Impaired Natural Killer Cell Phenotype and Function in Idiopathic and Heritable Pulmonary Arterial Hypertension

Running title: Ormiston et al; Impaired natural killer cells in PAH

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Journal Subject Codes: [18] Pulmonary circulation and disease; [130] Animal models of human disease
Abstract:

**Background** - Beyond their role as innate immune effectors, Natural Killer (NK) cells are emerging as important regulators of angiogenesis and vascular remodeling. Pulmonary arterial hypertension (PAH) is characterized by severe pulmonary vascular remodeling and has long been associated with immune dysfunction. Despite this association, a role for NK cells in disease pathology has not yet been described.

**Methods and Results** - Analysis of whole blood lymphocytes and isolated NK cells from PAH patients revealed an expansion of the functionally defective CD56/CD16+ NK subset that was not observed in patients with chronic thromboembolic pulmonary hypertension. NK cells from PAH patients also displayed decreased levels of the activating receptor NKp46 and the killer immunoglobulin-like receptors (KIRs) 2DL1/S1 and 3DL1, reduced secretion of the cytokine MIP-1β, and a significant impairment in cytolytic function associated with decreased KIR3DL1 expression. Genotyping patients (n=222) and controls (n=191) for KIR gene polymorphisms did not explain these observations. Rather, we show that NK cells from PAH patients exhibit increased responsiveness to TGF-β, which specifically downregulates disease-associated KIRs. NK cell number and cytotoxicity were similarly decreased in the monocrotaline rat and chronic hypoxia mouse models of PAH, accompanied by reduced production of IFNγ in NK cells from hypoxic mice. NK cells from PAH patients also produced elevated quantities of MMP-9, consistent with a capacity to influence vascular remodeling.

**Conclusions** - Our work is the first to identify an impairment of NK cells in PAH and suggests a novel and substantive role for innate immunity in the pathobiology of this disease.

**Key words:** immunology; pulmonary hypertension; natural killer cells; transforming growth factor-beta
Introduction

Pulmonary arterial hypertension (PAH) is characterized by a loss of pulmonary vasculature at the level of the pre-capillary arterioles. This pathological vascular remodeling is marked by excessive muscularization of the remaining pulmonary arteries and the formation of obstructive vascular lesions associated with the aberrant proliferation of endothelial cells, vascular smooth muscle cells and fibroblasts. The resulting elevation of pulmonary arterial pressure leads to right ventricular hypertrophy and death from right heart failure. Recently, members of the transforming growth factor-β (TGF-β) superfamily have been recognized as critical mediators of pulmonary vascular remodeling. Mutations in the gene encoding the bone morphogenetic protein (BMP) type II receptor (BMPR-II) account for over 70% of heritable PAH cases and 15-40% of cases of the sporadic form of the disease. Reduced BMP signaling and heightened TGF-β signaling have been reported in pulmonary vascular cells from PAH patients, whether or not a mutation is present, as well as in animal models of disease.

While PAH is primarily considered to be a vascular disease, there is a well-established link between PAH and immune dysfunction, both in the setting of autoimmune disease and viral infection. For example, the prevalence of PAH is over 300-fold higher in the HIV patient population (0.46%) than in the general population. Despite this link, there is limited understanding of the precise nature of altered immunity in PAH and how it might lead to vascular remodeling. Many studies have focused on circulating cytokines or elements of the adaptive immune system in animal models of PAH. In comparison, the contribution of innate immunity, and specifically the potential role of innate lymphocytes such as Natural Killer (NK) cells, has received considerably less attention.

NK cells are best known for their capacity to target and eliminate stressed, virally
infected or oncogene transformed cells independent of specific antigen recognition. However, a
growing body of literature supports a role for NK cells in a wide array of physiological
processes, including the regulation of vascular remodeling and regeneration\textsuperscript{8-10}. Much of this
work has centered on the critical role for decidual NK (dNK) cells in the remodeling of spiral
arteries at the maternal-fetal interface. During the first trimester of pregnancy, the spiral arteries
are transformed from narrow, highly muscularized vessels into amuscular, trophoblast-lined
channels that lack vasomotor responsiveness\textsuperscript{11}. The importance of NK cells in this process is
supported by studies demonstrating an association between preeclampsia, a disease of inadequate
spiral artery remodeling, and the interaction of certain members of the killer immunoglobulin-
like receptor (KIR) family with their class I major histocompatibility complex (MHC-I)
ligands\textsuperscript{12}. In PAH, recent work in the monocrotaline rat model of disease has also implicated a
role for NK cells in the mode of action of experimental, cell-based therapies\textsuperscript{13}. However, it is
hitherto unknown whether an abnormal NK cell phenotype exists in patients with idiopathic or
heritable PAH.

Natural Killer cells also feature in the pathogenesis of HIV, where several studies have
reported an altered NK cell phenotype and reduced function. HIV seropositive individuals
exhibit altered proportions of the CD56\textsuperscript{bright}/CD16\textsuperscript{-} and CD56\textsuperscript{dim}/CD16\textsuperscript{+} NK cell subsets, related
to a rise in circulating levels of functionally deficient, CD56/CD16\textsuperscript{+} NK cells\textsuperscript{14}. NK cells from
HIV patients also exhibit decreased surface expression of activating receptors and altered
inhibitory receptor expression, corresponding to an impaired capacity to lyse both tumor cell
lines and HIV infected autologous target cells\textsuperscript{14, 15}.

Considering the association between PAH and HIV, we sought to determine whether
similar defects were present in circulating NK cells from idiopathic and heritable PAH patients.
We performed a phenotypic and functional evaluation of NK cells from PAH patients and demonstrated marked differences in the absolute levels of circulating NK cell subsets, decreased surface expression of both activating and inhibitory receptors, a substantial impairment of functional capacity and elevated production of extracellular matrix-degrading enzymes. This dysfunction does not appear to be directly linked to genetic predisposition in the patient population, but instead may arise from altered TGF-β signaling. In the monocrotaline rat and chronic hypoxic mouse models of PAH, we were able to confirm defects in NK cell number and function that mirrored those found in human disease.

