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Original Article

A toxicology study of *Csf2ra* complementation and pulmonary macrophage transplantation therapy of hereditary PAP in mice

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Pulmonary macrophage transplantation (PMT) is a gene and cell transplantation approach in development as therapy for hereditary pulmonary alveolar proteinosis (hPAP), a surfactant accumulation disorder caused by mutations in *CSF2RA/B* (and murine homologs). We conducted a toxicology study of PMT of *Csf2ra* gene-corrected macrophages (mGM-Rα$^+$ Mϕs) or saline-control intervention in *Csf2ra*^{KO} or wild-type (WT) mice including single ascending dose and repeat ascending dose studies evaluating safety, tolerability, pharmacokinetics, and pharmacodynamics. Lentiviral-mediated *Csf2ra* cDNA transfer restored GM-CSF signaling in mGM-Rα$^+$Mϕs. Following PMT, mGM-Rα$^+$Mϕs engrafted, remained within the lungs, and did not undergo uncontrolled proliferation or result in bronchospasm, pulmonary function abnormalities, pulmonary or systemic inflammation, anti-transgene product antibodies, or pulmonary fibrosis. Aggressive male fighting caused a similarly low rate of serious adverse events in saline- and PMT-treated mice. Transient, minor pulmonary neutrophilia and exacerbation of pre-existing hPAP-related lymphocytosis were observed 14 days after PMT of the safety margin dose but not the target dose (5,000,000 or 500,000 mGM-Rα$^+$Mϕs, respectively) and only in *Csf2ra*^{KO} mice but not in WT mice. PMT reduced lung disease severity in *Csf2ra*^{KO} mice. Results indicate PMT of mGM-Rα$^+$Mϕs was safe, well tolerated, and therapeutically efficacious in *Csf2ra*^{KO} mice, and established a no adverse effect level and 10-fold safety margin.

INTRODUCTION

Mutations in either *CSF2RA* or *CSF2RB*, encoding the granulocyte/macrophage colony-stimulating factor (GM-CSF) receptor, cause hereditary pulmonary alveolar proteinosis (hPAP), a recessive disorder of alveolar macrophage dysfunction, pulmonary surfactant accumulation, and hypoxemia, typically presenting as progressive dyspnea of insidious onset in a previously healthy child.[1–4] The natural history also includes pulmonary fibrosis, respiratory failure, and death in some individuals.[5] Of the 55 people with hPAP diagnosed globally since it was first reported in 2008,[1,2] *CSF2RA* mutations were causative in 44 (80%) and *CSF2RB* mutations in 11 (20%). Mice homozygous for *Csf2ra* or *Csf2rb* gene ablation develop hPAP identical to the human disease with respect to clinical, physiological, histopathological, and biochemical abnormalities, disease biomarkers, and natural history.[6–8] Myeloid cells require constitutive stimulation by GM-CSF to maintain their differentiated state and functions.[3,9] Without GM-CSF, alveolar macrophages have impaired clearance of surfactant-derived cholesterol, which accumulates intracellularly, becomes esterified and sequestered in

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intracytoplasmic lipid droplets (as a cell protective mechanism), resulting in foamy-appearing macrophages with further impairment of surfactant clearance and other functions.[9–12] Absence of GM-CSF signaling also impairs neutrophil host defense functions[13] and increases susceptibility to a wide range of microbial pathogens.[11,13–19]

No Food and Drug Administration (FDA)-approved specific therapy of hPAP is available. Currently, hPAP is treated by periodic whole lung lavage (WLL), a procedure to physically remove surfactant by washing the lungs that does not correct the macrophage dysfunction or stop surfactant accumulation.[20] WLL requires general anesthesia, intubation of each lung, and mechanical ventilation of the non-treated lung while the other is washed with saline (up to 50 L per lung in adults). Although some patients require WLL every 1–2 months,[21] it is not widely available, especially at pediatric medical centers, and is more difficult in small children due to the unavailability of small double-lumen endotracheal tubes.[5,22] In mice, hPAP was successfully treated by *Csf2rb* gene correction and bone marrow transplantation.[23] In humans, lentiviral vector-mediated complementation of *CSF2RA* mutations in hPAP patient-derived induced pluripotent stem cell-derived macrophages restored GM-CSF signaling and surfactant clearance in a laboratory setting,[24] but gene therapy has not been attempted *in vivo*. Allogeneic bone marrow transplantation, stem cell transplantation, and lung transplantation have been performed with mixed results in part secondary to complications from infection related to myeloablation and immunosuppression and from graft rejection.[2,25–29]

Pulmonary macrophage transplantation (PMT) is a novel gene and cell therapy approach involving direct pulmonary instillation of autologous gene-corrected macrophages without myeloablation or immune suppression.[6] In mice, PMT of either wild-type (WT) or *Csf2rb* gene-corrected macrophages in *Csf2rb*^{KO} mice resulted in engraftment, replacement of endogenous dysfunctional alveolar macrophages by transplanted functional macrophages resulting in efficacious and durable correction of hPAP that increased survival and lifespan by 20%.[6] Preclinical studies in *Csf2ra*^{KO} mice yielded similar results for PMT of either WT or *Csf2ra* gene-corrected macrophages.[8,30]

We report a formal toxicology study conducted to support our investigational new drug (IND) application (no. 28593). Results inform the preclinical safety of lentiviral vector-mediated *Csf2ra* gene correction and PMT therapy of hPAP and establish a no observed adverse effect level (NOAEL) and safety margin in mice.

RESULTS

Study design and rationale

This study evaluated PMT of congenic *Csf2ra*^{KO} bone marrow-derived, *Csf2ra* gene-corrected macrophages (mGM-Rα^+Mϕs) in *Csf2ra*^{KO} or WT recipients, without preparative myeloablation, and included single ascending dose (SAD) studies conducted over 24 h (SAD-001a) or 14 days (SAD-001b) and a repeat ascending dose (RAD) study conducted over 6 months (RAD-002) (Figure 1; Table S1). Both SAD studies included three groups each of *Csf2ra*^{KO} and WT mice wherein each mouse received one administration of saline (50 µL) or mGM-Rα^+Mϕs at a dose of 500,000 or 5,000,000 cells/mouse—saline, PMT-500K, and PMT-5M groups, respectively. The RAD study included two groups of *Csf2ra*^{KO} mice wherein each mouse received three monthly intrapulmonary administrations of saline (50 µL) or mGM-Rα^+Mϕs (at sequential doses of 26,000, 105,000, or 500,000 cells/mouse in 50 µL saline)—saline and PMT groups, respectively. Room-specific and study-specific sentinel mice were housed alongside and studied in parallel to ensure the adequacy of housing and study conditions.

Production and characterization of recombinant *Csf2ra*-expressing SIN-LV vector

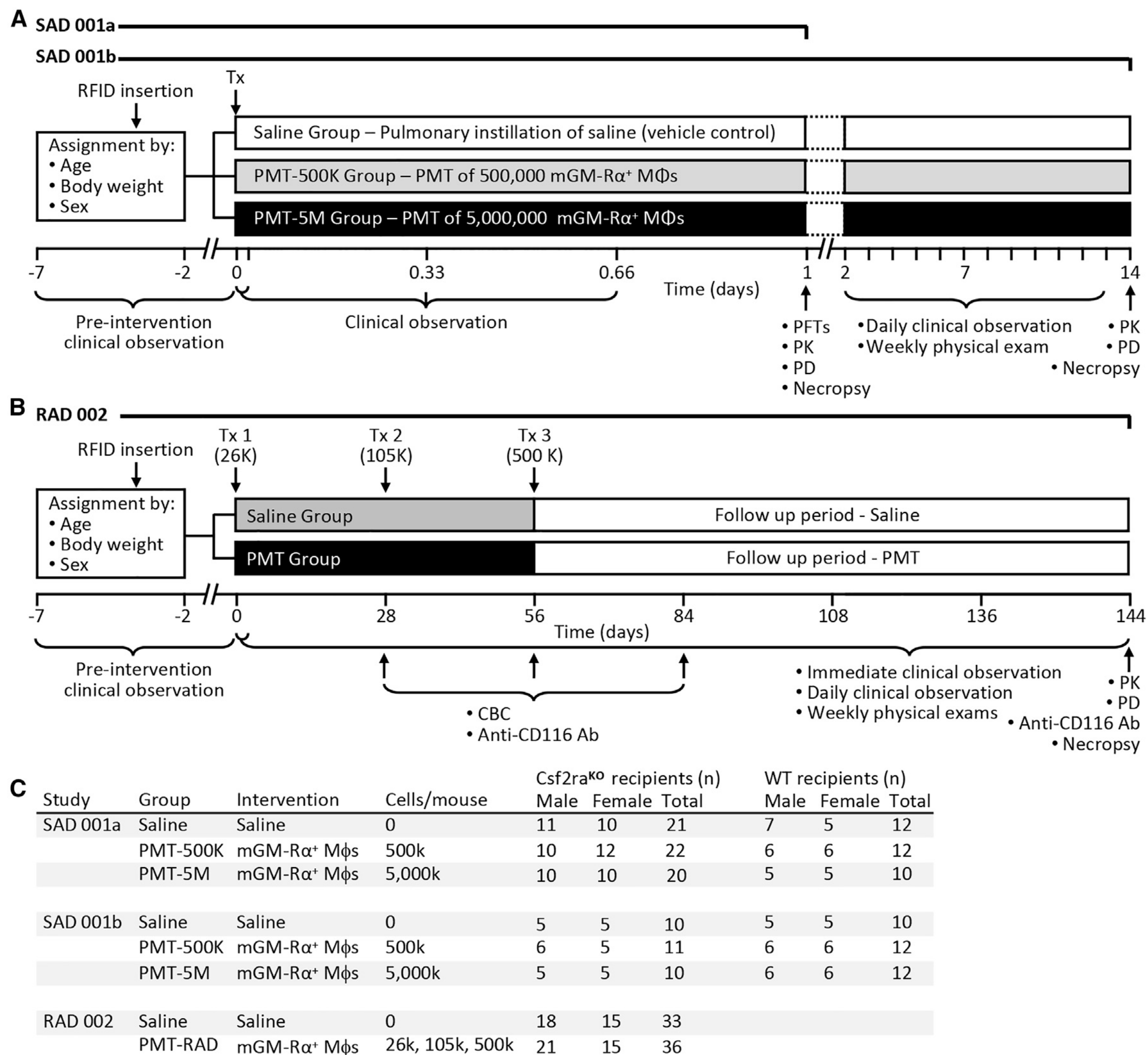
A third-generation, self-inactivating lentiviral vector was constructed to express the murine *Csf2ra* cDNA (mGM-Rα-LV) and was similar in design (Figure 2A) to the vector[31] for use in the human trial except for inclusion of a species-specific transgene, which was necessary because human and mouse GM-CSF are neither functionally interchangeable nor immunologically cross-reactive.[32–34] The vector was manufactured by transient transfection,[30] characterized extensively, and shown to have no microbial contamination, minimal endotoxin, an adequate titer (Table S2), and a DNA sequence identical to the reference vector (Figure S1) and to the human vector backbone (not shown).[31]

Production, characterization, and cryopreservation of mGM-Rα^+Mϕs

The mGM-Rα^+Mϕs used throughout this study were prepared from a single manufacturing lot of *Csf2ra*^{KO} hematopoietic lineage negative, Sca-1$^+$, c-kit$^+$ bone marrow cells by transduction with mGM-Rα-LV (lot no. VPF-2907) followed by expansion and differentiation into macrophages *ex vivo* using established protocols.[30] Extensive characterization demonstrated freshly harvested mGM-Rα^+Mϕs had a total cell count of 1.4 billion cells, viability of >98%, macrophage morphology of >98% (Figure 2B), good expression of macrophage phenotypic markers (Figure 2C) and CD116 (Figure 2D), restoration of GM-CSF signaling (Figure 2E), no baseline macrophage activation (Figure 2F), an acceptably low endotoxin level, and no bacterial, fungal, and mycobacterial contamination (Table S3). Transduction efficiency was determined by measuring the number of vector genome copies per cell (vector copy number or VCN) as reported,[30] which was 2.09 following transduction and 4.35 in the harvested cell product (Table S3). The mGM-Rα^+Mϕs were cryopreserved (as drug substance) at 4 or 10 million cells/mL and stored at -135°C to -190°C.

