± 6.41 pg/ml vs. 33.29 ± 4.38 pg/ml). Similar to murine

Figure 5. A_{2A} receptor agonist attenuates superoxide generation and $p47^{phox}$ phosphorylation in invariant natural killer T (iNKT) cells. (A) Total reactive oxygen species (ROS) production was quantified in murine iNKT cells using dichlorofluorescein dye and measuring relative fluorescence units (RFU). Compared with normoxia (Norm), hypoxia–reoxygenation (HR) markedly increased ROS production in wild-type (WT) iNKT cells, which was markedly reduced by ATL313 (ATL) or apocynin (Apo) treatment. ROS production was also prevented in HR-exposed iNKT cells from $p47^{phox-/-}$ mice. $^*P < 0.05$ versus Norm WT; $^{\#}P < 0.05$ versus HR WT; $n = 5$ /condition. (B) HR-exposed WT iNKT cells displayed significant superoxide anion generation compared with Norm cells, which was significantly attenuated by ATL313 or Apo treatment. Superoxide anion production by HR-exposed iNKT cells from $p47^{phox-/-}$ mice was significantly attenuated versus HR-exposed WT iNKT cells. $^*P < 0.05$ versus Norm WT; $^{\#}P < 0.05$ versus HR WT; $n = 5$ /condition; relative light units (RLU). (C) Phosphorylation of $p47^{phox}$ (ratio of phosphorylated to total $p47^{phox}$ in RLU) in WT iNKT cells was significantly elevated after HR, which was blocked by ATL313 treatment. $^*P < 0.05$ versus Norm; $n = 5$ /condition. (D) Representative confocal microscopy images of iNKT cells demonstrating $p47^{phox}$ localized to the cytosol of normoxic iNKT cells (*top*). HR induced translocation of $p47^{phox}$ to a predominantly membrane localization (*middle*; punctate staining indicated by white arrows), which was prevented by ATL313 treatment (*bottom*). Magnification, $\times 400$. All data are presented as mean $\pm$ SEM.

Figure 6. Activation of invariant natural killer T (iNKT) cells by hypoxia–reoxygenation (HR) is mediated by NADPH oxidase and attenuated by A_{2A} receptor agonist. (A) Exposure of wild-type (WT) iNKT cells to HR significantly increased IL-17 production compared with normoxia (Norm), which was significantly attenuated by ATL313 (ATL) or apocynin (Apo) pretreatment. KT 5720 (KT) pretreatment of iNKT cells significantly counteracted the ATL313-mediated mitigation of IL-17 production after HR. IL-17 production by iNKT cells from $p47^{phox-/-}$ mice ($p47^{-/-}$) was significantly attenuated after HR. $^*P < 0.05$ versus Norm WT; $^{\#}P < 0.05$ versus HR WT; $^{**}P < 0.05$ versus HR WT + ATL; $^{\delta}P < 0.05$ versus Norm $p47^{-/-}$; $n = 5$ –8/condition. (B) IL-17 production by primary human $CD3^{+}CD56^{+}$ NKT cells was significantly increased after HR, which was attenuated by ATL313 treatment. $^*P < 0.001$ versus Norm; $^{\#}P < 0.001$ versus HR; $n = 8$ /condition. (C) ATL313 treatment significantly attenuated superoxide anion production by HR-exposed human $CD3^{+}CD56^{+}$ NKT cells compared with HR alone. $^*P < 0.05$ versus Norm; $^{\#}P < 0.05$ versus HR; $n = 6$ /condition. Data are presented as mean $\pm$ SEM. RLU = relative light units.

iNKT cells, CD3⁺CD56⁺ human NKT cells exposed to HR also demonstrated a significant increase in IL-17 production (156.6 ± 18.4 pg/ml vs. 17.4 ± 0.4 pg/ml), which was attenuated by ATL313 (66.8 ± 19.3 pg/ml) (Figure 6B). Superoxide anion generation in human NKT cells after HR was also markedly attenuated by ATL313 (Figure 6C). These results suggest that HR-induced IL-17 production by iNKT cells is mediated by NADPH oxidase and that activation of A_{2A}Rs on iNKT cells blocks HR-induced iNKT cell-dependent IL-17 production.