Methods

Patient Selection

Flow cytometric analysis of whole blood lymphocytes was performed on 11 PAH patients, 9 chronic thromboembolic pulmonary hypertension (CTEPH) patients and 37 healthy donors (Supplemental Table I). PAH and CTEPH cohorts were matched for haemodynamic parameters. Analysis of magnetically isolated NK cells was performed on peripheral blood samples from 14 PAH patients, four of whom have identified mutations in the gene for BMPPR-II, and 15 healthy donors with an equivalent distribution of age, sex and ethnicity (Supplemental Table II). All subjects gave informed consent to participate in the study, which was approved by the institutional ethical review committee.

Characterization of whole blood lymphocytes and magnetically isolated NK cells

Whole blood lymphocytes were analysed by flow cytometry as described previously16. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare, Piscataway, NJ) and enriched
for NK cells by negative magnetic selection using the EasySep NK cell enrichment kit (StemCell Technologies, Vancouver, Canada) as per the manufacturers’ instructions. Freshly isolated NK cells were characterized using the antibodies listed in the supplementary methods.

**Human CD107a mobilization assay**

NK cells (5x10^4 cells/well) were cultured with or without K562 target cells at a ratio of 1:1 in round-bottom 96 well plates in the presence of 5μL of PE-Cy5-conjugated α-CD107a antibody (clone H4A3, BD Biosciences, Franklin Lakes, NJ). After 1 hour, 6μg/mL monensin (GolgiStop, BD Biosciences) was added to each well and the cultures were continued for an additional 4 hours. Following co-culture, cells were washed and stained with α-CD56-APC, α-CD16-FITC, and α-KIR3DL1-PE (all BD Biosciences) or α-KIR2DL1/S1-PE (Beckman Coulter, Brea, CA) prior to analysis.

**Human NK cell culture and gene expression**

NK cells (2x10^5 cells per well) were maintained in 0.2 mL RPMI 1640 media with L-glutamine, 10% FBS and 0.5 U/mL penicillin-streptomycin in round-bottom 96 well plates, with or without 10 ng/mL of recombinant human TGF-β (R&D Systems, Minneapolis, MN) for 72 hours, followed by staining with α-CD56-APC, α-CD16-FITC and PE-conjugated antibodies for KIR3DL1, KIR2DL1/S1 or KIR2DL2/3 (Beckman Coulter). For gene expression, RNA was obtained from 6x10^5 NK cells after 24 hours of culture and purified using RNeasy RNA isolation columns (Qiagen, Germantown, MD). The expression of plasminogen activator-inhibitor-1 (PAI-1) and urokinase plasminogen activator (uPA) were determined relative to the hypoxanthine phosphoribosyltransferase 1 (HPRT1) reference gene by real-time PCR, using Qiagen QuantiTect primer assays.

**Statistical Analysis**
All data are presented as mean +/- SEM, and a threshold of P=0.05 was used for declaring statistical significance. Unpaired, two-sided Student’s t-tests were used for comparisons between two groups. Paired, two-sided t-tests were used for the analysis of within-person comparisons. For the analysis of genotype frequency data, a Bonferroni correction was used to account for multiple independent comparisons between two groups. Comparisons between multiple groups were assessed by ANOVA, followed by a Dunnett’s post-hoc analysis. Statistical analysis was performed using Prism software (GraphPad Software Inc., La Jolla, CA). We acknowledge that many of the analyses in the current study involve the comparison of small group sizes, and that this factor limits our power to detect differences between groups.

For additional methods please see the online supplement. All primers used for genotyping studies are listed in Supplemental Table III.

Results

Circulating CD56<sup>dim</sup>/CD16<sup>+</sup> NK cells are decreased in PAH patients

Circulating NK cells can be divided into two main subsets, CD56<sup>dim</sup>/CD16<sup>+</sup> cells, which contain all the necessary cellular machinery for cytotoxicity, and CD56<sup>bright</sup>/CD16<sup>-</sup> cells, which have little to no cytotoxic capacity, but produce large quantities of inflammatory cytokines upon stimulation. As an initial screen, freshly isolated PBMCs were stained for CD56 and CD3 and analysed by flow cytometry (Figure 1A-C). As previously reported for HIV, the absolute number of circulating CD56<sup>dim</sup>/CD3<sup>-</sup> NK cells was significantly decreased in PAH patients when compared to healthy donors (Figure 1B). Samples from chronic thromboembolic pulmonary hypertension (CTEPH) patients, haemodynamically matched to the PAH cohort (Supplementary Table I), demonstrated no significant difference in the absolute number of
CD56\textsuperscript{dim}/CD3\textsuperscript{−} cells compared to healthy controls. No differences in the number of circulating CD56\textsuperscript{bright}/CD3\textsuperscript{−} NK cells were observed between the groups (Figure 1C).

Previous reports have attributed the decrease in CD56\textsuperscript{dim} NK cells in HIV patients to a rise of CD56/CD16\textsuperscript{+} NK cells in the circulation, a subset that is linked to impaired NK cell function\textsuperscript{14}. Analysis of magnetically isolated NK cells from PAH patients demonstrated a loss of CD56 on the CD16\textsuperscript{+} population, resulting in a proportional increase in circulating CD56/CD16\textsuperscript{+} NK cells when compared to healthy donors (Figure 1D-E).