Thawing, stability, formulation, and characterization of mGM-Rα^+Mϕs

On the day of administration, cryopreserved mGM-Rα^+Mϕs were thawed, washed, resuspended in 0.9% sodium chloride (formulated drug product) and evaluated. CD116 expression was readily detected (Figure 2G) and potency was maintained as shown by short-term (Figure 2H), long-term (Figure 2I), and in-use (Figure 2J) stability studies. Formulated drug product was prepared on 17 occasions with consistent results for (mean ± SD) cell viability (95% ± 1.9% of cells), endotoxin content (all <0.011 ± 0.001 endotoxin units/mL), Gram stain (all negative for microbial pathogens), and sterility



Study	Group	Intervention	Cells/mouse	Csf2ra^KO recipients (n) Male	Female	Total	WT recipients (n) Male	Female	Total
SAD 001a	Saline	Saline	0	11	10	21	7	5	12
	PMT-500K	mGM-Rα⁺ Mϕs	500k	10	12	22	6	6	12
	PMT-5M	mGM-Rα⁺ Mϕs	5,000k	10	10	20	5	5	10
SAD 001b	Saline	Saline	0	5	5	10	5	5	10
	PMT-500K	mGM-Rα⁺ Mϕs	500k	6	5	11	6	6	12
	PMT-5M	mGM-Rα⁺ Mϕs	5,000k	5	5	10	6	6	12
RAD 002	Saline	Saline	0	18	15	33			
	PMT-RAD	mGM-Rα⁺ Mϕs	26k, 105k, 500k	21	15	36			

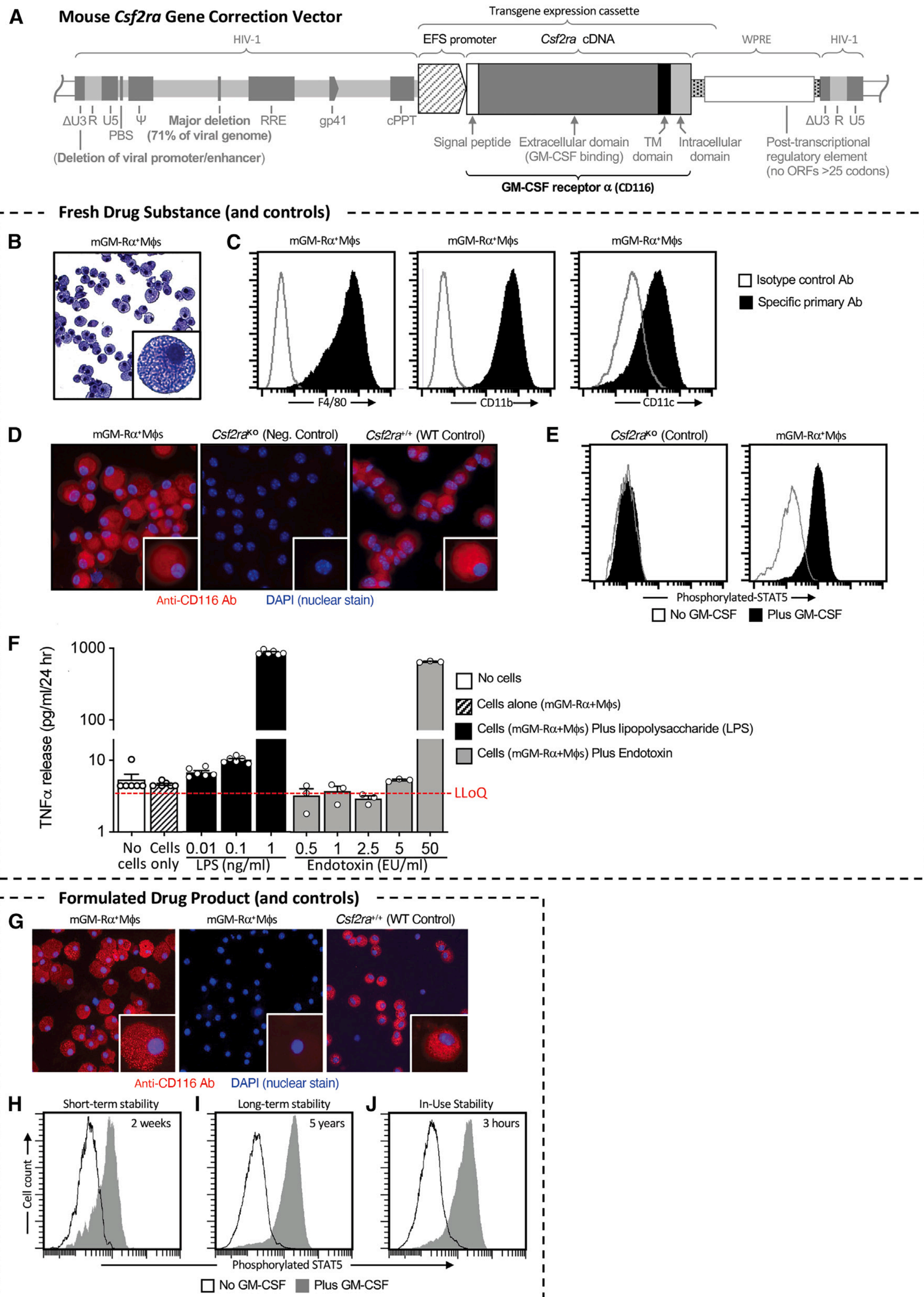
Figure 1. Schematic of the study design

(A) Schematic design of the single ascending dose (SAD) sub-studies evaluating *Csf2ra*^{KO} recipients (SAD-001a-1/b-1) or WT recipients (SAD-001a-2/b-2). In each, mice received one intratracheal instillation of normal saline without or with 500,000 or 5,000,000 mGM-Rα$^+$Mϕs (saline, PMT-500K, and PMT-5M groups, respectively). Recipients were then evaluated over 24 h (SAD-001a) or 14 days (SAD-001b) using the indicated outcome measures and times. (B) Schematic design of the repeat ascending dose (RAD-002) study evaluating *Csf2ra*^{KO} recipients after three intratracheal instillations of either normal saline or normal saline containing 26,000, 105,000, or 500,000 mGM-Rα$^+$Mϕs, respectively, at 1-month intervals (saline and PMT groups, respectively). Recipients were then evaluated over 6 months using the indicated outcome measures and times. (C) Numbers of male and female *Csf2ra*^{KO} and WT mice into each study and group. Ab, antibody; BAL, bronchoalveolar lavage; CBC, complete blood count; K, 1,000; M, 1,000,000; Mϕs, macrophages; PD, pharmacodynamics; PFTs, pulmonary function tests; PK, pharmacokinetics; PMT, pulmonary macrophage transplantation.

determined by immersion culture in tryptic soy broth (TSB) medium (all negative for microbial growth) (Table S4E). While 16 of the 17 formulations were negative by thioglycolate immersion culture, one (formulated on October 12, 2018) was turbid on day 14 (but not day 7) (Table S4D). Subsequent speciation identified *Bacillus halosaccharovorans* as the probable genus (Table S4D). This formulation was administered to WT mice (SAD-001b, sub-group 3), of which all completed the study as planned and none exhibited evidence of lung infection based on assessment of bronchoalveolar lavage (BAL) cell differential cytology (Table 2) by Diff-Quick (Figure S2A) and Gram staining (Figure S2B) or occurrence of adverse events (AEs) related to cage-side observations, body weight, physical examination, hematologic indices, or proinflammatory cytokines. Together, these results suggest the contamination may have occurred during inoculation of the thioglycolate culture bottle and not during formulation.

Pharmacokinetics

The formulated drug product (mGM-Rα$^+$Mϕs in 50 μL sterile saline) or control intervention (50 μL of sterile saline) was administered via



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endotracheal instillation as reported.[30] The cell dose—engraftment response, the relationship between the number of mGM-Rα^+Mϕs administered and number of copies of the vector genome detected in BAL cells, was similar in *Csf2ra*^{KO} and WT recipients at 24 h (Figures 3A and 3B). In WT recipients, a cell dose—engraftment response was present at 14 days but at reduced VCN (Figure 3C). In *Csf2ra*^{KO} recipients 6 months after initiating PMT of mGM-Rα^{+-} Mϕs as three sequential monthly escalating doses (26,000, 105,000, or 500,000 cells/administration, respectively), engraftment remained detectable (VCN = 1.560 ± 0.79; n = 19 mice) and compared favorably with values in *Csf2ra*^{KO} recipients 24 h after one 500,000k cell dose (VCN = 0.728 ± 0.289; n = 10 mice; p = 0.003) (Figures 3A and 3D). These results are consistent with the differential effects of the high level of GM-CSF in *Csf2ra*^{KO} (and *Csf2rb*^{KO}) mice and the low level in WT mice,[6,8,30] the effects of GM-CSF on macrophage proliferation,[6,35] and the increased proliferation, engraftment, and persistence of transplanted cells after PMT in *Csf2ra*^{KO} mice compared with WT mice.[30]

Importantly, mGM-Rα^+Mϕs were not detected in any extrapulmonary tissues in PMT-treated (Figure 3D, top) or in any tissues in saline-treated mice (Figure 3D, bottom).

Safety

Study completion

Of all mice enrolled, 98.3% (227 of 231) completed their assigned substudy as planned including 98% of mice receiving saline, 98% of mice receiving PMT at doses of up to 500,000 cells and 100% of mice receiving a dose of 5,000,000 cells.

Lung function

All mice were closely monitored for bronchospasm by cage-side observation after every administration of intervention. Of 86 saline-treated and 145 PMT-treated recipients, none (0%) exhibited respiratory distress during the 30 min after administration (Tables 1, 2, and 3). Furthermore, there were no changes in either airway resistance or dynamic lung compliance among saline- or PMT-treated *Csf2ra*^{KO} recipients 24 h after administration (Table 1).

Biologic effects and AEs

All study mice were observed cage-side to identify biologic effects related to appearance, behavior, movement, and viability. Observation of a single isolated biological effect event (BEE) without negative consequences for the mouse, for example, ruffled fur, was considered non-adverse. An observation comprising two or more biological effect events occurring simultaneously, for example, hunched back body habitus and abnormal movement, was considered an AE. Moribund behavior or death were considered serious adverse events (SAEs). Fighting was observed before administration of intervention in many males but not in any females. Consequently, aggressive males were separated and housed in isolation. Some females were housed separately for management purposes. Housing in isolation was balanced between saline- and PMT-treated mice (49% and 53%, respectively).