Lung IR Injury Is Mediated by NOX2-Dependent Activation of iNKT Cells

p47^{phox} is a critical regulatory subunit for NOX2 activity, whereas NOXO1, a p47^{phox} homolog, is the complementary regulatory subunit for NOX1. However, p47^{phox} has been shown to regulate NOX1 activity in vascular smooth muscle cells (32). Thus, use of p47^{phox}^{-/-} mice may not rule out a potential contribution of NOX1 in iNKT cell activation after IR. To confirm NOX2 as the specific isoform that mediates iNKT cell activation during lung IR injury, we evaluated lung IR injury in NOX2^{-/-} mice, which are deficient in the catalytic subunit gp91^{phox}. Similar to p47^{phox}^{-/-} mice, lungs of NOX2^{-/-} mice were markedly protected after IR compared with WT mice, as indicated by significantly reduced pulmonary artery pressure (5.56 ± 0.18 cm H₂O vs. 12.30 ± 0.28 cm H₂O), reduced airway resistance (1.42 ± 0.08 cm H₂O/μl/s vs. 1.83 ± 0.03 cm H₂O/μl/s), and increased pulmonary compliance (7.70 ± 0.76 μl/cm H₂O vs. 2.85 ± 0.13 μl/cm H₂O) (Figure 7A). Moreover, reconstitution of Jα18^{-/-} mice with NOX2^{-/-} iNKT cells resulted in improved pulmonary function after IR compared with reconstitution with WT iNKT cells, as shown by significantly reduced pulmonary artery pressure (6.30 ± 0.32 cm H₂O vs. 11.78 ± 0.34 cm H₂O), reduced airway resistance (1.22 ± 0.08 cm H₂O/μl/s vs. 2.05 ± 0.10 cm H₂O/μl/s), and increased pulmonary compliance (5.68 ± 0.15 μl/cm H₂O vs. 2.91 ± 0.13 μl/cm H₂O) (Figure 7B). In addition, neutrophil infiltration and IL-17 expression were significantly attenuated in NOX2^{-/-} mice after IR versus WT mice (Figure 8A). Similarly, neutrophil infiltration and IL-17 expression were significantly attenuated after IR in Jα18^{-/-} mice reconstituted with

Figure 7. Pulmonary dysfunction after ischemia-reperfusion (IR) is mediated by NADPH oxidase 2 (NOX2)-dependent activation of invariant natural killer T (iNKT) cells. (A) A significant improvement in lung function (reduced pulmonary artery pressure, reduced airway resistance, and increased pulmonary compliance) occurred in NOX2^{-/-} mice compared with wild-type (WT) mice after IR. * $P < 0.05$ versus Sham WT; ** $P < 0.05$ versus IR WT; $n = 5$ –6/group. (B) Reconstitution of Jα18^{-/-} mice with iNKT cells from NOX2^{-/-} mice (NOX2^{-/-}), but not from WT mice, resulted in significant attenuation of lung dysfunction (decreased airway resistance, decreased pulmonary artery pressure, and increased pulmonary compliance) after IR. # $P < 0.05$ versus IR Jα18^{-/-}; ## $P < 0.05$ versus IR Jα18^{-/-} (WT); $n = 5$ /group. Data are presented as mean $\pm$ SEM.

NOX2^{-/-}, but not WT iNKT cells (Figure 8B). These results confirm that NOX2 is the primary isoform of NADPH oxidase in iNKT cells that mediates IL-17 production and subsequent neutrophil infiltration and pulmonary dysfunction after lung IR.

