Circulating Myeloid-Derived Suppressor Cells Are Increased and Activated in Pulmonary Hypertension

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Background: Myeloid-derived suppressor cells (MDSCs) are increased in inflammatory and autoimmune disorders and orchestrate immune cell responses therein. Pulmonary hypertension (PH) is associated with inflammation, autoimmunity, and lung vascular remodeling. Immature myeloid cells are found in the lungs of humans and animals with PH, and we hypothesized that they would be increased in the blood of patients with PH compared with control subjects.

Methods: Twenty-six children with PH and 10 undergoing cardiac catheterization for arrhythmia ablation were studied. Five milliliters of fresh blood were analyzed using flow cytometry. Results were confirmed using magnetic bead sorting and immunofluorescence, while quantitative polymerase chain reaction and intracellular urea concentration assays were used as measures of MDSC arginase-1 activation.

Results: Flow cytometry demonstrated enrichment of circulating MDSCs among patients with PH ($n = 26$; mean, 0.271×10^6 cells/mL ± 0.17 ; 1.86% of CD45⁺ population ± 1.51) compared with control subjects ($n = 10$; mean, 0.176×10^6 cells/mL ± 0.05 ; 0.57% of CD45⁺ population ± 0.29 ; $P < .05$). Higher numbers of circulating MDSCs correlated to increasing mean pulmonary artery pressure ($r = 0.510$, $P < .05$). Among patients with PH, female patients had a twofold increase in MDSCs compared with male patients. Immunofluorescence analysis confirmed the results of flow cytometry. Quantitative reverse transcription polymerase chain reaction assay results for arginase-1 and measurement of intracellular urea concentration revealed increased activity of MDSCs from patients with PH compared with control subjects.

Conclusions: Circulating activated MDSCs are significantly increased in children with PH compared with control subjects. Further investigation of these cells is warranted, and we speculate that they might play significant immunomodulatory roles in the disease pathogenesis of PH.

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Abbreviations: Arg-1 = arginase-1; DC = dendritic cell; MDSC = myeloid-derived suppressor cell; PH = pulmonary hypertension; Treg = T regulatory

Pulmonary hypertension (PH) is a progressive syndrome with a poor prognosis that results in right-sided heart failure.¹ Although heterogeneous in its pathobiologic characteristics, it is distinguished in children and adults by cellular and structural changes in the full thickness of the walls of pulmonary arteries and by perivascular accumulation of inflammatory cells.^{2,3} Evidence for active, maladaptive inflammatory processes is manifest in both humans with PH and animal models of the disease.⁴ The scientific basis for inflammation in PH has recently been summarized⁵ and implicates innate and adaptive

immune cells, mediators and effectors of inflammation, and potential triggering events. Of particular interest to us is the collection of data implicating T and B cells in the pathobiologic aspects of PH and their interactions with mononuclear phagocytic cells. It has been suggested that a subpopulation of monocyte-derived dendritic cells (DCs) is functionally impaired in idiopathic PH⁶ and that the presence of immature DCs may alter the T and B cell responses in the hypertensive lung.⁷

Myeloid-derived suppressor cells (MDSCs) compose a phenotypically diverse subpopulation of cells

that includes mature (granulocytes, monocytes, macrophages, DCs) and immature (myelo-monocytic precursors) myeloid cells.⁸ MDSCs were described 20 years ago⁹ and regulate immune responses in both normal and disease settings.¹⁰ MDSCs participate in a multiplicity of processes, including innate and acquired immunity,¹⁰ autoimmunity,⁹ and nonimmunologic responses such as angiogenesis.¹¹ Immunohistochemical analysis of lung sections from patients with PH indicates that immature DCs are present in and around areas of vascular remodeling.⁷ In the rat model of monocrotaline-induced PH, the DCs recruited to remodeled vessels display an immature myeloid phenotype.⁷ Additional studies reveal that monocyte-derived DCs from patients with PH are defective in their ability to stimulate T cells in an allostimulatory mixed-leukocyte reaction assay.⁶ Indeed, abnormalities of T lymphocyte subsets have been documented in patients with PH.^{12,13} Although a direct mechanistic role for MDSCs was not established in these studies, these functionally impaired immature myeloid cells or DCs appeared prior to the formation of, but remained constantly present within, areas of vascular remodeling.