In the 24-h SAD study, BEEs were infrequent (<5% of observations) and limited to instances of isolated ruffled fur; no PMT-related AEs occurred (Table 1). Two saline-treated WT mice developed dehydration due to water dispenser dysfunction, which was identified at study completion; both mice exhibited decreased body weight (by 32% and 36%) and increased hematocrit (Hct) (to 46.7% and 49.9%, respectively). Dehydration was independently confirmed by the study veterinarian (Table 1).

In the 14-day SAD study, BEEs were infrequent (<5% of observations) and limited to instances of isolated ruffled fur; no PMT-related AEs occurred (Table 2).

In the 6-month RAD study, 18,129 cage-side observations of 69 mice made over 192 days resulted in health status data for 12,114 'mouse study days' reflecting the number of mice with observed BEEs, as well as their nature, frequency, duration, and the simultaneous occurrence of multiple effects in the same mouse.

The numbers of mice exhibiting BEEs were similar among saline-treated and PMT-treated mice and among males or females within each group (Table 3; Figure S3), but more frequent in males than

Figure 2. Characterization of the freshly manufactured and formulated mGM-Rα^+Mϕs

(A) Schematic illustration of the proviral form of the murine *Csf2ra*-expressing, third-generation, self-inactivating lentiviral vector used to manufacture *Csf2ra* gene-corrected macrophages from *Csf2ra*^{KO} lineage$^-$Sca$^+$Kit$^+$ bone marrow cells (mGM-Rα^+Mϕs). (B–F) Characterization of the freshly manufactured mGM-Rα^+Mϕs (intermediate drug substance) prior to cryopreservation (drug substance). (B) Photomicrograph of cells after cytocentrifugation, Diff-Quick staining, and microscopy at a magnification of 20× or 40× (inset). (C) Representative flow cytometry histograms showing flow cytometric detection of macrophage cell surface phenotypic markers F4/80, CD11b, and CD11c (filled histograms) or isotype control antibody (open histograms). (D) Immunofluorescence photomicrograph showing expression of *Csf2ra* (CD116) on freshly manufactured mGM-Rα^+Mϕs (left), unmanipulated *Csf2ra*^{KO} mouse macrophages (center), and WT macrophages (right). Original magnification, 20×; inset magnification, 40×. (E) Measurement by flow cytometry of GM-CSF-stimulated STAT5 phosphorylation in fresh mGM-Rα^+Mϕs and the absence of such stimulated expression in unmanipulated *Csf2ra*^{KO} macrophages (control). (F) Determination of macrophage activation in fresh mGM-Rα^+Mϕs using a novel TNF-α release assay in intermediate drug substance. Shown are TNF-α levels in fresh medium (clear bar), cells in culture medium without addition of LPS or endotoxin (striped bar), cells in culture medium 24 h after addition of LPS at the indicated concentrations (black bars), or cells in culture medium 24 h after addition of endotoxin at the indicated concentrations (gray bars). (G–J) Characterization of frozen mGM-Rα^+Mϕs after thawing, washing, and resuspension in sterile normal saline (formulated drug product). (G) Photomicrographs showing expression of *Csf2ra* (CD116) as described above (D). (H–J) Evaluation of cell potency after short-term (2 weeks) and long-term (5 years) cryostorage, or short-term (3 h) in-use stability at room temperature at the indicated times. Potency was measured as the difference in STAT5 phosphorylation in the absence (clear histograms) or after addition (gray histograms) of exogenous GM-CSF. Ab, antibody; CD, cluster of differentiation; GM-CSF, granulocyte macrophage colony-stimulating factor; LPS, lipopolysaccharide; pSTAT5, phosphorylated signal transducer and activator of transcription factor 5; TNF-α, tumor necrosis factor alpha; WT, wild-type.

Table 1. Selected outcome measures evaluated 24 h after one administration of PMT of mGM-Rα^{+}Mϕs

Recipient	Parameter[a]	Saline	PMT-500K	p value[b]	PMT-5M	p value[c]
	Pharmacokinetics					
	VCN, vector genome copies/cell; median (IQR) [n]	0.000 (0.000–0.000) [10]	0.624 (0.470–0.986) [10]	<0.0001	2.123 (1.152–2.964) [10]	<0.001
	Tolerability and safety					
	Biologic effect events, no. mice (no. occ./no. obs.)[d] [n]	0 (0/42) [21]	1 (44) [22]	0.331	0 (0/40) [20]	1.000
	Acute respiratory distress, no. mice[e] [n]	0 [21]	0 [22]	1.000	0 [20]	1.000
	Cage-side observation-related AEs (any), no. mice (no. occ./no. obs.)[f] [n]	0 (0/42) [21]	0 (0/44) [22]	1.000	0 (0/40) [20]	1.000
	Necropsy-related AEs, no. mice (no. occ./no. obs.)[g] [n]	0 (0/21) [21]	0 (0/22) [22]	1.000	0 (0/20) [20]	1.000
	Body weight at end of study, g; mean ± SD [n]	22.8 ± 2.8 [21]	21.9 ± 2.5 [22]	0.255	22.3 ± 2.8 [20]	0.583
	Lung airway resistance, cm H_2O/mL/s; median (IQR) [n]	0.571 (0.517–0.592) [10]	0.564 (0.522–0.639) [10]	0.684	0.556 (0.490–0.573) [10]	0.436
	Lung compliance, mL/cm H_2O; median (IQR) [n]	0.032 (0.029–0.036) [10]	0.032 (0.025–0.036) [10]	0.927	0.034 (0.030–0.047) [10]	0.166
	Lung neutrophilic histology score; median (IQR) [n]	2.00 (0.00–3.00) [11]	0.50 (0.00–1.75) [12]	0.210	2.00 (0.00–2.00) [10]	0.359
	Lung lymphocytosis histology score; median (IQR) [n]	2.00 (1.00–3.00) [11]	2.00 (1.00–2.75) [12]	0.717	2.00 (2.00–2.00) [10]	1.000
Csf2ra^{KO}	Lung fibrosis, no. mice (%) [n]	0 (0.0) [11]	1 (8.3) [12]	1.000	1 (10) [10]	1.000
	Unexpected gross or microscopic necropsy findings, no. mice (%) [n]	0 (0.0) [11]	0 (0.0) [12]	1.000	0 (0.0) [10]	1.000
	Hematologic index-related AEs (any), no. mice (%) [n][h]	0 (0.0) [11]	0 (0.0) [12]	1.000	0 (0.0) [10]	1.000
	IL-1β, pg/mL BAL; median (IQR) [n]	26.9 (26.9–26.9) [10]	26.9 (26.9–26.9) [10]	1.000	26.9 (26.9–26.9) [10]	1.000
	IL-6, pg/mL BAL; median (IQR) [n]	2.77 (2.07–5.07) [10]	5.51 (2.98–17.1) [10]	0.078	5.10 (4.09–9.27) [10]	0.036
	TNF-α, pg/mL BAL; median (IQR) [n]	0.59 (0.33–0.97) [10]	1.29 (0.80–2.53) [10]	0.024	0.97 (0.53–1.79) [10]	0.214
	Pharmacodynamics					
	Alveolar sediment score; median (IQR), [n]	2.00 (2.00–2.00) [11]	2.00 (2.00–2.00) [12]	1.000	2.00 (2.00–2.00) [10]	1.000
	BAL turbidity, OD_{600nm}; median (IQR) [n]	0.674 (0.609–1.124) [10]	0.811 (0.711–0.966) [10]	0.684	0.723 (0.493–1.00) [10]	0.796
	SP-D, μg/mL BAL; median (IQR) [n]	3.46 (3.16–3.94) [10]	4.20 (3.83–4.92) [10]	0.017	3.22 (2.07–3.99) [10]	0.353
	BAL GM-CSF, pg/mL BAL; mean ± SD [n]	20.9 ± 8.04 [10]	30.99 ± 16.3 [10]	0.102	10.4 ± 4.36 [10]	0.002
	BAL M-CSF, pg/mL BAL; mean ± SD [n]	1.51 ± 0.67 [10]	3.23 ± 1.69 [10]	0.011	1.71 ± 1.25 [10]	0.662
	BAL MCP-1, pg/mL BAL; median (IQR) [n]	63.1 (63.1–63.1) [10]	63.1 (63.1–63.1) [10]	0.737	80.3 (63.1–113.4.7) [10]	1.000

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Table 1. *Continued*

Recipient	Parameter[a]	Saline	PMT-500K	p value[b]	PMT-5M	p value[c]
Wild-type	**Pharmacokinetics**					
	VCN, vector genome copies/cell; median (IQR) [n]	0.000 (0.000–0.001) [12]	0.861 (0.623–1.013) [12]	<0.001	1.882 (1.631–2.009) [10]	<0.001
	Tolerability and safety					
	Biologic effect events, no. mice (no. occ./no. obs.) [n][d]	0 (0/24) [12]	1 (1/24) [12]	0.322	2 (2/20) [10]	0.131
	Acute respiratory distress, no. mice (%) [n][e]	0 (0.0) [12]	0 (0.0) [12]	1.000	0 (0.0) [10]	1.000
	Cage-side observation-related AEs, no. mice (no. occ./no. obs.) [n][f]	0 (0/24) [12]	0 (0/24) [12]	1000	0 (0/20) [10]	1.000
	Necropsy-related AEs, no. mice (no. occ./no. obs.) [n][g]	0 (0/12) [12]	0 (0/12) [12]	1.000	0 (0/10) [10]	1.000
	Body weight at end of study, g; mean ± SD [n]	19.0 ± 3.1 [12]	20.0 ± 1.6 [12]	0.326	20.6 ± 1.7 [10]	0.150
	Lung weight/body weight ratio, mean ± SD [n]	0.014 ± 0.002 [12]	0.015 ± 0.002 [12]	0.848	0.015 ± 0.002 [10]	0.914
	BAL cell count, $\times 10^6$/mouse; median (IQR) [n]	0.176 (0.129–0.216) [12]	0.200 (0.183–0.333) [12]	0.097	0.442 (0.245–0.534) [10]	0.001
	BAL macrophages, %; median (IQR) [n]	100 (97.5–100) [12]	99 (98.0–100) [12]	0.509	99 (97.3–100) [10]	0.418
	BAL neutrophils, %; median (IQR) [n]	0.0 (0.0–1.0) [12]	0.5 (0.0–1.0) [12]	0.893	0.8 (0.0–2.8) [10]	0.558
	BAL lymphocytes, %; median (IQR) [n]	0.0 (0.0–0.0) [12]	0.0 (0.0–1.0) [12]	0.640	0.0 (0.0–0.6) [10]	0.699
	SP-D, μg/mL BAL; mean ± SD [n]	0.18 ± 0.04 [12]	0.19 ± 0.03 [12]	0.340	0.35 ± 0.09 [12]	<0.001
	Hematologic index-related AEs (any), no. mice (%) [n][h]	2 (16.7) [12]	0 (0.0) [12]	0.478	0 (0.0) [10]	0.481
	IL-1β, pg/mL BAL; median (IQR) [n]	27.1 (27.1–27.1) [12]	27.1 (27.1–27.1) [12]	1.000	27.1 (27.1–27.1) [10]	1.000
	IL-6, pg/mL BAL; median (IQR) [n]	1.81 (1.45–4.23) [12]	1.07 (1.07–1.82) [12]	0.078	2.00 (1.45–2.54) [10]	0.807
	TNF-α, pg/mL BAL; median (IQR) [n]	0.31 (0.15–0.55) [12]	0.24 (0.12–0.40) [12]	0.318	0.33 (0.12–0.48) [10]	0.586

AE, adverse event; BAL, bronchoalveolar lavage; BEE, biologic effect event; cm H_2O/mL/s, centimeters of water per milliliter per second; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; K, thousand; M, million; MCP-1, monocyte chemoattractant protein 1; M-CSF, macrophage colony-stimulating factor; mL/cm H_2O, milliliter per centimeter of water; no., number; obs., observations; occ., occurrences; PMT, pulmonary macrophage transplantation; resp., respiratory; SAD, single ascending dose; SPD, surfactant protein D; TEAE, treatment emergent adverse event; TNF-α, tumor necrosis factor alpha.