Discussion

The present study defines several novel mechanisms important in iNKT cell-mediated lung IR injury and as a

mechanism for A_{2A}R agonist-mediated protection (summarized in Figure 9). First, NADPH oxidase-dependent activation of iNKT cells plays a key role in lung dysfunction, injury, and IL-17 production after IR. Second, selective activation of A_{2A}Rs on iNKT cells attenuates lung dysfunction, injury, and IL-17 production after IR. Furthermore, pharmacologic agonism of A_{2A}Rs on iNKT cells blocks p47^{phox} phosphorylation to inhibit NADPH oxidase activation (superoxide anion production) and IL-17 expression. Finally,

Figure 8. NADPH oxidase 2 (NOX2) activation of invariant natural killer T (iNKT) cells mediates neutrophil infiltration and IL-17 production after ischemia–reperfusion (IR). (A) Neutrophil infiltration (representative images, *top*; quantification of neutrophils/high-powered field [HPF], *middle*) and IL-17 production (*bottom*) were significantly attenuated in NOX2^{−/−} mice compared with wild-type (WT) mice after IR. **P* < 0.05 versus Sham WT; ***P* < 0.05 versus IR WT; *n* = 5–6/group. Scale bars = 50 μ m. (B) Reconstitution of Jα18^{−/−} mice with iNKT cells from NOX2^{−/−} mice [NOX2^{−/−}], but not from WT mice, significantly attenuated neutrophil infiltration (*top* and *middle*) and IL-17 production (*bottom*) after IR. #*P* < 0.05 versus IR Jα18^{−/−}; ##*P* < 0.05 versus IR Jα18^{−/−} (WT); *n* = 5/group. Data are presented as mean $\pm$ SEM.

we identified NOX2 as the primary NADPH oxidase isoform responsible for activation of iNKT cells after IR.

We have previously demonstrated that A_{2A}R agonism significantly attenuates lung IR injury (23, 33, 34) and have defined a pivotal role of iNKT cell-produced IL-17 in the initiation of lung injury and inflammation after IR (3). A_{2A}R is the predominant adenosine receptor on iNKT cells (35). Thus, in the present study, we investigated the signaling mechanisms of A_{2A}R-mediated immunoprotection via its actions on iNKT cell activation and IL-17 production. Several lines of evidence in the present study suggest that A_{2A}Rs on iNKT cells are the primary target for A_{2A}R agonist-mediated prevention of IR injury. Treatment of WT mice with ATL313 significantly abrogated lung inflammation and injury, but treatment of Jα18^{−/−} mice

with ATL313 after reconstitution with A_{2A}R^{−/−} iNKT cells failed to protect lungs after IR, indicating the importance of A_{2A}Rs on iNKT cells. Furthermore, *in vitro* studies using human and murine iNKT cells demonstrated a marked attenuation of IL-17 production by ATL313 after HR, indicating a potent immunomodulatory role of A_{2A}R agonism on iNKT cell activation. Taken together, these results demonstrate that A_{2A}Rs on iNKT cells are the primary target of A_{2A}R agonist-mediated attenuation of lung inflammation (especially IL-17 production), injury, and dysfunction after IR.

Several mechanisms of iNKT cell activation have been described, most notably through CD1d-mediated glycolipid presentation by antigen-presenting cells (36–38). The majority of murine iNKT cells express the invariant T-cell receptor (TCR),

Vα14Jα18, which is dependent on CD1d for positive selection in the thymus and subsequent activation in the periphery (39). Activation of iNKT cells by CD1d glycolipid presentation to TCRs has been shown to induce cytokines such as IFN- γ and IL-4 (40). However, antigen-independent mechanisms have also been described that rapidly facilitate iNKT cell activation (41–45). In support of this, Nowak-Machen and colleagues showed that pulmonary iNKT cells secreted significant levels of IFN- γ and IL-17 after exposure to hyperoxia (46). Our previous studies suggest that antigen-independent activation of iNKT cells contributes to lung IR injury by demonstrating that high mobility group box 1 (HMGB1), a damage-associated molecular pattern molecule, can rapidly activate iNKT cells to produce IL-17 *in vitro* and *in vivo* (26). In particular, ROS

Figure 9. Summary of key steps involved in the activation of invariant natural killer T cells by ischemia–reperfusion. Ischemia–reperfusion induces p47^{phox} phosphorylation (P) and activation of the NADPH oxidase 2 (NOX2) complex, leading to superoxide generation and IL-17 secretion. Increased IL-17 levels significantly contribute to neutrophil infiltration and subsequent lung injury. Binding of agonist to the A_{2A} receptor (A_{2A}R) leads to increased cAMP and protein kinase A (PKA) activation, which leads to blocking of p47^{phox} phosphorylation to inhibit NOX2 activation (dashed line) and IL-17 production, resulting in attenuation of lung injury.