**Analysis of activating and inhibitory receptors on isolated NK cells**

The ability of NK cells to lyse potential targets is determined through the integration of opposing signals from activating and inhibitory receptors that recognize ligands on the target cell surface. Magnetically isolated NK cells from PAH patients and healthy donors were analyzed for a panel of activating receptors, including DNAM-1, NKG2D and the natural cytotoxicity receptors (NCRs) NKp46 and NKp30 (Figure 2A). The surface expression of NKp46 was significantly decreased on NK cells from PAH patients when compared to controls. No significant differences were observed in surface expression of DNAM-1, NKp30 or NKG2D.

Isolated NK cells were also assessed for surface expression of activating and inhibitory KIRs (Figure 2B-C). NK cells from PAH patients exhibited a decreased percentage of cells that bound the EB6B monoclonal antibody, which recognizes both the inhibitory KIR2DL1 and activating KIR2DS1 receptors. In contrast, binding of the GL183 antibody, which recognizes the KIR2DL2 and KIR2DL3 inhibitory receptors and the activating KIR2DS2, was observed at similar levels on NK cells from both PAH patients and healthy donors. Analysis of the inhibitory receptor KIR3DL1 revealed dramatic decreases in the percentage of KIR3DL1\textsuperscript{+} cells and the mean surface expression of this receptor on NK cells from PAH patients (Figure
NK cells from PAH patients are functionally impaired

Recent reports have implicated a role for inhibitory, MHC-I binding receptors, including members of the KIR family, in the education of NK cells and the enhancement of their killing capacity. Having demonstrated decreased expression of certain KIRs in association with PAH, NK cell function was assessed by measuring degranulation via α-CD107a antibody uptake in the presence or absence of the MHC-I deficient, K562 target cell line (Figure 3A). Analysis of total CD107a+ NK cells demonstrated a significant decrease in the overall functional capacity of circulating NK cells from PAH patients when compared to healthy donors (Figure 3B).

Staining of NK cells for KIR3DL1 or KIR2DL1/S1 after co-culture allowed for a determination of the contribution of these KIR+ subsets to the total lytic response of the entire NK cell population. In order to control for differences in MHC-I type between individuals, all subjects were genotyped and only those expressing the appropriate HLA ligand for each KIR (HLA-Bw4 for KIR3DL1 and HLA-C2 for KIR2DL1/S1) were included in subsequent analyses. Division of CD107a-positivity into relative contributions from the KIR3DL1 positive and negative populations demonstrated that, in healthy individuals expressing the HLA-Bw4 ligand, expression of the inhibitory KIR3DL1 on a particular NK cell results in an increased propensity of that cell to degranulate when cultured with target cells (Figure 3C). In HLA-Bw4+ PAH patients, expression of KIR3DL1 provided no such enhancement of functional capacity, suggesting that decreased KIR3DL1 expression is responsible for at least part of the impaired functional capacity of NK cells in these individuals. In the case of KIR2DL1 and 2DS1, expression of at least one of these receptors, as indicated by binding of the EB6B antibody, did not result in enhanced lytic activity in either group (Figure 3D). However, this result may be
attributable to the lack of specificity of the EB6B antibody.

Cytokine secretion in response to K562 cell stimulation, as measured by MIP-1β production, was also significantly decreased in NK cells from PAH patients when compared to cells from healthy donors (Figure 3E), further confirming the functional impairment of these cells in PAH. Parallel cultures containing NK or K562 cells alone resulted in baseline MIP-1 β production that was below the detectable threshold of the assay (data not shown).

For PAH patients, no correlation was observed between NK cell dysfunction and disease severity, as determined by haemodynamic parameters or 6 minute walk distance (data not shown).

Decreased KIR levels are not associated with KIR or HLA gene polymorphisms

The KIR family of genes display a high level of diversity at the allelic level, as well as extensive locus-specific polymorphic variability. In the case of KIR3DL1, different polymorphic forms of the 3DL1 gene have been shown to result in distinct levels of NK cell surface expression. The most common of these polymorphisms have been categorized into forms that result in high (*001, *002, *0152, *020), low (*005, *007) and no (*004) surface expression19, 20. In addition to the many polymorphic forms of the inhibitory receptor, the 3DL1 locus can also encode an activating receptor, KIR3DS1.

In order to address the possibility that the decreased KIR3DL1 levels observed in our PAH patients were the result of a genetic predisposition in this group, a cohort of 222 British Caucasian PAH patients and 191 ethnicity-matched control subjects was typed for the KIR3DL1 and 3DS1 forms of the 3DL1 locus (Figure 4A). KIR3DL1-positive individuals were also typed for 7 of the most common polymorphic variants of KIR3DL1 (Figure 4B). This analysis failed to reveal any significant differences in the frequency of KIR3DL1, 3DS1 or any of the KIR3DL1
polymorphisms in PAH patients when compared to controls.

The complex regulation of KIR surface expression is further compounded by the influence of HLA ligands, which can induce clonal expansion of NK cells expressing complementary KIR molecules and are themselves highly variable. The ligands for KIR3DL1 are HLA-B molecules and a few HLA-A types that contain the Bw4 motif\textsuperscript{21,22}. Bw4 allotypes containing isoleucine at position 80 (Bw480I) are preferential ligands over types with threonine at this position (Bw480T)\textsuperscript{23,24}. KIR2DL1 and KIR2DS1 receptors bind the C2 form of the HLA-C molecule, whereas C1 allotypes are ligands for KIR2DL3 and weakly bind 2DL2\textsuperscript{25}. The PAH and control cohorts were also genotyped for HLA-Bw6, HLA-Bw4-80T and Bw4-80I (Figure 4C), as well as HLA-C1 and C2 (Figure 4D). Again, no significant differences in allelic frequencies were found between the groups. Taken together, these results suggest that the observed differences in KIR surface expression on NK cells from PAH patients are not the direct result of a genetic predisposition, but may instead result from \textit{in vivo} conditioning.