[a]Male and female *Csf2ra*^{KO} or WT mice received endotracheal intrapulmonary administration of either saline or PMT of 500,000, or 5,000,000 *Csf2ra* gene-corrected macrophages and were evaluated over the 24-h period as described in the Materials and methods. Data are the number (no.) of mice with the indicated biologic effect event or adverse event (AE), the no. of observations identifying an event/total no. observations made on all mice) for categorical data; mean ± standard deviation for parametric data, or median (interquartile range) for nonparametric data. The number of mice evaluated in each treatment group is indicated in square brackets.

[b]Comparisons between the saline and PMT-500K groups were made using Fischer's exact test (categorical data), Student's t test (parametric data), or Mann-Whitney test (nonparametric data).

[c]Comparison between the saline and PMT-5M groups were made using Fischer's exact test (categorical data), Student's t test (parametric data), or Mann-Whitney test (nonparametric data).

[d]Included ruffled coat, abnormal movement/reflexes, or hunched body habitus identified during cage-side observations made at 8 or 16 h after administration of the intervention.

[e]Included biologic effect events, respiratory distress, moribund appearance, or altered consciousness identified during continuous cage-side observation for 30 min after administration of intervention.

[f]Included cage-side observations with two or three concurrent biologic effect events, respiratory distress, moribund appearance, altered consciousness, or death identified during cage-side observations made at 8 or 16 h after administration of the intervention.

[g]Included rapid/shallow respiration, ruffled coat, lacerations, mass, eye stupor/lacrimation-secretion/corneal opacification, reduced visual placing, oral or nasal membrane lacerations/erythema or overbite, pale skin, reduced body tone/muscle strength, or abnormal movement/reflexes identified at necropsy.

[h]Included evaluations of white blood cell differential count (% neutrophils, % monocytes, % lymphocytes, % eosinophils, % basophils), red blood cell indices (red blood cell number, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width), and platelet count.

Table 2. Selected outcome variables evaluated 14 days after one administration of PMT of mGM-Rα⁺Mϕs

Recipient	Parameter[a]	Saline	PMT-500K	p value[b]	PMT-5M	p value[c]
*Csf2ra*KO	**Tolerability and safety**					
	Biologic effect events, no. mice (no. occ./no. obs.) [n][d]	0 (0/40) [20]	1 (1/44) [22]	0.331	0 (0/40) [20]	1.000
	Acute respiratory distress, no. mice (no. occ./no. obs.) [n][e]	0 (0/10) [10]	0 (0/11) [11]	1.000	0 (0/10) [10]	1.000
	Cage-side observation-related AEs, no. mice (no. occ./no. obs.) [n][f]	0 (0/210) [10]	0 (0/228) [11]	1.000	0 (0/212) [10]	1.000
	Weekly physical exam-related AEs, no. mice (no. occ./no. exams) [n][g]	0 (0/20) [10]	0 (0/22) [11]	1.000	0 (0/20) [10]	1.000
	Necropsy-related AEs, no. mice (no. occ./no. obs.) [n][h]	0/10 [10]	0/11 [11]	1.000	0/10 [10]	1.000
	Body weight at end of study, g; mean ± SD [n]	23.5 ± 2.6 [10]	24.1 ± 2.0 [11]	0.580	23.4 ± 2.5 [10]	0.904
	Lung neutrophil histology score; median (IQR) [n]	0.00 (0.00–0.00) [10]	0.00 (0.00–1.00) [11]	0.214	2.00 (1.75–2.00) [10]	<0.001
	Lung lymphocytosis histology score; median (IQR) [n]	1.00 (1.00–2.00) [10]	1.00 (1.00–2.00) [11]	0.853	3.00 (1.75–3.00) [10]	0.015
	Lung fibrosis, no. mice (%) [n]	0 (0.0) [10]	0 (0.0) [11]	1.000	0 0.0) [10]	1.000
	Unexpected gross or microscopic necropsy findings, no. mice (%) [n]	0 (0.0) [11]	0 (0.0) [12]	1.000	0 (0.0) [10]	1.000
	Hematologic index-related AEs (any), no. mice (%) [n][i]	0 [10]	0 [11]	1.000	0 [10]	1.000
	Pharmacodynamics					
	Alveolar sediment score; median (IQR) [n]	2.00 (2.00–2.00) [10]	2.00 (2.00–2.00) [11]	1.000	2.00 (2.00–2.00) [10]	1.000
Wild-type	**Pharmacokinetics**					
	VCN, vector genome copies/cell median (IQR) [n]	0.000 (0.000–0.000) [10]	0.141 (0.113–0.211) [12]	<0.0001	0.414 (0.381–0.476) [12]	<0.001
	Tolerability and safety					
	Biologic effect events, no. mice (no. occ./no. obs.) [n][d]	0 (0/20) [10]	1 (1/24) [12]	0.322	2 (2/20) [10]	0.131
	Acute respiratory AEs, no. mice (no. occ./no. obs.) [n][e]	0 [10]	0 [12]	1.000	0 [12]	1.000
	Cage-side observation-related AEs, no. mice (no. occ./no. obs.)[f] [n]	0 (0/212) [10]	0 (0/252) [12]	0.903	0 (0/252) [12]	1.000
	Weekly physical exam-related AEs, no. mice (no. occ./no. exams)[g] [n]	0 (0/20) [10]	0 (0/24) [12]	0.366	0 (0/24) [12]	1.000
	Necropsy-related AEs, no. mice (no. occ./no. obs.)[h] [n]	0 (0/10) [10]	0 (0/12) [12]	1.000	0 (0/12) [12]	1.000
	Body weight at end of study, g; mean ± SD [n]	23.7 ± 2.1 [10]	23.4 ± 2.6 [12]	0.778	23.6 ± 2.0 [12]	0.929
	Lung weight/body weight ratio; mean ± SD [n]	0.014 ± 0.002 [10]	0.019 ± 0.004 [12]	0.007	0.015 ± 0.003 [12]	0.429
	BAL cell count, $\times 10^6$ cells/mouse; median (IQR) [n]	0.245 (0.127–0.279) [10]	0.273 (0.250–0.379) [11]	0.034	0.241 (0.230–0.292) [12]	0.710
	BAL macrophages, %; median (IQR) [n]	100 (99.0–100) [10]	99.5 (97.3–100) [12]	0.242	99 (98.0–99) [12]	0.019
	BAL neutrophils, %; median (IQR) [n]	0.0 (0.0–0.0) [10]	0.0 (0.0–1.0) [12]	0.286	0.0 (0.0–1.0) [12]	0.323
	BAL lymphocytes, %; median (IQR) [n]	0.0 (0.0–1.0) [10]	0.0 (0.0–1.8) [12]	0.626	1.0 (0.0–1.8) [12]	0.147
	SP-D, μg/mL BAL; median (IQR) [n]	0.15 (0.14–0.16) [10]	0.17 (0.16–0.19) [12]	0.007	0.17 (0.15–0.19) [12]	0.131
	Hematologic index-related AEs (any), no. mice (%) [n][i]	0 (0.0) [10]	0 (0.0) [12]	1.000	0 (0.0) [12]	1.000
	IL-1β, pg/mL BAL; median (IQR) [n]	8.40 (8.40–8.40) [10]	8.40 (8.40–8.40) [12]	1.000	8.40 (8.40–8.40) [12]	1.000

(*Continued on next page*)

Table 2. *Continued*

Recipient	Parameter[a]	Saline	PMT-500K	p value[b]	PMT-5M	p value[c]
Wild-type	IL-6, pg/mL BAL; median (IQR) [n]	3.86 (3.86–3.86) [10]	3.86 (3.86–3.86) [12]	1.000	3.86 (3.86–3.86) [12]	1.000
	TNF-α, pg/mL BAL; median (IQR) [n]	0.31 (0.31–0.31 [10]	0.31 (0.31–0.31 [12]	1.000	0.31 (0.31–0.31 [12]	1.000

AE, adverse event; BAL, bronchoalveolar lavage; BE, biologic events; IL, interleukin; K, thousand; M, million; no., number; occ., occurrences; obs., observations; PMT, pulmonary macrophage transplantation; resp., respiratory; SAD, single ascending dose; SP-D, surfactant protein D; TEAE, treatment emergent adverse event; TNF-α, tumor necrosis factor alpha.

[a]Male and female *Csf2ra*^{KO} or WT mice received on endotracheal intrapulmonary administration of either saline or PMT of 500,000, or 5,000,000 *Csf2ra* gene-corrected macrophages and were evaluated over the 14-day period as described in the Materials and methods. Data are the number (no.) of mice with the indicated biologic effect event or adverse event (AE), the no. of observations identifying an event/total no. observations made on all mice) for categorical data; mean ± standard deviation for parametric data, or median (interquartile range) for nonparametric data. The number of mice evaluated in each treatment group is indicated in square brackets.

[b]Comparisons between the saline and PMT-500K groups were made using Fischer's exact test (categorical data), Student's t test (parametric data), or Mann-Whitney test (nonparametric data).

[c]Comparison between the saline and PMT-5M groups were made using Fischer's exact test (categorical data), Student's t test (parametric data), or Mann-Whitney test (nonparametric data).

[d]Included ruffled coat, abnormal movement/reflexes, or hunched body habitus identified during cage-side observations conducted twice daily (weekdays) or once daily (weekend days and holidays).

[e]Included biologic effect events, respiratory distress, moribund appearance or altered consciousness identified during continuous cage-side observation for 30 min after administration of intervention.

[f]Included cage-side observations with two or three concurrent biologic effect events, respiratory distress, moribund appearance, altered consciousness, or death identified during cage-side observations conducted twice daily (weekdays) or once daily (weekend days and holidays).

[g]Evaluations made weekly during the 14-day period following administration of the intervention include (system/assessment [specific AE]): respiratory (rapid/shallow respiration), skin (ruffled coat [scruffy fur], laceration(s)/scratches, mass), eyes (stupor, lacrimation, secretion [cleanliness], corneal opacification, reduced visual placing), mouth (lacerations, membrane/lips, nares; overbite), ears and toes (pale skin), musculoskeletal (reduced body tone, reduced muscle strength), movement and reflexes (abnormal movement/reflexes).

[h]Included rapid/shallow respiration, ruffled coat, lacerations, mass, eye stupor/lacrimation-secretion/corneal opacification, reduced visual placing, oral or nasal membrane lacerations/erythema or overbite, pale skin, reduced body tone/muscle strength, abnormal movement/reflexes identified at necropsy.