production can modulate the activation of inflammatory cells, including iNKT cells, by directly activating nuclear factor- κ B or oxidation of cysteine residues on protein tyrosine phosphatases (47). We have previously demonstrated that HR directly activates iNKT cells to produce IL-17 (31), signifying a rapid, antigen-independent process. Our results demonstrate that this is mediated by NADPH oxidase-dependent superoxide generation in iNKT cells after HR (*in vitro*) or IR (*in vivo*). Treatment of mice with anti-CD1d antibody before IR did not significantly alter lung dysfunction or IL-17 production after IR (see Figure E2). In contrast, primary iNKT cells from WT mice (thus, in the absence of CD1d presentation) demonstrated significantly induced IL-17 expression after HR, which was markedly attenuated by apocynin or by p47^{phox} deficiency (Figure 6). These results suggest that an alternate, antigen-independent mechanism of rapid iNKT cell activation and IL-17 production largely contributes to lung IR injury. Our unpublished data also suggest an important nexus between iNKT cells,

alveolar macrophages, and endothelial cells that is mediated by NOX2-dependent ROS production. It is possible that iNKT cell recruitment and/or expansion is modulated by endothelial- or macrophage-dependent ROS production. We have previously described a crosstalk between macrophages and iNKT cells via the HMGB1/receptor for advanced glycation endproducts (RAGE) axis that mediates iNKT cell activation after lung IR (26). Ongoing studies reveal that HMGB1 release by alveolar macrophages is dependent on NOX2, which can then influence iNKT cell activation after IR. In addition, preliminary data and unpublished results suggest that pannexin-1 channel-mediated ATP release by endothelial cells may mediate iNKT cell transmigration and activation after IR (48).

Previous studies suggest that NADPH oxidase is critically involved in the redox regulation of pulmonary injury and inflammation after IR (31, 49–52). A variety of stimuli, including IR, can lead to superoxide production by NADPH oxidase,

especially in phagocytes in which a strong response is achieved through ligands that activate Gq-type G protein-coupled receptors (53, 54). Upon cell stimulation, PKC-mediated phosphorylation of p47^{phox} results in its translocation to the bound cytochrome b558 complex at the membrane (gp91^{phox} and p22^{phox}) (55). Previous studies have shown that p47^{phox} can be phosphorylated by a multitude of protein kinases including PKA and PKC. A_{2A}R agonism has been shown to inhibit human neutrophil oxidative burst by activating cAMP/PKA (56). Various isoforms of PKC, depending upon the requirement of Ca²⁺ and phospholipids, have also been implicated in p47^{phox} phosphorylation under varying conditions (57, 58). In addition, several other kinases (e.g., Akt, MAPK, c-Src) have been implicated in p47^{phox} phosphorylation (14, 16, 59). For the first time, the present study demonstrates that HR-stimulated iNKT cells undergo p47^{phox} phosphorylation and translocation to the membrane, thereby forming an active

NADPH oxidase complex that leads to superoxide generation and subsequent IL-17 production. This process was blocked by ATL313, an A_{2A}R agonist, signifying the inhibitory effect of ATL313 on NADPH oxidase activity in iNKT cells as an effective mechanism of attenuating IL-17 production (Figure 9). A previous study by Sullivan and colleagues showed that A_{2A}R-mediated suppression of neutrophil oxidative burst was counteracted by a PKA inhibitor (56). Similarly, our results confirm that ATL313-mediated attenuation of iNKT cell activation after IR is also counteracted by a selective PKA inhibitor.