We reasoned that MDSCs are participating in the immunomodulatory process apparently active in PH, and therefore might be in greater quantity in the peripheral blood of patients with PH. We found significant increases in MDSCs in these patients compared with control subjects and found correlation with increasing mean pulmonary artery pressure. We conclude that PH is associated with elevated levels of circulating active MDSCs. A more complete

understanding of their putative roles in PH might lead to novel therapies.

MATERIALS AND METHODS

Subjects, Clinical Data, and Blood Collection

The Colorado Multiple Institutional Review Board approved the study (10-0259), and consent (assent where appropriate) was obtained from all patients. Demographic data for all subjects are presented in Tables 1 and 2. Five milliliters of blood were drawn from the femoral vein during initial cardiac catheterization in all patients. The pulmonary arterial hypertension group consisted of children and young adults < 18 years of age at the time of diagnosis with PH, with World Health Organization classification group 1 defined as either idiopathic pulmonary arterial hypertension, heritable pulmonary arterial hypertension, or pulmonary arterial hypertension associated with congenital heart disease. The control group included children < 18 years of age with a structurally normal heart undergoing heart arrhythmia ablation, before the onset of the procedure. One control patient was included who was undergoing repair for a ventricular septal defect. All patients undergoing ablation were in normal sinus rhythm at the start of the catheterization when the blood was drawn and had a normal echocardiogram.

Hemodynamics and echocardiographic data were obtained at the time of initial catheterization, then later at therapeutic assessment. Fresh, never-frozen blood (5 mL) was prepared without Ficoll gradient as described by Hong et al.¹⁴ Buffy coat cells were removed from blood centrifuged for 10 min at 1,200 rpm at room temperature. Following RBC lysis by the addition of ammonium chloride (0.15 M, pH 7.3), leukocytes were washed, verified for viability using trypan blue exclusion, and prepared to a concentration of 1×10^6 cells/mL for antibody labeling and flow cytometry.

Flow Cytometric Analysis

Cells were labeled using antibodies against CD45-Pacific Blue (PB986; BD Biosciences), CD33-FITC (340533; BD Biosciences), HLA-DR-PE (FAB4869P; R&D Systems), and CD11b-APC (340936; BD Biosciences), per manufacturer's protocol. For flow cytometry, CXP software and an FC500 instrument (Beckman) were used. Negative thresholds for gating were set using isotype control-labeled cells from both patients with PH and control subjects, as described by Mathai et al.¹⁵ All samples were gated for the CD45⁺ region, then for expression of CD33, then analyzed for CD11b expression and absence of HLA-DR (MHCII). MDSC numbers (CD45⁺/CD33⁺/MHCII⁻/CD11b⁺) were calculated and also expressed as a percentage of total leukocytes. More than 200,000 events were used to generate histograms.

Cytospin and Immunofluorescence

Flow-sorted cells from three patients with PH and three control subjects were centrifuged at 800 rpm and resuspended in 200 μ L. Following cytopins, cells were fixed in cold acetone/methanol for 10 min. MDSCs were assessed as MHCII⁻/CD33⁺/CD11b⁺. Antibodies used were pSTAT3 (Tyr705, Cell Signaling 9131), CD33 (Millipore WM53), CD11b (Abcam 2Q902), and MHCII (eBioscience 14-9956). Antibody isotype-negative controls were included with each sample group, as was preadsorption of antibody to its cognate antigen. Slides were mounted in Vectashield plus 4',6-diamidino-2-phenylindole medium (Vector), and images were acquired at room temperature using an Axiovert S100 (Zeiss) fitted with Zeiss 20 $\times$ 0.4 numerical aperture, and 10 $\times$ 0.3 numerical aperture objectives.