**TGF-\(\beta\) regulates KIR surface expression**

Considering the central role for the TGF-\(\beta\) superfamily in the pathogenesis of PAH, we questioned whether the impaired NK phenotype in PAH could be attributed to altered TGF-\(\beta\) signaling in these individuals. Treatment of freshly isolated NK cells from healthy donors with 10 ng/mL TGF-\(\beta\) induced a substantial drop in the surface expression of KIR3DL1 and KIR2DL1/S1, the two markers that were decreased in PAH (Figure 5A). In contrast, surface levels of KIR2DL2/3, which was unaltered in PAH patients, was relatively insensitive to TGF-\(\beta\) stimulation. A comparison of changes in KIR surface expression did not show greater reductions in KIRs following TGF-\(\beta\) stimulation in the patient group compared with controls (data not shown). However, this was likely due to the fact that NK cells from patients already exhibited
depressed KIR expression at the time of isolation. In support of this, NK cells from PAH patients exhibited a marked recovery in KIR3DL1 levels after 72 hours ex vivo (Figure 5B). This recovery, which was not observed for any healthy control subjects or for KIR2DL1/S1 expression in either patients or controls, was blocked if TGF-β was added to the culture medium.

Gene expression of plasminogen activator-inhibitor-1 (PAI-1) and urokinase plasminogen activator (uPA) were used as downstream measures of TGF-β signaling in cultured NK cells. Cells from PAH patients demonstrated increased baseline expression of PAI-1 and uPA. A greater increase in PAI-1 gene expression was also observed in NK cells from PAH patients versus controls following stimulation with TGF-β (Figure 5C). No such effect was observed with uPA, for which gene expression was likely maximal in both groups following TGF-β stimulation. Together, these findings suggest that the impaired NK cell phenotype observed in PAH is the result of excessive TGF-β signaling. In agreement with previous reports investigating TGF-β signaling in pulmonary vascular smooth muscle cells from PAH patients³, division of the PAH cohort based on the presence or absence of a mutation in the gene for BMPR-II revealed similarly increased of TGF β signaling in both groups (Supplemental Figure 1).

**Decreased NK cell number and function in rodent models of PAH**

To determine the relevance of these findings in vivo, we examined NK cell function in both the monocrotaline rat model and chronic hypoxic mouse model of PAH. Rats given monocrotaline developed pulmonary hypertension by 21 days after injection, as evidenced by elevated right ventricular systolic pressures (RVSP) and right ventricular (RV) hypertrophy (Figure 6A-B). In agreement with previous findings in the rat model², monocrotaline-induced pulmonary hypertension was associated with increased TGF-β signaling, as demonstrated by increased PAI-1 gene expression in the lungs of monocrotaline-challenged rats (Figure 6C). Consistent with
findings in human NK cell cultures, increased PAI-1 gene expression was accompanied by significantly decreased expression of rat kir3dl1 (Figure 6D). Analysis of peripheral blood and splenic lymphocytes demonstrated decreased numbers of NKR-P1A+/CD3− NK cells in both tissues for MCT-treated animals (Figure 6E,F). In addition, splenic NK cells isolated from monocrotaline-challenged rats were functionally impaired, as demonstrated by a decreased capacity to lyse the YAC-1 murine lymphoma target cell line (Figure 6G, Supplemental Figure 2).

Mice with chronic hypoxia-induced pulmonary hypertension also exhibited NK cell impairment. Mice maintained at 10% O2 for 21 days developed significantly elevated RVSP (Figure 7A) and RV hypertrophy (Figure 7B), associated with increased pulmonary arteriolar muscularization (Figure 7C-E). Pulmonary hypertension was accompanied by decreased numbers of NKR-1.1+/CD3− NK cells in the blood, spleen and lungs of mice maintained under hypoxia (Figure 7F,G), as well as diminished NK cell function in response to stimulation with YAC-1 cells or interleukin-12 (IL-12) (Figure 7H). NK cells from hypoxia exposed mice exhibited decreased degranulation in response to both stimuli, as measured by the CD107a mobilization assay (Figure 7I). As in humans, naïve murine NK cells have a limited ability to produce INFγ when co-cultured with YAC-1 target cells. However, stimulation of NK cells with IL-12 revealed a decreased capacity of NK cells from hypoxic mice to secrete INFγ, further highlighting the reduced functional capacity of these cells (Figure 7J).

**Increased MMP production in NK cells from PAH patients**

Previous studies examining the mechanism by which dNK cells influence spiral artery remodeling in pregnancy suggested a role for matrix metalloproteinases (MMPs) in this process. Examination of MMP gene expression in *ex vivo* NK cell cultures revealed no
significant difference in MMP-2 gene expression in PAH NK cells, but did show a significant increase in the expression of MMP-9 when compared with healthy donors (Figure 8A, B). Quantification of active enzyme secretion in NK cell culture supernatants by gelatin zymography revealed no significant increase in active MMP-2, but did demonstrate increased levels of active MMP-9 in supernatants from PAH NK cell cultures (Figure 8C). High molecular weight forms of MMP-9 were also observed in 8 of the 12 patient samples, but none of the control samples. These high molecular weight bands have previously been identified as a complex of MMP-9 with neutrophil gelatinase-associated lipocalin (NGAL), which has been shown to preserve MMP-9 activity by protecting the enzyme from degradation. Further examination of extracellular matrix metalloproteinase inducer (EMMPRIN) and tissue inhibitors of MMP (TIMP-1 and -3) gene expression revealed no significant differences between patients and controls (Supplemental Figure 3). However, a significant increase in TIMP-2 gene expression was observed in NK cells from PAH patients.

**Discussion**

Our findings identify for the first time an impairment of NK cell phenotype and function in human idiopathic and heritable PAH and two widely used animal models of the disease. In patients, this abnormal NK cell phenotype was marked by a rise in the functionally deficient CD56-/CD16+ subset. This change was not detected in a disease control group of CTEPH patients, suggesting that the observed NK dysfunction is specific to PAH and is not a secondary effect of altered pulmonary haemodynamics.