In conclusion, the present study defines several key mechanisms involved in lung IR injury and in A_{2A}R agonist-mediated protection. First, p47^{phox} phosphorylation and subsequent activation of NOX2 is a key mechanism for iNKT cell activation and IL-17 production after IR. Second, the protective effect of A_{2A}R agonist is largely attributed to the attenuation of iNKT cell activation. Finally, our study demonstrates the robust ability of A_{2A}R activation to inhibit NOX2 activity in iNKT cells by inhibiting p47^{phox} phosphorylation to effectively block downstream proinflammatory cytokine

production (especially IL-17) and thereby attenuate lung injury, dysfunction, and neutrophil infiltration after IR. This study underlines the importance of NOX2-mediated IL-17 production by iNKT cells in lung IR injury and suggests that selective agonism of A_{2A}Rs on iNKT cells is a novel therapeutic strategy for the treatment or prevention of IR injury and primary graft dysfunction after lung transplantation. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Lee I, Dodia C, Chatterjee S, Zagorski J, Mesaros C, Blair IA, Feinstein SI, Jain M, Fisher AB. A novel nontoxic inhibitor of the activation of NADPH oxidase reduces reactive oxygen species production in mouse lung. *J Pharmacol Exp Ther* 2013;345:284–296.
- Fiser SM, Tribble CG, Long SM, Kaza AK, Kern JA, Jones DR, Robbins MK, Kron IL. Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome. *Ann Thorac Surg* 2002;73:1041–1047, discussion 1047–1048.
- Sharma AK, LaPar DJ, Zhao Y, Li L, Lau CL, Kron IL, Iwakura Y, Okusa MD, Laubach VE. Natural killer T cell-derived IL-17 mediates lung ischemia-reperfusion injury. *Am J Respir Crit Care Med* 2011;183:1539–1549.
- Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ, et al. Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress. *Circulation* 2007;115:1408–1416.
- Hoidal JR. Reactive oxygen species and cell signaling. *Am J Respir Cell Mol Biol* 2001;25:661–663.
- Brown DM, Hutchison L, Donaldson K, Stone V. The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1444–L1451.
- Rahman I, MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* 1998;53:601–612.
- Leonard MO, Kieran NE, Howell K, Burne MJ, Varadarajan R, Dhakshinamoorthy S, Porter AG, O'Farrelly C, Rabb H, Taylor CT. Reoxygenation-specific activation of the antioxidant transcription factor Nrf2 mediates cytoprotective gene expression in ischemia-reperfusion injury. *FASEB J* 2006;20:2624–2626.
- Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007;87:245–313.
- Sumimoto H. Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. *FEBS J* 2008;275:3249–3277.
- Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004;4:181–189.
- El-Benna J, Dang PM, Gougerot-Pocidalo MA, Marie JC, Braut-Boucher F. p47^{phox}, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. *Exp Mol Med* 2009;41:217–225.
- Sheppard FR, Kelher MR, Moore EE, McLaughlin NJ, Banerjee A, Silliman CC. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol* 2005;78:1025–1042.
- Chowdhury AK, Watkins T, Parinandi NL, Saatian B, Kleinberg ME, Usatyuk PV, Natarajan V. Src-mediated tyrosine phosphorylation of p47^{phox} in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. *J Biol Chem* 2005;280:20700–20711.
- Leverence JT, Medhora M, Konduri GG, Sampath V. Lipopolysaccharide-induced cytokine expression in alveolar epithelial cells: role of PKC ζ -mediated p47^{phox} phosphorylation. *Chem Biol Interact* 2011;189:72–81.
- Dewas C, Fay M, Gougerot-Pocidalo MA, El-Benna J. The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced p47^{phox} phosphorylation in human neutrophils. *J Immunol* 2000;165:5238–5244.
- Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 2006;5:247–264.
- Eckle T, Koeppen M, Eltzschig HK. Role of extracellular adenosine in acute lung injury. *Physiology (Bethesda)* 2009;24:298–306.
- Schepp CP, Reutershan J. Bench-to-bedside review: adenosine receptors—promising targets in acute lung injury? *Crit Care* 2008;12:226.
- Chhabra P, Linden J, Lobo P, Okusa MD, Brayman KL. The immunosuppressive role of adenosine A_{2A} receptors in ischemia reperfusion injury and islet transplantation. *Curr Diabetes Rev* 2012;8:419–433.
- Day YJ, Huang L, Ye H, Li L, Linden J, Okusa MD. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: the role of CD4⁺ T cells and IFN- γ . *J Immunol* 2006;176:3108–3114.
- Day YJ, Marshall MA, Huang L, McDuffie MJ, Okusa MD, Linden J. Protection from ischemic liver injury by activation of A_{2A} adenosine receptors during reperfusion: inhibition of chemokine induction. *Am J Physiol Gastrointest Liver Physiol* 2004;286:G285–G293.
- Sharma AK, Linden J, Kron IL, Laubach VE. Protection from pulmonary ischemia-reperfusion injury by adenosine A_{2A} receptor activation. *Respir Res* 2009;10:58.
- Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science* 1997;278:1623–1626.
- Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* 1999;19:9192–9200.
- Sharma AK, LaPar DJ, Stone ML, Zhao Y, Kron IL, Laubach VE. Receptor for advanced glycation end products (RAGE) on iNKT cells mediates lung ischemia-reperfusion injury. *Am J Transplant* 2013;13:2255–2267.
- Anvari F, Sharma AK, Fernandez LG, Hranjec T, Ravid K, Kron IL, Laubach VE. Tissue-derived proinflammatory effect of adenosine A_{2B} receptor in lung ischemia-reperfusion injury. *J Thorac Cardiovasc Surg* 2010;140:871–877.