[i]Included evaluations of white blood cell differential count (% neutrophils, % monocytes, % lymphocytes, % eosinophils, % basophils), red blood cell indices (red blood cell number, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width), and platelet count.

females when compared independent of the intervention (62% vs. 43%; n = 39 or 30, respectively; p = 0.151, Fisher's exact test). The rate of occurrence of BEEs in individual mice (% of observations per mouse) was similar in saline- and PMT-treated mice, negligible (<2%) in all females, small (<5%) in most males, and higher (∼5%–50%) in one-third of males (Figure S4A). Only 5% of the 18,129 observations were associated with any BEE, and among them, ruffled fur was far more frequent than abnormal movement or hunched body habitus (93%, 4.0%, and 2.7% of 969 observations, respectively; $p < 0.001$, chi-squared test). Ruffled fur was identified in 5.0% of observations and 61% of mice, occurred at similar frequency in saline- and PMT-treated mice (Table 3), and was more common in males (Figure S4B). The rate of occurrence (% of observations per mouse) was negligible in all females, small in most males and higher in one-third of males (Figure S4B). Abnormal movement was identified in 0.22% of observations and 25% of mice, occurred at similar frequency in saline- and PMT-treated mice (Table 3), and at similar frequency in males and females (Figure S4C). The rate of occurrence of abnormal movement was negligible in 94% of mice, including all females, and was small in 6% males (Figure S4C). Hunched body habitus was identified in 0.12% of observations and 25% of mice, and at similar frequency in saline- and PMT-treated mice (Table 3). The rate of occurrence was negligible in 94% of mice, including all females, and small in 6% of male mice (Figure S4D).

A daily health status (DHS) score was determined from cage-side observations for each mouse on each day of study participation (mouse study day or MSD) with values ranging from 0 to 4 indicating (0) no observed events, (1) one isolated BEE, (2 or 3) two or three simultaneous BEEs, respectively, and (4) moribund behavior or death. DHS scores of 0–1 were judged to be non-adverse and scores from 2 to 4 to indicate an AE graded as mild, moderate, or severe, respectively. AEs of any severity (DHS = 2–4) occurred on <1% of the 12,114 mouse study days (Figure S5), were slightly more frequent in PMT-treated than saline-treated mice (0.48% vs. 0.22% of MSDs; n = 6,299, 5,815, respectively; p = 0.0215, Fisher's exact test). AEs (DHS = 2–4) occurred slightly more frequently in males than females when compared independent of the intervention (0.58% vs. 0.09% of MSDs; n = 6,544, 5,571, respectively; p = 0.0001, Fisher's exact test) but were similar among male or female PMT-treated or saline-treated mice (males: 0.75% vs. 0.43% of MSDs; n = 3,325, 3,033; p = 0.1047; females: 0.17% vs. 0% of MSDs; n = 2,974, 2,782; p = 0.0631; both comparisons performed using Fisher's exact test).

Body weight, an important vital sign in mouse studies,[36,37] increased with age and was similar among saline- and PMT-treated mice at study completion in all studies (Tables 1, 2, and 3; Figures S7–S9). Weekly physical examinations resulted in a similarly low rate of AEs in saline- and PMT-treated mice in all studies (Tables 1, 2, and 3). Laboratory AEs related to hematologic indices were infrequent and similar among saline-treated and PMT-treated mice in the 24-h, 14-day, and 6-month studies (Tables 1, 2, and 3). Serum chemistry was evaluated in the 6-month study, but no AEs were identified (Table 3).

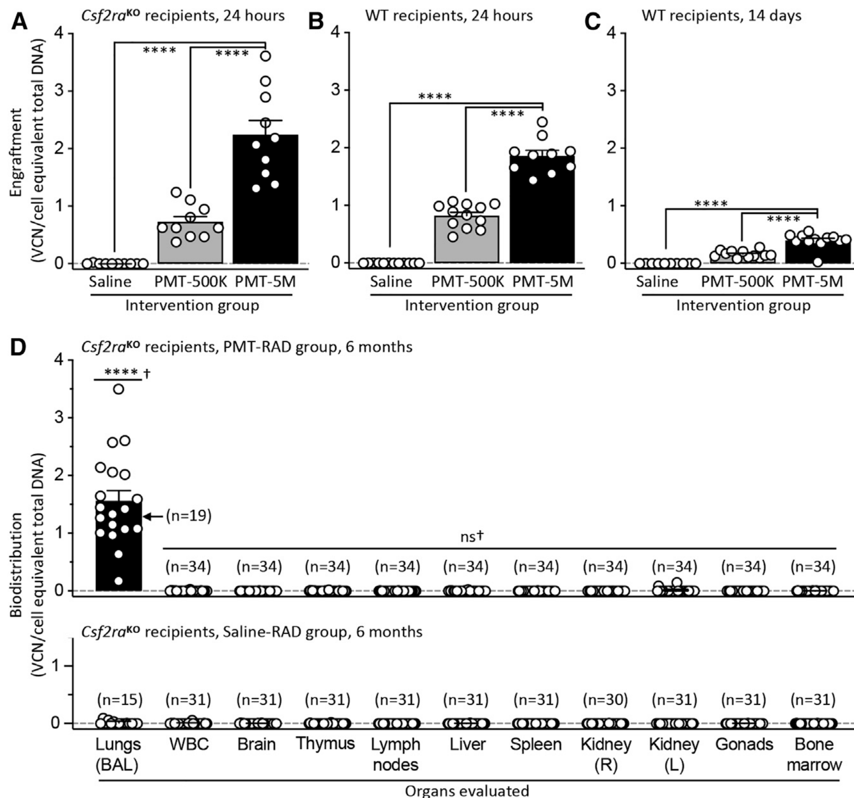

Figure 3. Engraftment and biodistribution of transplanted macrophages after PMT

Detection of vector DNA was assessed in multiple tissues using qPCR. (A) VCN /cell in BAL cells of *Csf2ra*^{KO} recipients (saline [n = 10], PMT-500K [n = 10], and PMT-5M [n = 10]) 24 h after PMT. (B) VCN /cell in BAL cells of WT recipients (saline [n = 12], PMT-500K [n = 12], and PMT-5M [n = 10]) 24 h after PMT. (C) VCN /cell in BAL cells of WT recipients (saline [n = 10], PMT-500K [n = 12], and PMT-5M [n = 12]) 14 days after PMT. (D) Biodistribution of *Csf2ra* gene-corrected macrophages was assessed in multiple organs 6 months (RAD study) after PMT in *Csf2ra*^{KO} recipients. The bottom graph shows VCN in multiple organs from *Csf2ra*^{KO} recipients that received saline intervention. The number of mice evaluated per/organ is indicated within parentheses. NS, not significant; ****p < 0.0001. BAL, bronchoalveolar lavage fluid; K, thousand; L, left; M, million; n, number of mice; PMT, pulmonary macrophage transplantation; R, right; VCN, vector copy number; WT, wild-type.

significantly increased in the PMT-5M group while the percentages of macrophages, neutrophils, and lymphocytes were similar (99%–100%, 0%–0.5%, and 0%, respectively) (Table 1) consistent with administration of mGM-Rα^{+-} Mϕs 1 day earlier.

SAEs

SAEs were infrequent, affected only 4 of 231 the enrolled mice, occurred only in males and in a similar number of saline- and PMT-treated mice (Tables 1, 2, and 3). SAEs did not occur in any SAD study. In the 6-month RAD study, 6% (2 of 33) saline-treated mice and 5.5% (2 of 36) PMT-treated mice exhibited an SAE (one mouse, ID: 3D90, was found dead on day 6 and three mice, IDs: F634, 584B, and 3226, were found moribund on days 4, 11, and 92, respectively, and euthanized). Thus, the rate of SAEs was similar among saline- and PMT-treated mice (Table 3; Figure S6). All four mice with SAEs were observed to be fighting before administration of intervention and were separated and housed in isolation and following comprehensive evaluations, all SAEs were judged to have resulted from fighting-related injuries (Necropsy reports S1–S4, supplemental appendix, Figures S19–S25).

Lung inflammation

In the 24-h SAD study, histologic findings in *Csf2ra*^{KO} recipients (Figure S10) were hPAP-related, occurred at levels (Figures S11–S13) similar to untreated, age-matched *Csf2ra*^{KO} mice,[8] and were similar in saline- and PMT-treated mice (Table 1). Among *Csf2ra*^{KO} recipients, the lung neutrophil and lymphocyte scores were not different in comparisons of PMT-500K or PMT-5M groups to the saline group (Table 1). The potential for lung inflammation was evaluated in WT recipients by enumeration of BAL cells and differential cytology. Compared with the saline group, the BAL total cell count was numerically (but not significantly) increased in the PMT-500K group and

In the 14-day SAD study, histologic findings in *Csf2ra*^{KO} recipients (Figure S14) were typical of untreated, age-matched *Csf2ra*^{KO} mice.[8] Among *Csf2ra*^{KO} recipients, the respective lung neutrophil and lymphocyte scores were not different in the PMT-500K group, and both were increased in the PMT-5M group, both compared with the saline group (Table 2). Among WT recipients, the BAL total cell count was increased in the PMT-500K group but not in the PMT-5M group compared with the saline group (Table 2). The respective percentages of lung neutrophils and lymphocytes were similar in all groups, while, compared with the saline group, the percentage of macrophages was similar in the PMT-500K and decreased trivially in the PMT-5M group (100% vs. 99%, respectively) (Table 2).

In the 6-month RAD study, histologic findings in *Csf2ra*^{KO} recipients (Figure 5) were typical of untreated, age-matched *Csf2ra*^{KO} mice[8] and lung histology-based neutrophil and lymphocytes scores were similar in the saline and PMT groups (Table 3).

Lung fibrosis

In the 24-h SAD study, pulmonary fibrosis was trivial and not statistically different among saline- and PMT-treated mice (Table 1); one small perivascular focus was observed in one lobe in one PMT-500K-treated mouse and in one PMT-5M mouse. In the 14-day SAD study, pulmonary fibrosis was not detected in any mice (Table 2). In the RAD-002 study, pulmonary fibrosis was trivial and not statistically different among saline- and PMT-treated mice (Table 3); small perivascular

Table 3. Selected outcome variables evaluated during and 6 months after repeated administration of PMT in *Csf2ra*^{KO} mice