Beyond altered circulating subsets, NK cells from PAH patients also exhibited decreased surface levels of activating and inhibitory receptors, including a decrease in KIR3DL1 that we
directly linked to impaired NK cytolytic function in these individuals. The observation of decreased KIR levels on NK cells from PAH patients differs from previous findings in HIV patients, where the functional deficiency of circulating NK cells was attributed to decreased levels of activating receptors and increased expression of inhibitory KIRs\textsuperscript{14}. However, our findings are in agreement with a more recent model of NK cell education\textsuperscript{18}. Under this model, increased expression of inhibitory receptors in the presence of the appropriate MHC-I ligand induces a dependency on inhibitory receptor-ligand interactions to maintain a resting state, accompanied by more robust cytotoxic responses when that inhibition is lacking, such as when an NK cell encounters an MHC-I deficient target.

Previous studies investigating the role of KIRs in disease pathogenesis have primarily focused on the capacity of KIR and HLA gene polymorphism to influence receptor levels and alter NK cell function. Disease association studies have shown that KIR-HLA genotype interactions can influence the outcome of viral infections, including HIV\textsuperscript{28}. Certain combinations of maternal KIR2D receptors and fetal HLA-C genes can also influence the risk of pre-eclampsia and dictate reproductive success\textsuperscript{12}. In comparison, relatively little attention has been given to the possible role of in vivo conditioning in the regulation of KIR levels. Here we show that signaling through the TGF-\(\beta\) pathway, known to be perturbed in PAH, specifically downregulates disease-associated KIRs in vitro and contributes to the abnormal NK cell phenotype in patients. Previous studies in pulmonary arterial smooth muscle cells from PAH patients, with and without BMPR-II mutations, have highlighted altered responses to TGF-\(\beta\) stimulation\textsuperscript{3}. In agreement with our current findings, increased TGF-\(\beta\) signaling has also been reported in the monocrotaline rat model of PAH, where it was specifically linked to increased TGF-\(\beta\) production by pulmonary artery smooth muscle cells and macrophages surrounding remodeled pulmonary arteries\textsuperscript{2}. 

Inhibition of the type-I TGF-β receptor, activin-like kinase 5 (ALK5), in this model attenuates and reverses the disease. In the context of NK cell function, TGF-β is has been shown to decrease surface expression of the activating receptors NKp30 and NKG2D and reduce cytotoxic activity. There are also limited reports of the capacity of TGF-β to alter KIR expression on cultured NK cells. However, our study is the first to demonstrate a role for TGF-β in the regulation of KIR expression in the context of a human disease.

As in PAH patients, our findings in the monocrotaline rat and chronic hypoxic mouse models of PAH demonstrate similar reductions in NK cell number, cytolytic activity and cytokine production. These observations support a role for NK dysfunction in the pathogenesis of PAH and help address the limitation of examining NK cells from patients that are at different stages in disease progression and are receiving treatment with a range of therapies. In addition to reduced NK cell number and function, increased PAI-1 gene expression was also observed in the lungs of monocrotaline-challenged rats, consistent with increased TGF-β signaling. This increased PAI-1 expression was accompanied by decreased transcription of kIR3dl1, the only KIR-like sequence that has been identified in the rat genome. Despite the limited and ambiguous nature of the KIR repertoire in rodents, further support for a role of KIR3DL1 in PAH was provided by a recent study identifying kIR3dl1 as one of a small number of genes that is significantly downregulated in the PBMCs of cattle that develop pulmonary hypertension at high altitude.

Having demonstrated an impairment of NK cell function in both human PAH and animal models of disease, the question remains as to how NK cell dysfunction may contribute to pathological vascular remodeling. During pregnancy, the extensive demuscularization of spiral arteries, coupled by a loss of the endothelial monolayer, represents a precise reversal of the
vascular remodeling observed in the PAH lung. While the role of decidual NK cells in this process is now commonly accepted, the mechanism by which this remodeling is achieved remains uncertain. A recent report by Hanna and colleagues suggested that secreted factors, including dNK-derived angiogenic factors and chemokines, induce vascular remodeling and trophoblast migration. Other work using human tissues has implicated a role for dNK cell-derived MMPs in the induction of vascular cell apoptosis and the demuscularization of spiral arteries. Although dNK cells are thought to be a distinct subset to circulating NK cell populations, with a unique capacity to influence vascular structure, circulating NK cells have also been implicated in the support of collateral artery formation in a murine model of hindlimb ischemia and in cardiac angiogenesis following myocardial infarction.

Our finding of increased MMP-9 gene expression and enzymatic activity in circulating NK cells from PAH patients provides a potential mechanism by which these cells can directly influence pathological pulmonary vascular remodeling. This finding is supported by previous work reporting elevated MMP-2 and MMP-9 in the lungs of rats with monocrotaline-induced pulmonary hypertension. In addition to being a surrogate measure for enhanced TGF-β signaling, the identification of increased urokinase gene expression in NK cells from PAH patients also supports a role for these cells in the turnover of the extracellular matrix. In the monocrotaline rat model, it was suggested that MMPs allow for increased smooth muscle cell motility and the muscularization of the pulmonary arteries, as has been described previously for MMPs in the enhancement of tumor invasion and metastasis. However, in pregnancy MMP production is associated with the demuscularization of arteries and smooth muscle cell apoptosis, suggesting that increased MMP production in NK cells from PAH patients may be a protective response to increased pulmonary vascular muscularization in the diseased lung.
Taken together this study proposes a potentially important role for NK cells in the regulation of vascular remodeling beyond the context of pregnancy and provides novel insights into the complex mechanisms underlying PAH.