28. Sharma AK, Fernandez LG, Awad AS, Kron IL, Laubach VE. Proinflammatory response of alveolar epithelial cells is enhanced by alveolar macrophage-produced TNF- α during pulmonary ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L105–L113.
29. Dai W, Hale SL, Nayak R, Kloner RA. ATL 313, a selective A(2A) adenosine receptor agonist, reduces myocardial infarct size in a rat ischemia/reperfusion model. *Open Cardiovasc Med J* 2009;3:166–172.
30. Day YJ, Li Y, Rieger JM, Ramos SI, Okusa MD, Linden J. A2A adenosine receptors on bone marrow-derived cells protect liver from ischemia-reperfusion injury. *J Immunol* 2005;174:5040–5046.
31. Sharma AK, Mulloy DP, Le LT, Laubach VE. NADPH oxidase mediates synergistic effects of IL-17 and TNF- α on CXCL1 expression by epithelial cells after lung ischemia-reperfusion. *Am J Physiol Lung Cell Mol Physiol* 2014;306:L69–L79.
32. Drummond GR, Selemidis S, Griendling KK, Sobey CG. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 2011;10:453–471.
33. Gazoni LM, Laubach VE, Mulloy DP, Bellizzi A, Unger EB, Linden J, Ellman PI, Lisle TC, Kron IL. Additive protection against lung ischemia-reperfusion injury by adenosine A2A receptor activation before procurement and during reperfusion. *J Thorac Cardiovasc Surg* 2008;135:156–165.
34. LaPar DJ, Laubach VE, Emaminia A, Crosby IK, Hajzus VA, Sharma AK, Sumner HM, Webb DV, Lau CL, Kron IL. Pretreatment strategy with adenosine A2A receptor agonist attenuates reperfusion injury in a preclinical porcine lung transplantation model. *J Thorac Cardiovasc Surg* 2011;142:887–894.
35. Nowak M, Lynch L, Yue S, Ohta A, Sitkovsky M, Balk SP, Exley MA. The A2aR adenosine receptor controls cytokine production in iNKT cells. *Eur J Immunol* 2010;40:682–687.
36. Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, Kozuka Y, Porcelli SA, Cardell S, Brenner MB, et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* 2000;12:211–221.
37. Zhou D, Mattner J, Cantu C III, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu YP, Yamashita T, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 2004;306:1786–1789.
38. Nagarajan NA, Kronenberg M. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J Immunol* 2007;178:2706–2713.
39. Lappas CM, Day YJ, Marshall MA, Engelhard VH, Linden J. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. *J Exp Med* 2006;203:2639–2648.
40. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol* 2005;23:877–900.
41. Brigl M, Tatituri RV, Watts GF, Bhowruth V, Leadbetter EA, Barton N, Cohen NR, Hsu FF, Besra GS, Brenner MB. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J Exp Med* 2011;208:1163–1177.
42. Holzapfel KL, Tyznik AJ, Kronenberg M, Hogquist KA. Antigen-dependent versus -independent activation of invariant NKT cells during infection. *J Immunol* 2014;192:5490–5498.
43. Rachitskaya AV, Hansen AM, Horai R, Li Z, Villasamil R, Luger D, Nussenblatt RB, Caspi RR. Cutting edge: NKT cells constitutively express IL-23 receptor and ROR γ and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. *J Immunol* 2008;180:5167–5171.