Parameter[a]	Saline group	PMT group	p value[b]
Pharmacokinetics			
VCN, vector genome copies/cell median (IQR) [n]	0.000 (0.000–0.002) [17]	1.373 (1.021–2.047) [20]	<0.0001
Cage-side observation-related biologic effect events[c]			
Any biologic effect event, number (no.) of mice affected (%) [n]	20 (61) [33]	27 (75%) [36]	0.301
Any biologic effect event in females, no. mice (%) [n]	6 (40%) [15]	7 (47%) [15]	1.000
Any biologic effect event in males, no. mice (%) [n]	14 (78%) [18]	20 (95%) [21]	0.162
Ruffled fur, no. mice (no. occ./no. obs.) [n]	16 (320/8707) [33]	26 (584/9422) [36]	0.052
Abnormal movement/reflexes, no. mice (no. occ./no. obs.) [n]	9 (18/8707) [33]	8 (21/9422) [36]	0.781
Hunched-back body habitus, no. mice (no. occ./no. obs.) [n]	5 (12/8707) [33]	9 (14/9422) [36]	0.378
Cage-side observation-related adverse events			
Acute respiratory distress, no. mice (no. occ./no. obs.)[d] [n]	0 (0/96) [33]	0 (0/108) [36]	1.000
Any AE, no. mice, (%), [n]	7 (21%) [33]	12 (33%) [36]	0.293
Any AE in female mice, (%), [n]	0 (0%) [15]	3 (20%) [15]	0.224
Any AE in male mice, (%), [n]	7 (47%) [18]	8 (73%) [21]	1.000
Serious AEs, no. mice, (%), [n][e]	2 (6%) [33]	2 (5.5%) [36]	1.000
Female mice, (%), [n]	0 (0%) [15]	0 (0%) [15]	1.000
Male mice, (%), [n]	2 (11) [18]	2 (9.5%) [21]	1.000
Physical exam-related biologic effect events and AEs[f]			
Rapid/shallow respiration, no. mice (no. occ./no. obs.) [n]	1 (1/846) [33]	1 (1/916) [36]	1.000
Ruffled fur, no. mice (no. occ./no. obs.) [n]	15 (96/846) [33]	21 (127/916) [36]	0.339
Skin lacerations/scratches, no. mice (no. occ./no. obs.) [n]	4 (4/846) [33]	4 (4/916) [36]	1.000
Ocular stupor, no. mice (no. occ./no. obs.) [n]	2 (4/846) [33]	0 [(0/916)36]	0.225
Ocular secretions, no. mice (no. occ./no. obs.) [n]	0 (0/846) [33]	1 (1/916) [36]	1.000
Corneal opacification, no. mice (no. occ./no. obs.) [n]	4 (21/846) [33]	1 (1/916) [36]	0.186
Reduced muscle tone, no. mice (no. occ./no. obs.) [n]	1 (1/846) [33]	0 (0/916) [36]	0.478
Reduced muscle strength, no. mice (no. occ./no. obs.) [n]	1 (1/846) [33]	1 (1/916) [36]	1.000
Abnormal movement/reflexes, no. mice (no. occ./no. obs.) [n]	3 (3/846) [33]	1 (1/916) [36]	0.343
Laboratory test-related AEs at end of study (6 months)			
Serum chemistry-related AEs, no. mice (%) [n][g]	0 (0%) [33]	0 (0%) [33]	1.000
Hematologic index-related AEs (any), no. mice (%) [n][h]	0 (0%) [33]	0 (0%) [36]	1.000
Necropsy-related AEs at end of study (6 months)[i]			
Physical exam-related AEs, no. mice (%) [n]	0 (0%) [31]	0 (0%) [34]	1.000
Organ weight/body weight ratio abnormalities, no. mice (%) [n]	0 (0%) [31]	0 (0%) [34]	1.000
Extramedullary hematopoiesis, no. mice, (%), [n]	0 (0%) [33]	1 (2.8%) [34]	1.000
Lymph node hyperpigmentation, no. mice, (%), [n]	1 (3%) [33]	0 (0%) [34]	1.000
Lymph node enlargement, no. mice, (%), [n]	0 (0%) [33]	1 (2.8%) [34]	1.000
Lung lymphocytosis histology score, median (IQR) [n]	2.00 (2.00–2.75) [16]	2.00 (2.00–3.00) [15]	0.849
Lung neutrophilic histopathology score; median (IQR) [n]	1.50 (0.00–2.00) [16]	2.00 (0.00–2.00) [15]	0.357
Lung fibrosis, no. mice [n] (%)	2 (12.5) [16]	0 (0.0) [15]	0.484
IL-1β, pg/mL BAL; median (IQR) [n]	8.73 (8.73–8.73) [15]	8.73 (8.73–8.73) [19]	1.000
IL-6, pg/mL BAL; median (IQR) [n]	4.79 (1.06–15.96) [15]	2.25 (0.82–7.00) [19]	0.196
TNF-α, pg/mL BAL; median (IQR) [n]	0.57 (0.44–0.89) [15]	0.48 (0.34–0.72) [19]	0.170
Liver inflammation histopathology score at EOS; median (IQR) [n]	1.00 (0.00–1.00) [31]	1.00 (0.00–1.00) [34]	1.000
Body weight at end of study, g; median (IQR) [n]	29.3 (25.3–33.7) [31]	29.9 (26.0–34.6) [34]	0.325

(*Continued on next page*)

Table 3. *Continued*

Parameter[a]	Saline group	PMT group	p value[b]
Change in body weight (EOS, baseline), g; median (IQR) [n]	7.2 (6.10–10.10) [31]	8.4 (6.40–10.90) [34]	0.312
Anti-GM-CSF receptor α antibodies at EOS, ng/mL; median (IQR) [n]	0 (0-0)[j] [31]	0 (0-0)[j] [34]	1.000
Pharmacodynamics			
Lung weight/body weight ratio; mean ± SD	0.021 ± 0.003 [15]	0.018 ± 0.002 [19]	0.008
Alveolar sediment score; median (IQR) [n]	4.00 (4.00–4.00) [16]	2.00 (1.00–4.00) [15]	<0.001
BAL turbidity, $OD_{600\ nm}$; median (IQR) [n]	1.347 (1.187–1.752) [15]	0.817 (0.464–0.957) [19]	<0.001
SP-D, μg/mL BAL; mean ± SD	8.53 ± 1.36 [15]	2.93 ± 1.74 [19]	<0.001
Lung GM-CSF, pg/mL BAL; median (IQR) [n]	22.03 (14.63–31.97) [15]	7.99 (3.93–14.20) [19]	<0.001
Lung M-CSF, pg/mL BAL; median (IQR) [n]	2.01 (1.58–4.49) [15]	1.06 (0.68–2.23) [19]	0.026
Lung MCP-1, pg/mL BAL; median (IQR) [n]	41.88 (28.94–49.04) [15]	28.94 (28.94–28.94) [19]	0.023

AE, adverse event; BAL. bronchoalveolar lavage; BE, biologic events; EOS, end of study; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; IQR, interquartile range; MCP, monocyte chemoattractant factor; M-CSF, macrophage colony-stimulating factor; no., number of mice evaluated; n, number of mice; occ., occurrences; obs., observations; PMT, pulmonary macrophage transplantation; RAD, repeat ascending dose (study); resp., respiratory; SAE, serious adverse event; SD, standard deviation; TEAE, treatment emergent adverse event; TNF-α, tumor necrosis factor alpha.

[a]*Csf2ra*^{KO} mice received three monthly endotracheal intrapulmonary administrations of either saline or *Csf2ra* gene-corrected macrophages (at sequentially escalating doses of 26,000, 105,000, and 500,000 cells per mouse) and were evaluated over a 6-month period as described in the Materials and methods.

[b]Comparisons of mice in the PMT and saline groups for the indicated outcome measure were made using Fischer's exact test (categorical data), Student's t test (parametric data), or Mann-Whitney test (nonparametric data) as appropriate.

[c]Biologic effect events included ruffled coat, abnormal movement/reflexes, hunched body habitus identified during cage-side observations conducted twice daily (weekdays) or once daily (weekend days and holidays).

[d]Included assessment of biologic effect events (hunched body habitus, ruffled coat, abnormal movement/reflexes), respiratory distress, moribund appearance or altered consciousness during 30 min after administration.

[e]These (and other) male mice were observed engaged in fighting before administration of intervention and subsequently either died spontaneously (n = 1) or became moribund (n = 3) due to fighting-related injuries requiring euthanasia.

[f]Included assessment for rapid/shallow respiration, ruffled coat, lacerations, mass, eye stupor/secretion/corneal opacification, reduced visual placing, oral or nasal lacerations/erythema, overbite, pale skin, body tone/muscle strength, movement/reflexes during weekly exams.

[g]Included blood urea nitrogen, creatinine, glucose, aspartate aminotransferase, alanine transaminase, total protein, total bilirubin, and lactate dehydrogenase.

[h]Included evaluations of white blood cell differential count (% neutrophils, % monocytes, % lymphocytes, % eosinophils, % basophils), red blood cell indices (red blood cell number, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width), and platelet count.

[i]Included rapid/shallow respiration, ruffled coat, lacerations, mass, eye stupor/lacrimation-secretion/corneal opacification, reduced visual placing, oral or nasal membrane lacerations/erythema or overbite, pale skin, reduced body tone/muscle strength, abnormal movement/reflexes identified at necropsy.

[j]All measurement results were below the lower limit of quantitation and censored to zero to enable between-group statistical comparison.

foci were detected in 1 or 2 lobes, respectively, in 2 of 16 saline-treated mice (Figure S15) and none of 15 PMT-treated mice.

Liver and spleen histology

In the 6-month RAD study, mild mononuclear and neutrophilic inflammatory infiltrates of varying degrees (Figure S16) were observed in the livers of 61% (19 of 31) saline-treated mice and 62% (21 of 34) PMT-treated mice (Figure S17) and were of similar severity as determined by the liver inflammation score (Table 3).

Spleen histology was unremarkable in all 31 saline-treated mice and 33 of 34 PMT-treated mice (Figure S18). Extramedullary hematopoiesis was observed in one PMT-treated mouse. No vector DNA was present in the spleen of this mouse, but moderate inflammation was present (Necropsy report S5, supplemental appendix, section S3.5, Figure S23). This finding was judged to be related to the impaired host defense[11,13,17] and increased risk of infection caused by disruption of GM-CSF signaling[14–16,19,38,39] and unrelated to the intervention.

Cytokines

BAL was evaluated for proinflammatory cytokines interleukin-1beta (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) in all studies to assess molecular evidence of inflammation. Cytokines were present at trivial levels in all studies and no important between-group differences were observed at 24 h, 14 days, or 6 months (Tables 1, 2, and 3).

Unexpected gross and microscopic findings

Unexpected gross or microscopic findings were not observed in *Csf2ra*^{KO} or WT mice in either SAD study and were similarly infrequent among saline- and PMT-treated mice in the RAD study (Table 3). In the 6-month study, one PMT-treated mouse had a dark-colored lymph node caused by excess melanin pigmentation (Necropsy report S6, Figure S24) and another had an enlarged mesenteric lymph node with benign histology (Necropsy report S7, supplemental appendix, section S3.7, Figure S25). Following extensive evaluations, both were judged to be unrelated to the intervention.

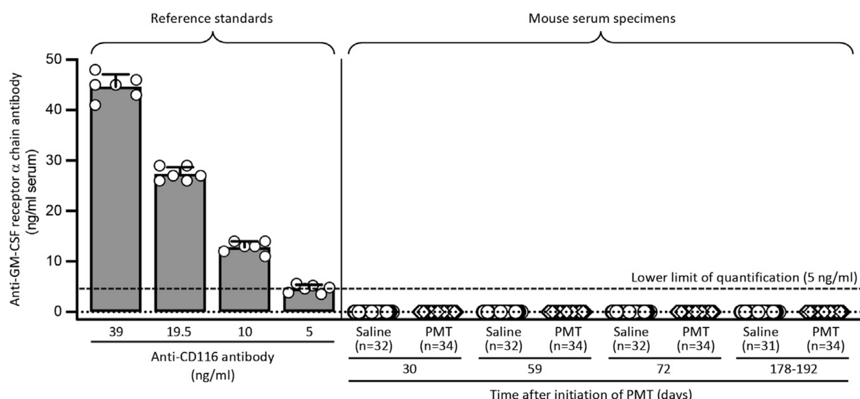

Figure 4. Antibodies to the *Csf2ra* transgene were not detected after PMT in *Csf2ra*^{KO} mice

Detection of anti-GM-CSF receptor α chain (CD116) antibodies in the plasma of *Csf2ra*^{KO} recipients after PMT of *Csf2ra*-expressing macrophages. Data from saline intervention and PMT intervention at four time points (days) after the initiation of the interventions are represented. Time points evaluated were 24 h after second PMT dose, 24 h and 14 days after third PMT dose, and at the end of the study. The number of mice evaluated is indicated within parentheses. Filled bars are anti-CD116 antibody reference standards.

No anti-GM-CSF receptor α (CD116) antibody response

Because *Csf2ra*^{KO} mice do not express CD116[8,30] but it is abundantly expressed on the mGM-Rα$^+$Mϕs (Figures 2D and 2G) administered to *Csf2ra*^{KO} recipients, we evaluated recipients for development of anti-CD116 antibodies. Despite administration of mGM-Rα$^+$Mϕs by PMT on three separate occasions at 1-month intervals and measurements four times over a 6-month period (on study days 30, 59, 72, and 178–192), no PMT-treated mice developed detectable anti-CD116 antibodies (Figure 4).