Acknowledgements: The authors thank Dr. M. Weale, X. Yang and Dr. P. Jurasz for assistance with statistical analyses, animal studies and zymography, respectively. Dr. P. Stevenson provided YAC-1 cells.

Funding Sources: This work was funded by the British Heart Foundation, Canadian Institutes of Health Research and Fondation Leducq. JT is supported by the Wellcome Trust and MRC. FC is supported by the Wellcome Trust, MRC and AICR. Research infrastructure was provided by the Cambridge NIHR Biomedical Research Centre.

Conflict of Interest Disclosures: None.

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Figure Legends:

**Figure 1.** Quantification of circulating NK cell subsets. (A) Whole blood mononuclear cells, stained for CD56 and CD3 and gated on the lymphocyte population. Absolute quantification of (B) CD3+/CD56dim and (C) CD3+/CD56bright NK cells in the circulation of healthy donors (n=37), chronic thromboembolic pulmonary hypertension (CTEPH) patients (n=9) and PAH patients (n=11). (D) Magnetically-isolated NK cells from healthy donors and PAH patients, stained for CD56 and CD16. (E) Quantification of CD56+/CD16+ cells as a percentage of total NK cells. n=14-15, * P<0.05.

**Figure 2.** Analysis of NK surface receptor expression. Flow cytometric analysis of (A) activating receptors and (B) KIRs on magnetically isolated NK cells. Receptor expression quantified as (A) shift in mean fluorescence intensity versus isotype (grey) or (B) percent positive NK cells. (C) Percent positivity and mean fluorescence intensity of KIR3DL1 on magnetically isolated NK cells from PAH patients (red) and healthy donors (green). n=11-15 per group, * P<0.05.

**Figure 3.** Functional analysis of circulating NK cells. (A) Representative flow plots gated on CD16+ NK cells following incubation with or without K562 target cells (1:1) in the presence of α-CD107a MAb (B) Quantification of total CD107a+ NK cells from all subjects (n=12-14) after co-culture with K562 cells. (C-D) Quantification of CD107a positivity in NK cells subdivided for expression of (C) KIR3DL1 and (D) KIR2DL1/S1. Significance determined by paired t-test comparing KIR positive and negative subsets from the same individual. Only individuals expressing both the KIR of interest and the appropriate HLA ligand for each KIR were assessed.
(E) Quantification of MIP-1β secretion by isolated NK cells (n=12-14) incubated with K562 target cells. * P<0.05.

**Figure 4.** Genotype frequencies of KIR3DL1, KIR3DS1, HLA-B and HLA-C variants in PAH and control cohorts. (A) A cohort of British, Caucasian healthy controls (□, n=191) and PAH patients (■, n=222) were genotyped for allelic frequency of KIR3DL1 and 3DS1, as well as (B) polymorphic variants of KIR3DL1. All subjects were also typed for variants of (C) HLA-B and (D) HLA-C. Significance was determined by Fisher’s exact test (*P<0.05), with a Bonferroni correction for comparisons of 3DL1 subtype frequency. No significant differences were found between the groups.

**Figure 5.** Response of isolated NK cells to TGF-β stimulation. (A) Representative plots of magnetically isolated NK cells from a healthy donor cultured for 72 hours with or without 10 ng/mL TGF-β. (B) After 72 hours *ex vivo*, NK cells from a subset of PAH patients exhibited a recovery in KIR3DL1 expression that was blocked by the addition of TGF-β. Gene expression of (C) PAI-1 and (D) uPA in NK cells after 24 hours of *ex vivo* culture with or without TGF-β stimulation. n=12-14, * P<0.05.

**Figure 6.** NK cell number and function are decreased in monocrotaline-induced pulmonary hypertension. (A) Right ventricular systolic pressure and (B) right ventricular hypertrophy 21 days after intraperitoneal injection of monocrotaline or an equivalent volume of saline. (C,D) Gene expression of (C) PAI-1 and (D) kir3dll1 in the lungs of rats treated with monocrotaline or saline control. (E) Representative flow plots of peripheral blood and splenic lymphocytes,
isolated from monocrotaline (MCT)-challenged or control rats and stained for CD3 and NKR-P1A. (F) Quantification of NKR-P1A+/CD3− NK cells in the blood and spleen of rats treated with monocrotaline or saline control. (G) Functional assessment of NK cells isolated from monocrotaline or saline-treated rats. Percent lysis of YAC-1 target cells was determined by ⁵¹Cr release following 6 hour co-culture at an effector to target ratio of 80:1. n=6, * P<0.05.

**Figure 7.** NK cell number and function are decreased in mice exposed to chronic hypoxia. (A) Right ventricular systolic pressure and (B) right ventricular hypertrophy after 21 days of exposure to hypoxia (10% O₂) or normoxia. (C,D) Lung sections from (C) normoxic and (D) hypoxic mice stained for vonWillebrand factor (green) smooth muscle α-actin (red) and DAPI nuclear counterstain (blue). (E) Quantitative assessment of pulmonary arterial muscularization, divided into non-, partial- and fully-muscularized arteries (n=6). (F) Representative flow plots of peripheral blood, splenic and lung lymphocytes from hypoxic and normoxic mice stained for CD45, CD3 and NK1.1. (G) NK1.1+/CD3− NK cells in the blood, spleen and lung were quantified as a percentage of CD45+ lymphocytes (n=5-8). (H-J) Functional assessment of NK cells from normoxic and hypoxic mice, determined by (I) α-CD107a binding and (J) intracellular IFNγ production as a percentage of total NK cells (n=3-5). * P<0.05.