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Table 1—Data for Subjects With PH

Sex	PH, WHO Classification	Present Age, y	Date of Diagnosis	mPAP at Diagnosis, mm Hg	Date of Sample	mPAP at Date of Sample, mm Hg	MDSC, %	MDSC, $\times 10^6$ cells/mL	Medications at Transition
F	iPAH, 1	20	2/7/07	32	7/14/10	30	1.76	0.18	Diltiazem
M	iPAH, 2	15	9/25/95	n/a	7/14/10	37	1.4	0.21	Sildenafil
									+ Remodulin (IV)
									+ Ambrisentan
F	iPAH, 2	9	6/29/05	90	7/14/10	28	5.6	0.45	Flolan
									+ Ambrisentan
F	sPAH, 2	15	5/21/99	n/a	7/15/10	54	2.4	0.34	Tadalafil
									+ Remodulin (IV)
									+ Ambrisentan
M	sPAH, 4	19	3/21/02	54	7/15/10	55	1.9	0.16	Tadalafil
									+ Flolan
									+ Ambrisentan
F	sPAH, 2	15	5/15/02	71	7/21/10	51	2.65	0.27	Tadalafil
									+ Treprostinil
F	iPAH, 2	10	10/7/08	58	7/21/10	35	1.65	0.15	Tadalafil
									+ Ambrisentan
M	sPAH, 3	17	10/6/92	n/a	8/11/10	43	2.37	0.29	Tadalafil
									+ Treprostinil
F	sPAH, 1	12	11/21/01	19	8/11/10	23	0.88	0.13	Nifedipine
F	sPAH, 1	9	2/1/07	56	8/11/10	18	0.98	0.11	Sildenafil
									+ Ambrisentan
									+ Nifedipine
M	iPAH, 2	2	8/20/08	n/a	8/18/10	17	0.65	0.12	Sildenafil
									+ Remodulin (IV)
M	iPAH, 1	15	8/1/00	n/a	8/18/10	20	0.25	0.16	Tadalafil
									+ Iloprost
									+ Ambrisentan
F	sPAH, 2	12	5/19/05	n/a	8/18/10	37	5.7	0.47	Sildenafil
									+ Bosentan
F	sPAH, 1	5	8/26/09	38	8/25/10	26	2.51	0.29	Nifedipine
F	iPAH, 1	3	9/20/07	n/a	8/25/10	24	3.15	0.39	Sildenafil
M	sPAH, 2	13	10/11/01	73	8/25/10	56	2.85	0.31	Sildenafil
									+ Remodulin (IV)
									+ Ambrisentan
F	sPAH, 3	18	3/1/96	n/a	9/8/10	61	5.1	0.71	Sildenafil
									+ Remodulin (IV)
									+ Bosentan
M	iPAH, 1	15	10/26/05	70	9/8/10	44	2.1	0.24	Tadalafil
									+ Ambrisentan
M	sPAH, 4	17	2/19/97	n/a	10/4/10	80	1.95	0.19	Tadalafil
									+ Flolan
									+ Bosentan
M	sPAH, 2	22	12/19/95	n/a	10/13/10	32	1	0.11	Ambrisentan
M	iPAH, 2	10	12/17/04	106	10/13/10	74	3	0.63	Remodulin (IV)
									+ Tadalafil
M	sPAH, 2	4	5/29/07	20	10/13/10	14	0.1	0.12	None
M	sPAH, 2	2	10/29/09	28	10/21/10	28	0.5	0.21	Sildenafil
M	sPAH, 1	1	12/22/08	n/a	11/10/10	19	0.1	0.11	Sildenafil
M	sPAH, 1	3	3/8/10	n/a	11/10/10	32	0.9	0.31	Sildenafil
F	iPAH, 1	11	10/10/05	48	11/10/10	41	1.5	0.36	Tadalafil
									+ Bosentan

Values are presented as mean $\pm$ SD. F = female; iPAH = idiopathic pulmonary arterial hypertension; M = male; MDSC = myeloid derived suppressor cell; mPAP = mean pulmonary artery pressure; n/a = not catheterized at the time; PH = pulmonary hypertension; + = medication taken; sPAH = secondary pulmonary arterial hypertension; WHO = World Health Organization.

Immunomagnetic Bead Separation

Buffy coat cells were sorted per manufacturer's instructions using the following separation sequences: CD33⁺ (130-045-501; Miltenyi), MHCII⁻ (130-046-101; Miltenyi), and CD11b⁺ (130-049-

601; Miltenyi). MDSCs were then immediately processed for either quantitative polymerase chain reaction or urea assay, as described in the "Quantitative Polymerase Chain Reaction" section.