Pharmacodynamics

Progressive accumulation of surfactant, a cardinal manifestation of hPAP in humans[3,5] and mice,[8,30] is caused by disruption of GM-CSF signaling in alveolar macrophages[8] and can be corrected by PMT.[6,8,30] Consequently, the degree of alveolar surfactant accumulation was evaluated in saline- and PMT-treated mice using multiple methods as reported previously.[6,8,30] In the 24-h SAD study, no between-group differences were observed in the microscopic appearance of the lungs (Figure S10), alveolar sediment score, BAL turbidity, or BAL concentrations of M-CSF, or MCP-1 (Table 1). Interestingly, BAL GM-CSF concentration was reduced in the PMT-5M group compared with saline group but not in the PMT-500K group (Table 1) consistent with the larger GM-CSF clearance from a larger number of GM-Rα$^+$Mϕs administered in the former. In the 14-day SAD study, similar comparisons did not identify between-group differences in the microscopic appearance of the lungs (Figure S14) or in the alveolar sediment score (Table 2). In the 6-month RAD study, comparisons to saline-treated controls demonstrated PMT-treated *Csf2ra*^{KO} recipients had a significantly reduced extracellular surfactant sediment within alveoli and increased numbers of alveolar macrophages containing internalized sediment, as well as significant reductions in the alveolar sediment score, lung to body weight ratio, BAL turbidity, and BAL concentrations of SP-D, GM-CSF, M-CSF, and MCP-1 ($p < 0.05$ for all between group comparisons) (Figures 5A–5K; Table 3).

DISCUSSION

This toxicology study, conducted after consultation with the United States FDA, evaluated the safety, tolerability, pharmacokinetics, and pharmacodynamics of PMT of mGM-Rα$^+$Mϕs in *Csf2ra*^{KO} mice, a validated model of human hPAP, and WT mice, a model without hPAP-related lung abnormalities. Myeloablation was not used due to the strong survival advantage of mGM-Rα$^+$Mϕs over *Csf2ra*^{KO} macrophages.[6] Congenic *Csf2ra*^{KO} donor macrophages modeled autologous cell transplantation and eliminated the need for immune suppression. SAD and RAD studies permitted assessments at immediate, acute, intermediate, and late times. A target dose of 500,000 cells was chosen as the lowest dose with efficacy indistinguishable from higher doses[6] and a dose of 5,000,000 cells permitted assessment of a 10-fold safety margin. Administration of three escalating monthly doses modeled the split-dose administration into an increasing number (1, 4, 14) of lung segments planned for the human trial. Saline controlled for PMT administration and study-specific and room-specific sentinel mice controlled for the study environment. Results demonstrate PMT of mGM-Rα$^+$Mϕs was not associated with bronchospasm, lung function abnormalities, pulmonary inflammation, vector-related genotoxicity, uncontrolled proliferation of transplanted cells, or an antibody response to the "novel" transgene product. Transplanted macrophages engrafted and remained confined to the lungs. Pharmacodynamic effects included restoration of GM-CSF signaling in gene-corrected macrophages and, following PMT, reduction in pulmonary surfactant accumulation and multiple pulmonary biomarkers of hPAP lung disease.

Absence of a safety signal for the target dose (500,000 cells/mouse) is an important finding of the study supported by multiple lines of evidence. A "safety margin" dose (5,000,000 cells/mouse) was well tolerated in both *Csf2ra*^{KO} and WT mice and associated with only a transient increased pulmonary neutrophilia and exacerbation of pulmonary lymphocytosis in *Csf2ra*^{KO} mice at 14 days that was not present at 24 h or at 6 months. None of the PMT-treated WT mice exhibited pulmonary neutrophilia or lymphocytosis at any time in any study. Furthermore, proinflammatory cytokines were not increased in the lungs of *Csf2ra*^{KO} or WT mice at either dose or any time. SAEs resulting in early termination were uncommon, occurred only in males, were attributable to aggressive male fighting, observed before administration of the intervention, and balanced in the saline and PMT groups.[40] A NOAEL dose was established at 631,000 cells/mouse. These studies establish a target dose, NOAEL dose, and safety margin dose.

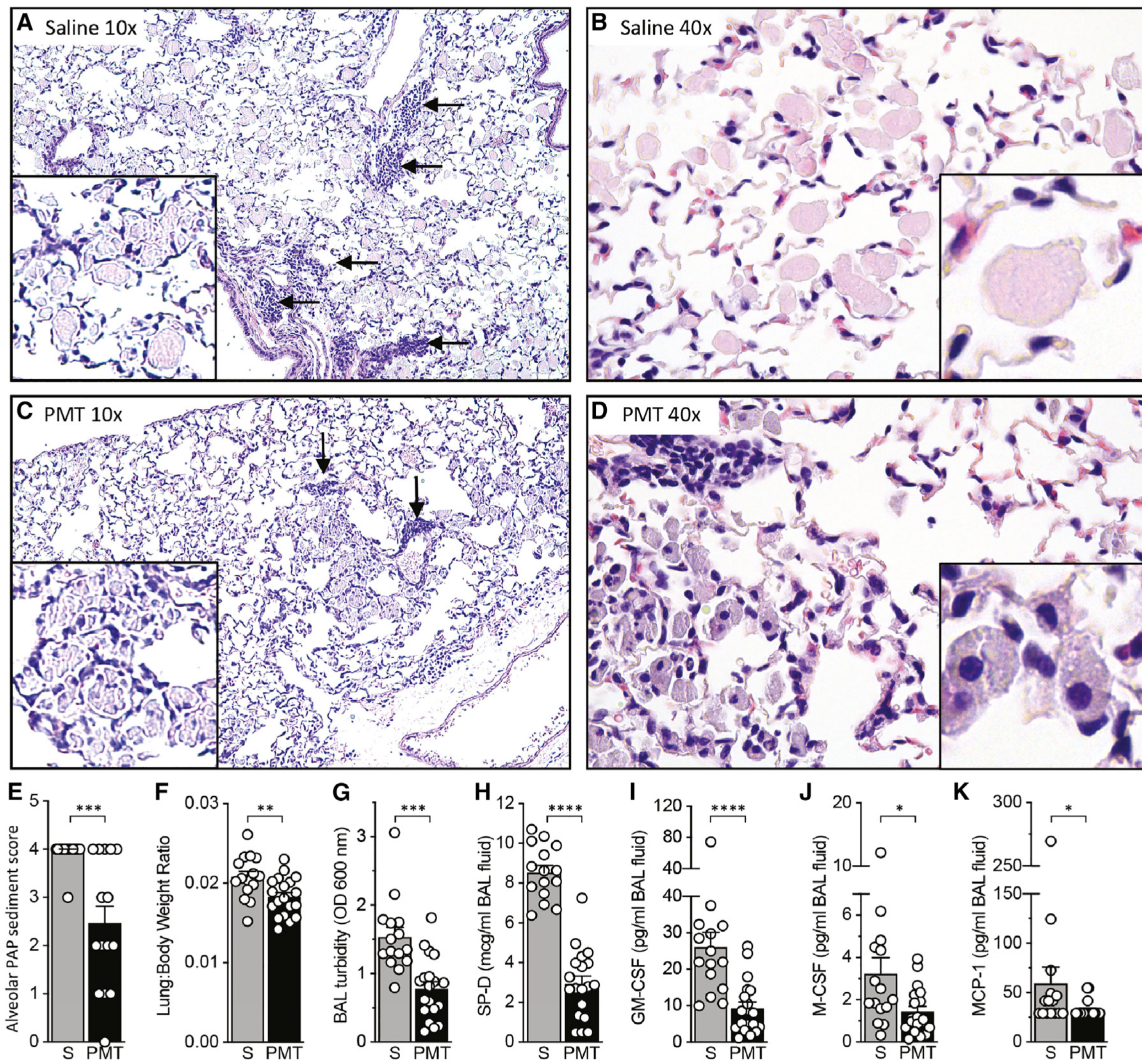

Figure 5. Pharmacodynamics of PMT of *Csf2ra* gene-corrected macrophages

Lung histology after staining with hematoxylin and eosin (H&E) and BAL PAP biomarker analysis of *Csf2ra*^{KO} mice receiving either saline or PMT in the RAD-002 study. (A) Low-power view of the lung of a saline-treated mouse illustrating the typical histopathology of hPAP caused by GM-CSF signaling deficiency including well-preserved walls, intra-alveolar accumulation of eosinophilic surfactant (inset), and focal parenchymal lymphocytosis (arrows). (B) High-power view of the saline-treated mouse lung showing the primarily extracellular location of the eosinophilic intra-alveolar sediment (inset). (C) Low-power view of the lung of a PMT-treated mouse showing well-preserved alveolar walls, intra-alveolar accumulation of eosinophilic sediment (inset), and focal parenchymal lymphocytosis (arrows). (D) High-power view of a PMT-treated mouse lung showing the eosinophilic material is primarily located within enlarged foamy macrophages with little present as extracellular material (inset). Original magnification, 10× (left panels), 40× (right panels). (E) Alveolar PAP sediment score. (F) Lung-body weight ratio. (G) BAL turbidity measured at OD 600 nm. (H) SP-D levels. (I) GM-CSF levels. (J) M-CSF levels. (K) MCP-1 levels. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. BAL, bronchoalveolar lavage; GM-CSF, granulocyte macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; PMT, pulmonary macrophage transplantation; S, saline; SP-D, surfactant protein D.

Absence of an antibody response to the transgene product was unexpected given that CD116 is not expressed in *Csf2ra*^{KO} mice and CD116^{+} mGM-Rα^{+}Mϕs were administered three times at 1-month intervals with multiple evaluations done over 6 months.[41,42] Potential explanations include (1) mild immune deficiency due to absence of GM-CSF signaling[13,14,38] in any cells other than mGM-Rα^{+}Mϕs, (2) impaired antigen presentation due to the lack of GM-CSF signaling in dendritic cells,[43] (3) absence of circulating CD116 or molecular fragments due to lack of receptor shedding,[44,45] and (4) "hiding" of CD116 epitopes due to unfavorable cell-surface positioning resulting in steric hindrance to immune receptors.[46] GM-CSF-deficient mice have impaired T cell responses to ovalbumin, delayed immunoglobulin G generation, T cell proliferation, and lower interferon-gamma (IFN-γ) production in response to limpet hemocyanin

exposure.[47] They also produce less IFN-γ in response to lipopolysaccharide exposure,[48] which is partly explained by reduced IL-12 and IL-18 secretion.[18] Furthermore, homeostatic expansion of B cells following pharmacologically induced lymphopenia was blocked by anti-GM-CSF antibodies in passively immunized non-human primates.[49] Additional studies will be needed to determine why PMT does not elicit an antibody response to the transgene product.

Important pharmacokinetic observations included that transplanted macrophages engrafted, persisted long-term, and remained confined to the lungs. These observations are supported by prior studies of PMT of WT or gene-corrected macrophages in *Csf2rb*^{KO} and *Csf2ra*^{KO} mice demonstrating transplanted macrophages remain in the lungs for at least 1 year and possibly for the life of the mouse.[6,8,30] They are also consistent with the concept of alveolar macrophage as a self-renewing cell population,[50–52] the size of which is regulated by the pulmonary GM-CSF concentration.[6] The observation that vector DNA was not detected in gonadal tissue suggests PMT has a low risk of transmission of the vector DNA to germline tissues.