**Figure 8.** NK cells from PAH patients exhibit elevated MMP-9 production. Gene expression of (A) MMP-2 and (B) MMP-9 in isolated NK cells after 24 hours of ex vivo culture. (C) Active MMP-2 (62 kDA), MMP-9 (92 kDA) and MMP-9/NGAL complexes (125 kDa) in supernatant from NK cell cultures, as measured by gelatin zymography. Quantification of active (D) MMP-2 and (E) MMP-9 secretion by densitometry. n=12-14, * P<0.05.
Impaired Natural Killer Cell Phenotype and Function in Idiopathic and Heritable Pulmonary Arterial Hypertension

Mark L. Ormiston, Chiwen Chang, Lu L. Long, Elaine Soon, Des Jones, Rajiv Machado, Carmen Treacy, Mark R. Toshner, Kate Campbell, Alex Riding, Mark Southwood, Joanna Pepke-Zaba, Andrew Exley, Richard C. Trembath, Francesco Colucci, Mark Wills, John Trowsdale and Nicholas W. Morrell

_Circulation._ published online July 25, 2012;

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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**Supplemental Methods**

*Characterization of magnetically isolated NK cells*

The surface receptor expression of magnetically isolated NK cells was characterized by staining with the following primary antibodies α-CD56-APC (clone B159), α-CD16-FITC (clone 3G8), α-KIR3DL1-FITC (clone DX9), α-KIR2DL2/3-FITC (clone GL183), α-NKG2D (clone 1D11), α-DNAM1 (clone DX11, all from BD Biosciences, Franklin Lakes, NJ), α-KIR2DL1/S1-FITC (clone EB6, Beckman Coulter, Brea, CA), α-NKp46 (clone 9E2, Miltenyi Biotec, Gladbach, Germany), α-NKp30 (clone AF29-4D12, Miltenyi Biotec) and mouse IgG1 isotype control (BD Biosciences). Unconjugated primary antibodies were used in conjunction with a goat-a-mouse IgG-APC secondary (R&D Systems, Minneapolis, MN). For CD56/CD16 dual staining, enriched NK cells were fixed and residual red blood cells were lysed after staining using BD Biosciences FACS lysis solution. All other samples were analyzed fresh.

*Human NK cell cytokine secretion*

For the measurement of NK cell cytokine secretion, parallel cultures were conducted as described for the CD107a mobilization assay without the addition of monensin or α-CD107a antibody. After 5 hours, supernatants were collected and macrophage inflammatory protein-1β (MIP-1β) secretion was quantified using a DuoSet ELISA kit (R&D Systems) as per manufacturer’s instructions.

*KIR and HLA genotyping*
As described previously\(^1\), genomic DNA samples, isolated from British, Caucasian PAH patients (n=222) or ethnically-matched blood donors (n=191) were genotyped for KIR3DL1, KIR3DS1, HLA-B and HLA-C using the sequence specific primers in Supplemental Table III. Samples were also typed for common polymorphisms of 3DL1, as reported by Yawata and colleagues\(^2\). Of the 222 samples in the PAH cohort, 13 possessed mutations in the gene for BMPR-II.

*Rodent models of pulmonary hypertension*

Male Sprague-Dawley rats, weighing 350-425 grams (Charles River, Margate, UK) were randomized and given intraperitoneal injections of either monocrotaline (60mg/kg, Sigma Aldrich, Saint Louis, MO) or an equivalent volume of sterile saline. On day 21, rats were anaesthetized with fentanyl, fluanisone and midazolam and right ventricular systolic pressure (RVSP) was measured by a 1.4F Millar catheter (Millar Instruments, Houston, TX). Blood was collected from the abdominal aorta. Following sacrifice, spleens, hearts and lung tissue were harvested and right ventricular (RV) hypertrophy was measured as a ratio of RV to left ventricular and septal weight (RV/L+S). RNA was isolated from snap frozen lung tissue by trizol extraction. Gene expression levels of PAI-1 and kir3dl1 were determined relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene by real-time PCR, using Qiagen QuantiTect primer assays.

In the chronic hypoxic model, male C57/Bl6 mice, aged 11 weeks (Charles River), were randomized and placed in either hypoxic (10% O\(_2\)) or normoxic (21% O\(_2\)) conditions. On day 21, RVSP was measured by direct cardiac puncture, as previously
3. Blood and tissues were collected and RV hypertrophy was assessed as detailed above for rats.

Assessment of rat NK cell number and function

For enumeration of NK cells in the blood and splenocytes, samples were treated with BD Pharm Lyse (BD Biosciences) to eliminate red blood cells and analyzed by flow cytometry following staining with PE-conjugated α-CD3 (clone 1F4) and APC-conjugated α-NKR-P1A (clone 10/78, both Biolegend, San Diego, CA). Untouched splenic NK cells were magnetically isolated from splenocyte suspensions by negative selection using the method described by Poli and colleagues, resulting in a yield that was >98% NKR-P1A⁺/CD3⁻ NK cells (data not shown). Following isolation, assessment of NK cell activity was determined by as a measure of chromium release following a 6 hour incubation with ⁵¹Cr-loaded YAC-1 murine lymphoma target cells at the specified effector to target ratios.

Assessment of mouse NK cell number and function

Spleens were homogenized and filtered through a 40 µm cell strainer to yield single-cell suspensions. For enumeration of NK cells in the blood and spleen, lymphocytes were treated with BD Pharm Lyse cells and analyzed by flow cytometry following staining with FITC-conjugated α-CD45 (clone 30-F11), PE-conjugated α-CD3 (clone 17A2) and APC-conjugated α-NK1.1 (clone PK136, all BD Biosciences). For lung NK cell enumeration, fresh lung tissue was minced into 1mm³ segments and digested in PBS (with Ca²⁺ and Mg²⁺) containing 1 mg/mL collagenase type IV, 0.1 mg/mL DNase I (both from Sigma) and 2% FBS at 37°C for 1 hour under gentle agitation. Following
digestion, cell suspensions were filtered through a 40 µm cell strainer and stained with the antibodies listed above.