Table 2—Data for Control Subjects

Sex	Diagnosis	Present Age, y	Date of Diagnosis	Date of Sample	MDSC, %	MDSC, $\times 10^6$ cells/mL
M	WPW	8	2/7/07	7/14/10	0.7	0.23
M	WPW, closing VSD, no PH	13	9/25/95	7/14/10	0.25	0.15
F	Arrhythmia (SVT)	10	6/29/05	7/14/10	0.9	0.2
F	Arrhythmia (SVT)	17	5/21/99	7/15/10	0.3	0.11
M	Arrhythmia (SVT)	14	3/21/02	7/15/10	0.5	0.17
F	WPW	9	5/15/02	7/21/10	0.25	0.12
M	Arrhythmia (SVT)	13	10/7/08	7/21/10	1.1	0.2
M	WPW	14	10/6/92	8/11/10	0.3	0.14
F	Arrhythmia (SVT)	17	11/21/01	8/11/10	0.5	0.3
M	Arrhythmia (SVT)	14	2/1/07	8/11/10	0.9	0.14

Values are presented as mean $\pm$ SD. SVT = supra ventricular tachycardia; VSD = ventricular septal defect; WPW = Wolff-Parkinson-White syndrome. See Table 1 legend for expansion of other abbreviations.

Quantitative Polymerase Chain Reaction

Active MDSC expression increased transcripts for arginase-1 (Arg-1).¹⁶ Primer sequences used to assess Arg-1 gene transcripts were F: 5'-GGAACCCAGAGAGCATGA-3'; and R: 5'-TTTTCCAGCAGACCAGCTT-3'. Bead-sorted MDSC total RNA isolation, RNA concentration, complementary DNA transcription, polymerase chain reaction cycling, and normalization were performed as previously described.¹⁷ Cells recovered from bead separations were pooled ($n = 5$ patients per group) prior to RNA analysis.

Urea Concentration Measurement

Stimulated MDSCs increase Arg-1 activity, as measured by urea production from the arginase metabolism of L-arginine to L-ornithine and urea.¹⁸ Pooled, bead-sorted MDSCs were counted, and an equivalent cell number (10^4) of lysates (in 10 mM Tris-HCl, pH 7.4 with 0.4% Triton X-100) from control subjects ($n = 7$) and from patients with PH ($n = 21$) were assayed colorimetrically in duplicate for urea, as previously described¹⁹ (Quantichrom Arginase Kit, DARG-200, Gentaur). Forty microliters of pooled sample were added to substrate buffer, and the difference in absorbance (520 nm) with and without pretreatment with urease (Sigma U1500) was compared with a urea standard curve.

Statistical Analysis

Values were expressed as mean $\pm$ SEM using Prism5 (GraphPad). Unpaired t tests were performed for two group comparisons, while Pearson correlation analysis was used to compare MDSC suppressor cell numbers with clinical and hemodynamic parameters. Significance was defined as $P < .05$.

RESULTS

MDSC Quantification

Circulating MDSCs expand in settings of autoimmunity and inflammation¹⁰ and following immunization with ovalbumin.²⁰ PH is associated with inflammation²¹ and autoimmunity,²² and can be induced by antigenic challenge via airway ovalbumin.²³ We hypothesized that individuals with PH would have increased circulating MDSCs. We found that they had increased MDSCs, expressed as cells per milliliter or as a percentage of CD45⁺ cells, compared with control subjects

($n = 26$; mean, 0.271×10^6 cells/mL ± 0.17 ; $1.86\% \pm 1.51\%$; vs $n = 10$; mean, 0.176×10^6 cells/mL ± 0.05 ; $0.57\% \pm 0.29\%$, respectively; $P < .05$). Analysis is shown in Figure 1, and quantification in Figures 2A, 2B. No differences were observed in MDSC numbers among those patients with idiopathic PH compared with those with secondary PH. Female patients with PH had higher MDSC percentages, but not cells per milliliter, compared with male subjects with PH (female subjects, $n = 12$; mean, 0.326×10^6 cells/mL ± 0.19 ; $2.68\% \pm 1.72\%$; vs male subjects, $n = 14$; mean, 0.226×10^6 cells/mL ± 0.13 ; $1.36\% \pm 0.97\%$; $P < .01$ for percentage data and $P < .13$ for cells per milliliter data) (Figs 2C, 2D).