44. Reilly EC, Wands JR, Brossay L. Cytokine dependent and independent iNKT cell activation. *Cytokine* 2010;51:227–231.
45. Rieder SA, Nagarkatti P, Nagarkatti M. CD1d-independent activation of invariant natural killer T cells by staphylococcal enterotoxin B through major histocompatibility complex class II/T cell receptor interaction results in acute lung injury. *Infect Immun* 2011;79:3141–3148.
46. Nowak-Machen M, Schmelzle M, Hanidziar D, Junger W, Exley M, Otterbein L, Wu Y, Csizmadia E, Doherty G, Sitkovsky M, et al. Pulmonary natural killer T cells play an essential role in mediating hyperoxic acute lung injury. *Am J Respir Cell Mol Biol* 2013;48:601–609.
47. Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat Immunol* 2002;3:1129–1134.
48. Sharma AK, Zhao Y, Kron IL, Isakson BE, Laubach VE. Pannexin-1 channel inhibition attenuates lung ischemia-reperfusion injury [abstract]. *Am J Respir Crit Care Med* 2015;191:A6132.
49. Yang Z, Sharma AK, Marshall M, Kron IL, Laubach VE. NADPH oxidase in bone marrow-derived cells mediates pulmonary ischemia-reperfusion injury. *Am J Respir Cell Mol Biol* 2009;40:375–381.
50. Weissmann N, Sydykov A, Kalwa H, Storch U, Fuchs B, Mederos y Schnitzler M, Brandes RP, Grimminger F, Meissner M, Freichel M, et al. Activation of TRPC6 channels is essential for lung ischaemia-reperfusion induced oedema in mice. *Nat Commun* 2012;3:649.
51. Zhu C, Bilali A, Georgieva GS, Kurata S, Mitaka C, Imai T. Salvage of nonischemic control lung from injury by unilateral ischemic lung with apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, in isolated perfused rat lung. *Transl Res* 2008;152:273–282.
52. Pearse DB, Dodd JM. Ischemia-reperfusion lung injury is prevented by apocynin, a novel inhibitor of leukocyte NADPH oxidase. *Chest* 1999;116(1 Suppl):55S–56S.
53. van der Vliet A. NADPH oxidases in lung biology and pathology: host defense enzymes, and more. *Free Radic Biol Med* 2008;44:938–955.
54. Jackson SH, Devadas S, Kwon J, Pinto LA, Williams MS. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol* 2004;5:818–827.
55. Bokoch GM, Zhao T. Regulation of the phagocyte NADPH oxidase by Rac GTPase. *Antioxid Redox Signal* 2006;8:1533–1548.
56. Sullivan GW, Rieger JM, Scheld WM, Macdonald TL, Linden J. Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A(2A) receptor agonists. *Br J Pharmacol* 2001;132:1017–1026.
57. Cheng N, He R, Tian J, Dinan MC, Ye RD. A critical role of protein kinase C δ activation loop phosphorylation in formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of p47(phox) and rapid activation of nicotinamide adenine dinucleotide phosphate oxidase. *J Immunol* 2007;179:7720–7728.
58. Huang X, Chen LY, Doerner AM, Pan WW, Smith L, Huang S, Papadimos TJ, Pan ZK. An atypical protein kinase C (PKC ζ) plays a critical role in lipopolysaccharide-activated NF- κ B in human peripheral blood monocytes and macrophages. *J Immunol* 2009;182:5810–5815.
59. Dang PM, Stensballe A, Boussetta T, Raad H, Dewas C, Kroviarski Y, Hayem G, Jensen ON, Gougerot-Pocidalo MA, El-Benna J. A specific p47phox -serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J Clin Invest* 2006;116:2033–2043.