For assessment of murine NK cell function, PBMCs were isolated from splenocyte suspensions using Ficoll Paque Plus density gradient centrifugation and NK cells were enriched using the mouse NK cell isolation kit from Miltenyi Biotec. Ficoll centrifugation is necessary in this protocol to control for an altered hematocrit in hypoxic mice. The CD107a mobilization assay was conducted as described for human cells, with the following changes. NK cells were incubated with YAC-1 target cells in the presence of 1µL FITC-conjugated α-CD107a (clone 1D4B) and 50 µM β-mercaptoethanol. Following a 5-hour co-culture, cells were counterstained with α-CD3 and α-NK1.1, fixed and permeabilized using the fix-perm kit from BD Biosciences and stained for intracellular IFNγ using PE-Cy-7-conjugated α-IFNγ antibody (clone XMG1.2, BD Biosciences). In order to obtain a robust measure of IFNγ production, murine NK cells were also cultured with or without 10 ng/mL recombinant murine IL-12 (R&D systems), in complete RPMI, containing 1.4 µg/mL monensin and 50 µM β-mercaptoethanol. Following 4-hour cultures, cells were stained for surface markers and intracellular IFNγ as described above.

**Immunofluorescence**

Sections of fixed mouse lung tissue (6 µm) were labeled with polyclonal rabbit-α-mouse von Willebrand factor (Dako, Glostrup, Denmark), followed by NL637-conjugated donkey-α-rabbit IgG secondary antibody (R&D Systems), Cy3-conjugated mouse-α-rat smooth muscle α-actin (clone 1A4, Sigma) and nuclear staining with DAPI. Vessels with
diameters ranging from 25 to 75 µm were counted from 4 randomly chosen fields and categorized as either fully-, partially- or non-muscularized.

**MMP zymography and gene expression**

Cell culture supernatants and RNA were collected from 24-hour NK cell cultures as described above. For zymography, 10µL of supernatant was loaded onto a 10% polyacrylamide gel containing 2mg/mL gelatin. Following electrophoretic separation, gels were washed three times with 2.5% Triton-X100 for 20 minutes, followed by two 20 minute washes with matrix metalloproteinase (MMP) digestion buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl and 5 mM CaCl$_2$). Gels were then incubated for 16 hours in fresh digestion buffer prior to staining with 0.4% Coomassie blue R250 in 45% methanol and 10% acetic acid. Gels were destained with 30% methanol and 10% acetic acid prior to visualization. Gene expression of MMPs -2 and -9, tissue inhibitors of MMPs (TIMPs) -1, -2 and -3, and extracellular matrix metalloproteinase inducer (EMMPRIN) were determined relative to HPRT1 by real-time PCR, using Qiagen QuantiTect primer assays.
Supplemental References


### Supplemental Table I: Subjects for Whole Blood Flow Cytometry

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*n=37*

*a. Treatment class abbreviations: ERA - Endothelin Receptor Antagonists, PGI - Prostacyclin Analogues, PDEI - Phosphodiesterase Inhibitors*
## Supplemental Table II: Subjects for Isolated NK Cell Experiments

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**Summary**  
45.4 +/- 3.4  64% female  
59.2 +/- 4.1  2.5 +/- 0.3  12.4 +/- 1.5  428 +/- 22

### Healthy Donors

| H1$^b$ | 37  | F   | Caucasian |
| H2      | 45  | F   | Asian     |
| H3      | 50  | F   | Caucasian |
| H4      | 35  | F   | Caucasian |
| H5      | 40  | M   | Caucasian |
| H6      | 58  | F   | Caucasian |
| H7      | 30  | M   | Caucasian |
| H8      | 45  | F   | Caucasian |
| H9      | 55  | M   | Caucasian |
| H10     | 47  | M   | Caucasian |
| H11     | 28  | F   | Oriental  |
| H12$^c$| 53  | M   | Caucasian |
| H13$^c$| 54  | F   | Caucasian |
| H14$^c$| 59  | F   | Caucasian |
| H15$^c$| 51  | F   | Caucasian |

**Summary**  
45.8 +/- 2.6  67% female

---

*a. Treatment class abbreviations: ERA - Endothelin Receptor Antagonists, PGI - Prostacyclin Analogues, PDEI - Phosphodiesterase Inhibitors*

*b. Donors only used for phenotypic characterization of NK cells and not subsequent functional or mechanistic studies. One patient transplanted, one relocated. One healthy donor relocated.*

*c. Donors not characterized for surface expression of activating receptors or KIR2DL1/S1 due to changes in cytometer and antibody availability.*
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<th>Antisense Primers (sequence 5’-3’)</th>
<th>Concentration (µM)</th>
<th>Amplicon size (bp)</th>
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*Control Primers (added to each primer mix to verify successful DNA amplification)*

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<td>GCATCTTGCTGTGAGAT</td>
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Supplemental Figures

Supplemental Figure 1. TGF-β stimulation in NK cells from PAH patients subdivided for the presence of a mutation in the gene for BMPR-II. Gene expression of (A) PAI-1 and (B) uPA in NK cells after 24 hours of ex vivo culture with or without TGF-β stimulation.
Supplemental Figure 2. Assessment of rat NK cell function by chromium release at all E:T ratios. Percent lysis of YAC-1 target cells by NK cells isolated from monocrotaline or saline-treated rats was determined by $^{51}$Cr release following 6-hour co-culture at the indicated effector to target ratios. Test for significance was performed at an E:T ratio of 80:1. n=6, * P<0.05.
Supplemental Figure 3. Assessment of EMMPRIN and TIMP gene expression in isolated NK cells. Gene expression of (A) EMMPRIN and (B-D) TIMPs -1, -2 and -3 in isolated NK cells after 24 hours of ex vivo culture. Quantification of active (D) MMP-2 and (E) MMP-9 secretion by densitometry. n=9-12, * P<0.05.