MDSC Correlation to Clinical and Hemodynamic Parameters

MDSCs are known to correlate to tumor burden, infection, inflammation, and autoimmunity.¹⁰ In our cohort of patients with PH, MDSC counts positively correlated to mean pulmonary artery pressure ($r = 0.417$ for percentage and 0.510 for cells per milliliter; $P < .05$) (Fig 2E), but not increasing age or duration of treatment.

Immunophenotyping and Activity Assessment of MDSC

We verified the sorted MDSC phenotype using immunofluorescence. MDSCs sorted from the blood of patients with PH and blood from control subjects strongly expressed CD33 and CD11b, but not MHCII, confirming the cytometry results (Figs 3A, 3B). Additionally, phospho-STAT3 was detected in MDSCs sorted from the blood of patients with PH but not blood from control subjects (Fig 3C). PhosphoSTAT3 is associated with the survival and proliferation of MDSCs.²⁴ Arg-1 activity, along with that of inducible nitric oxide synthase, mediates the suppression of T-cell responses by MDSCs.¹⁵ To investigate differences in Arg-1 expression and activity, we performed

FIGURE 1. Scatter diagrams show flow cytometric analysis for myeloid-derived suppressor cells (MDSCs). A, Side scatter (SSC) vs forward scatter (FSC) analysis of $\geq 200,000$ cells from representative control subject blood sample. B, SSC vs CD45 Pacific Blue (PB) isotype sample from control subject for gating. C, Isotype control used to set gating for the CD45⁺/CD33⁺ population from control subject blood sample. D, Isotype control used to set gating for the MHCII⁺/CD11b⁺ population from control subject blood sample. E, This control subject had 0.5% of total CD45⁺ cells labeling as MHCII⁺/CD11b⁺ (quadrant B4), corresponding to 0.17×10^6 cells/mL. F, Isotype control used to set gating for the MHCII⁺/CD11b⁺ population from blood sample of patient with pulmonary hypertension (PH). G, This patient with PH had 2.1% of total CD45⁺ cells labeling as MHCII⁺/CD11b⁺ (quadrant B4), corresponding to 0.24×10^6 cells/mL. Isotype controls were performed to set gates on each sample from control subjects and patients with PH, as described by Mathai et al.¹⁵ and all samples were run in triplicate. APC = allophycocyanin; FITC = fluorescein isothiocyanate; FL = channel; FS = forward scatter; INT LIN = integration linear; INT LOG = integration logarithmic; PE = phycoerythrin; SS = side scatter.

quantitative polymerase chain reaction assays for Arg-1 transcripts and measured urea concentrations¹⁹ on pooled lysates from bead-sorted MDSCs. MDSCs from control subjects had minimal Arg-1 gene expression and activity, while those from patients with PH showed enhanced expression and activity of Arg-1 (Figs 3D-3E).

DISCUSSION

We have previously shown that cells of mononuclear phagocytic origin accumulate in peribronchovascular regions in animal models of PH and contribute to vascular remodeling.⁴ Others have described immature myeloid cells in similar regions in humans and in animal models, often in proximity with T and B lymphocytes.⁷ Furthermore, observations of impaired DC function⁶ and increases in T regulatory (Treg) lymphocytes^{12,13} convinced us to test whether MDSC levels would be elevated in patients with PH compared with control subjects. We demonstrate an increase in circulating CD45⁺/CD33⁺/MHCII⁺/CD11b⁺ MDSCs

in patients with PH compared with control subjects. We found correlation between MDSC counts and mean pulmonary artery pressure. Our observations support the notion that the mobilization of MDSCs contributes to pathologic pulmonary vascular remodeling, since MDSCs are potent immunomodulatory cells functionally implicated in the pathobiologic aspects of chronic inflammatory disorders, cancer, and immunosuppression phenomena. This work extends (in humans) our previous findings of, in hypoxic models of PH, an abundance of circulating mononuclear cells that also appear in the pulmonary vasculature, are actively proliferating, and are profibrotic and pro-inflammatory. The current study identifies a unique subset of circulating mononuclear cells as myeloid suppressor cells with an activated phenotype.

MDSCs are a heterogeneous population of cells, and local contexts of inflammatory microenvironments greatly affect their tissue recruitment, retention, and immunomodulatory capabilities.²⁵ There are significant differences between the pulmonary microvasculature and larger lung vessels in terms of cell

FIGURE 2. Scatter diagrams show that circulating MDSC counts are enriched in patients with PH compared with control subjects and correlate to mean pulmonary artery pressure. A and B, Mean MDSC counts in 26 patients with PH were mean, 0.271×10^6 cells/mL ± 0.17 , $1.86\% \pm 1.51\%$; and counts in 10 control subjects were mean, 0.176×10^6 cells/mL ± 0.05 ; $0.57\% \pm 0.29\%$; $P < .05$ vs control subjects. C and D, Female subjects with PH ($n = 12$; mean, 0.32×10^6 cells/mL ± 0.19 ; $2.68\% \pm 1.72\%$) had higher MDSC counts than male subjects with PH ($n = 14$; mean, 0.23×10^6 cells/mL ± 0.13 ; $1.36\% \pm 0.97\%$); $P < .05$ vs control for percentage. E, MDSC numbers per milliliter in patients with PH positively correlate to mean PAP ($r = 0.510$; $P < .05$ vs control subjects). PAP = pulmonary artery pressure. See Table 1 legend for expansion of other abbreviations.

constituency and specialization of cell function.²⁶ Microenvironmental cues in these compartments may facilitate the recruitment and differentiation of unique MDSC subsets. Wang et al⁶ observed decreased levels of blood myeloid DCs in patients with PH compared with control subjects, which would appear to be in conflict with our data showing increased levels of MDSCs in patients with PH. However, the myeloid DCs in their study were HLA-DR⁺/CD11c⁺ cells, while we focused on MDSCs defined by several groups¹⁰ as HLA-DR⁻/CD11b⁺. Furthermore, Perros et al⁷ determined that immature myeloid cells in lungs from the monocrotaline rat model of PH had an OX-62⁺/CD68⁻/MHCII⁻(HLA-DR⁻)/CD11b⁺ phenotype characteristic of MDSC. Thus, unique circulating subsets of immature myeloid cells could vary in number and/or simultaneously differentiate and home in to distinct lung niches.

To effect immunosuppression, MDSCs act in proximity to T cells, perhaps requiring direct contact.²⁷ Although we did not directly address MDSC-mediated immunosuppression, several lines of evidence offer clues as to how this might occur in the setting of PH. First, plasma IL-6 levels appear to be elevated in PH,²⁸ and overexpression of IL-6 in mice induces PH.²⁹ IL-6 potently activates pSTAT3 and augments survival in MDSCs,²⁴ and we found increased pSTAT3 in MDSCs from patients with PH compared with control subjects. Second, expression of S100 proteins such as S100A4 contributes to cell motility and

plexogenic arteriopathy in PH.³⁰ Interestingly, the S100 family of inflammatory mediators, particularly S100A8/A9, participates in an autocrine feedback loop that sustains accumulation of MDSCs in mouse tumor models.³¹ Once retained in the lung, MDSCs may be able to induce FOXP3⁺ Treg lymphocytes, as they do in several models of inflammation and cancer.³² Two groups showed increased Treg cells in the peripheral blood of patients with idiopathic pulmonary arterial hypertension.^{12,13} Finally, while IL-4 upregulates immunosuppressive activity of MDSCs via Arg-1,³³ it also drives Th2 immune responses in PH.²³ Arg-1, in turn, is important for the development of secondary lymphoid organs such as bronchus-associated lymphoid tissues,³⁴ which are critical sites for immune tolerance and self-antigen presentation potentially involving MDSCs. Intriguingly, bronchus-associated lymphoid tissues are more conspicuous in the lung tissues of humans with PH¹³ and in lungs from both the chronic hypoxia and monocrotaline rat models of PH (Michael E. Yeager, PhD, February 2011, unpublished data). Important next steps will be to determine whether the MDSCs in patients with PH are similar (or different) than the MDSCs in patients with cancers and autoimmune diseases, whether increases in blood MDSCs translate to increases in lung MDSCs, and what are the mechanisms of influx and retention.

In cancer, MDSCs inhibit innate and adaptive immunity and subvert the elimination of transformed

FIGURE 3. Photomicrographs and charts display the expression and activity analysis of circulating MDSCs. A, Buffy coat cells were sorted using immunomagnetic beads conjugated to anti-CD33, CD11b, and MHCII antibodies. Cells were pooled ($n =$ three per group) and probed using antibodies against CD33 or CD11b (red, Cy3) and either MHCII or pSTAT3 (green, Alexa-488). Neither CD33⁺ cells (B) nor CD11b⁺ cells (C) appreciably coexpress MHCII. D, Cells from patients with PH, but not cells from control subjects, contained detectable pSTAT3. E, Pooled ($n =$ five per group) sorted CD33⁺/CD11b⁺/MHCII⁻ cells were analyzed using quantitative polymerase chain reaction assays for arginase-1 (Arg-1) mRNA. Patients with PH had increased Arg-1 levels compared with control subjects, normalized to the housekeeping gene HPRT. F, Arginase activity, as measured by intracellular urea concentration, was significantly enhanced in MDSCs from patients with PH compared with pooled control subjects. For F, the data represent the mean $\pm$ SD, $n = 21$ samples from patients with PH and $n =$ seven samples from control subjects that were pooled following bead separation, from a representative group of three independently performed experiments. White scale bar = 10 μ m. CTL = control sample; DAPI = 4',6-diamidino-2-phenylindole; n.d. = below level of detection. See Figure 1 legend for expansion of other abbreviations.

cells.³⁵ MDSCs suppress CD4⁺ and CD8⁺ T cells by mechanisms involving arginase, nitric oxide, and local depletion of cysteine.⁸ Further, MDSCs induce Treg cells, which then depress cell-mediated immunity,³⁶ and they skew local immune responses from a T helper 1 to a T helper 2 program by IL-10 secretion.¹⁶ Last, MDSCs promote angiogenesis in models of cancer.^{37,38} In the context of PH associated with autoimmunity, MDSCs may be required to delimit the activation of autoreactive T and B cells. Some forms of PH have an autoimmune component, including production of autoantibodies.^{22,39} Our data showing increased levels of MDSCs in female patients with PH compared with male patients with PH may be related to the increased predilection for female patients to develop autoimmune disorders as well as PH.

Several potential limitations of our study should be pointed out. First, although our finding of increased activated MDSCs is novel, we lack lung-specific data. Therefore, the increase in MDSCs may be associative and not causal. We were unable to confirm the presence of MDSCs in the lungs of our patient cohort as a result of the scarcity of available tissue. Second, MDSCs are admittedly a heterogeneous population. Therefore, the marker set we chose may not encom-

pass the chief subset (or subsets) of MDSCs influencing inflammation in the lungs of patients with PH. Third, we did not directly use assays to test for the immunosuppressive capability of the MDSCs because we had insufficient numbers to carry out such assays.

All-*trans*-retinoic acid has been used to attempt to mature MDSCs to attenuate their immunosuppressive function in patients with cancer.¹⁰ In the face of the extensive vascular remodeling present in patients with PH, it seems unlikely that therapeutically “braking” or modulating persistent inflammation would, by itself, lead to the restoration of normal lung vasculature. A better understanding of which subsets of mononuclear phagocytic cells (eg, fibrocytes, MDSCs, etc) are contributing to the pathologic characteristics of PH, and by what mechanisms, should greatly facilitate the future success of unique combinatorial therapies. Future studies will determine the extent of MDSC infiltration in the lungs of patients with PH, and using animal models, we will attempt to unravel their putative mechanistic contribution to inflammation-associated vascular remodeling, with particular emphasis on immune suppression phenomena.

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Dr Yeager: conceived of the study, conducted experiments, interpreted data, wrote and revised the manuscript, and approved the final article for publication.

Ms Nguyen: conducted experiments, helped draft and revise the manuscript, and approved the final article for publication.

Mr Belchenko: conducted experiments, helped draft and revise the manuscript, and approved the final article for publication.

Ms Colvin: conducted experiments, helped draft and revise the manuscript, and approved the final article for publication.

Dr Takatsuki: conducted experiments, helped draft and revise the manuscript, and approved the final article for publication.

Dr Ivy: provided patient access, interpreted data, provided funding, helped draft and revise the manuscript, and approved the final article for publication.

Dr Stenmark: interpreted data, provided funding, helped draft and revise the manuscript, and approved the final article for publication.

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