Although focused to safety, results demonstrated effective complementation of the *Csf2ra* gene defect in *Csf2ra*^{KO} macrophages. Furthermore, although pharmacodynamic effects were not observed at 24 h or 14 days after PMT in *Csf2ra*^{KO} recipients, by 6 months, marked improvement was seen across multiple measures of hPAP lung disease including reduction in pulmonary surfactant burden, lung to body weight ratio, and BAL turbidity, SP-D, GM-CSF, M-CSF, and MCP-1, consistent with prior reports.[6,30]

Study limitations included use of a single animal species (although both *Csf2ra*^{KO} mice[8] and WT mouse models were studied), the mouse vector used here was not identical to the human vector[50] to be used in the clinical trial (they differ only in the species-specific transgene), VCN in murine gene-corrected macrophages (4.3 vector copies per cell) is anticipated to be slightly higher than in human gene-corrected macrophages (VCN = 1.44 ± 0.53), based on our unpublished preliminary studies and the absence of any evidence of clonal expansion, myeloproliferation, or leukemia, vector insertion site analysis was not performed (because uncontrolled proliferation of mGM-Rα^+Mϕs was not observed).

In conclusion, results demonstrate PMT was well tolerated and safe in *Csf2ra*^{KO} and WT recipient mice and establish a total dose of 631,000 mGM-Rα^+Mϕs as the NOAEL in mice. Based on the lowest effective total murine cell dose (500,000 mGM-Rα^+Mϕs), the human equivalent dose for a 70 kg adult human is 778,000,000 *CSF2RA* gene-corrected human macrophages (hGM-Ra$^+$Mϕs), which is well below the calculated human equivalent NOAEL dose (981,836,000) and safety margin dose (7,780,000,000) for a 70 kg adult human. Together, these results support translational testing of PMT of *CSF2RA* gene-corrected macrophages in humans with hPAP.

MATERIALS AND METHODS

This section is an excerpt of the toxicology report submitted to the FDA in support of our IND application (no. 28593). Study-related source data have been retained by the principal investigator/sponsor (B.C.T.).

Study design, rationale, and oversight

The study of *Csf2ra* gene complementation and PMT therapy in *Csf2ra*^{KO} and WT mice was designed after consultation with the FDA to support a first-in-human clinical trial (NCT05761899). These animals were chosen because: (1) mice are an acceptable species for toxicology studies, (2) *Csf2ra*^{KO} mice develop hPAP[6–8] with the same clinical, physiological, histopathological, and biochemical manifestations, disease biomarkers, and natural history as children with hPAP,[1–4,6,8,20,30,51] (3) GM-CSF deficiency-related alveolar macrophage and neutrophil dysfunction is strikingly similar in humans and mice,[10,13] and (4) in humans, hPAP is caused more commonly by mutations in *CSF2RA* than *CSF2RB* (B.C.C. and B.C.T., unpublished data). Mice homozygous for WT *Csf2ra* alleles (WT mice) were included to permit evaluation of potential lung inflammation in the absence of the pulmonary manifestations of hPAP in *Csf2ra*^{KO} mice (alveoli filled with granular eosinophilic (surfactant) sediment, fragile foamy alveolar macrophages, uncleared apoptotic neutrophils, uncleared necrotic cell debris, and marked peribronchiolar/perivascular lymphocytosis[8,30]).

SAD sub-studies were conducted over 24-h (SAD-001a) and 14-days (SAD-001b) to evaluate potential acute and intermediate safety associated with administration of *Csf2ra* gene-complemented macrophages (mGM-Rα^+Mϕs) by PMT to *Csf2ra*^{KO} (SAD-001a/b-1) or WT (SAD-001a/b-2) mice, respectively. Each SAD sub-study included 3 groups of *Csf2ra*^{KO} mice and 3 groups of WT mice receiving saline, PMT of 500,000 or 5,000,000 mGM-Rα^+Mϕs—saline, PMT-500K, and PMT-5M groups, respectively. A RAD study was conducted over 6 months (RAD-002) to mimic the planned clinical trial. The RAD-002 study included 2 groups of *Csf2ra*^{KO} mice each receiving PMT of 26,000, 105,000, or 500,000 mGM-Rα^+Mϕs (each suspended in 50 µL of sterile saline) or saline alone (50 µL of sterile saline without cells) at 1-month intervals (Figure 1; Tables S1A–S1E).

The study was conducted in the Translational Pulmonary Science Center (TPSC) at Cincinnati Children's Hospital Medical Center (CCHMC) in collaboration with the CCHMC Vector Production Facility, Viral Vector Core, Pathology Research Core, and Division of Veterinary Services, Flow Cytometry Core, Translational Trials Development and Support Lab, Pathology Department, and Office of Clinical and Translational Research. The study strictly adhered to a sponsor-approved protocol approved by the Institutional Animal Care and Use Committee, which was compliant with the Final Rules of the Animal Welfare Act regulations (9 CFR: parts 1, 2, and 3) and the Guide for the Care and use of Laboratory Animals (National Research Council, National Academy Press, Washington, D.C., Copyright 2011). The vivarium was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All procedures involving cell manufacturing, animal manipulations, exposures, and assessments were performed using standard operating procedures (SOPs) or written procedures (Figure S5) approved by the sponsor (B.C.T.). All animals, animal-derived specimens, investigational product and specified

records were accounted for in written records retained at the testing facility.

Study animals

Rationale for the animal species and models chosen

In most hPAP patients, the lung disease is caused by biallelic function-disrupting mutations in *CSF2RA—about* four times more common than mutations in *CSF2RB*.[20] Consequently, our planned gene/PMT therapy of human hPAP patients will comprise administration of autologous *CSF2RA* gene-complemented macrophages (hGM-Rα^{+}Mϕs). Accordingly, *Csf2ra*^{KO} mice, a validated model of human hPAP caused by *CSF2RA* mutations,[8,30] was chosen to serve as recipients of the interventions to be administered.

In some sub-studies (see below, SAD-001a-2, SAD-001b-2), WT congenic mice were used to ensure the accuracy of the assessment of potential lung inflammation assessed by evaluation of lung leukocytes obtained by BAL—for two reasons. First, the excess alveolar surfactant in the lungs of *Csf2ra*$^{\mathrm{KO}}$ mice hinders the enumeration and differential counting of lung leukocytes obtained by BAL. Second, we wanted to evaluate potential lung inflammation independent of the lung pathology associated with PAP.

Origin

WT mice expressing the leukocyte marker CD45.2 (Ly5.2) on a C57BL background (C57BL/B6.SJL-*Ptprc*^{b}) were purchased from Charles River Laboratories (C57BL/6NCrl). Mice lacking GM-CSF receptor α chain expression (*Csf2ra*$^{\mathrm{KO}}$) mice were created at the Translational Pulmonary Science Center (TPSC) as reported previously.[8,30] In brief, C57BL/6.SJL-*Ptprc*^{b} mice (Charles River Laboratories) were used to introduce a 220 nucleotide (nt) deletion in the *Csf2ra* gene encompassing the 3′ end of the signal peptide and 5′ end of the mature peptide (encoded by *Csf2ra* exons 2 and 3) by gene editing. *Csf2ra*$^{\mathrm{KO}}$ mice were genotyped by qualitative polymerase chain reaction (qPCR) amplification to detect the 220 nt *Csf2ra* gene deletion identified by gel electrophoresis resulting in a 157 nt band for the *Csf2ra*$^{\mathrm{KO}}$ allele and a 382 nt band for the WT allele.[8]

Housing and environmental conditions

WT mice quarantined and acclimated in the CCHMC Vivarium for 5–7 days before enrollment in this study. *Csf2ra*$^{\mathrm{KO}}$ mice were bred according to a SOP (Table S5). All mice were maintained in the CCHMC Vivarium under a protocol approved by our Institutional Animal Care and Use Committee (IACUC2016-0079). Mice were housed in gas-sterilized polycarbonate, microisolator cages lined with premium corncob bedding on racks in rooms maintained at temperatures between 18°C and 26°C, humidity between 30% and 70%, and a night/day cycle that included 14 h of light per day according to a SOP (Table S5). Males and females were maintained separately at a density of not more than four mice per cage. Any mice initially housed together that exhibited aggressive fighting behavior (typically only males) were separated and housed in individual cages for the duration of the study to minimize fighting-related injury.

Food and water

Mice had continuous access to food (Purina PicoLab Rodent diet no. 5053 pellets sterilized by irradiation) dispensed from the microisolator cage lid food tray. Mice also had continuous access to water (municipal water sterilized by ultraviolet light) dispensed from a tube (Lixit) fitted to each microisolator cage. Food and water dispensers were checked daily, replenished and/or maintained by vivarium staff according to a SOP (Table S5).

Monitoring

All mice were monitored daily to ensure access to food and water and to evaluate health and well-being. In addition, all mice also underwent study-specific monitoring twice daily (weekdays) or daily (weekends and holidays). TPSC staff performed cage-side observations to monitor for BEEs, AEs, or SAEs related to appearance, movement/responses to stimuli, and body habitus. All mice underwent weekly physical examinations to assess appearance, body weight, vital signs, changes in fur/coat, eyes, ears, mouth, mucous membranes, signs of respiratory, circulatory, or neurological dysfunction, and muscle strength and tone.

Sentinel mouse program

A program including both room-specific and study-specific sentinel mice was instituted to ensure the adequacy of environmental conditions for this study. Study sentinels comprised male and female *Csf2ra*$^{\mathrm{KO}}$ mice that were housed (same room and rack) together with study mice (in separate cages), were evaluated in parallel with study mice by TPSC staff and underwent additional serological screening surveillance for a range of microbial pathogens according to a SOP (Table S5). Study sentinels underwent necropsy in parallel to study mice with retention of tissues for potential future analyses. At necropsy, blood was drawn from study sentinels and sent for detailed mouse serology by multiplex fluorometric immunoassay, which was performed at Charles River Laboratories after confirming that each specimen contained an adequate amount of immunoglobulin for valid screening testing. Serological screening for microbial pathogens included Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvo virus (MPV-1, MPV-2), parvovirus NS-1 (NS-1), murine norovirus, Theiler's murine encephalomyelitis virus (GDVII), reovirus, murine rotavirus EDIM (ROTA-A) and mycoplasma pulmonis. Results from the evaluation of study sentinels are included with the source documents maintained by the sponsor. Room sentinels comprised 3- to 6-week-old male and female Swiss Webster or CD1 mice that were housed in the same room as study mice but were evaluated separately from study mice by CCHMC Vivarium staff as part of their routine facility monitoring practice. Room sentinels were evaluated for the same microbial pathogens as described for study sentinels. Results from the evaluation of room sentinels are included with the source documents maintained by the sponsor.

Identification of study animals

Prior to assignment to an intervention group, Study mice were redundantly marked using an ear tag (or ear punch) and a subcutaneous

radio-frequency identification chip. The identity of each mouse was checked by delegated staff according to a SOP (Table S5).

Assignment of animals to intervention groups and identification marking

Mice were weighed and underwent clinical evaluation 2 to 4 days before assignment to intervention groups (and sub-groups), which was done via stratification by age, body weight, and gender to ensure the comparability of the mice in each group. The sequence of events was as follows. First, the mouse colony was genotyped to confirm that the mice for enrollment were of *Csf2ra*^{KO} genotype. Second, the age of the mice was calculated using the date of birth listed on the cage cards. Third, each mouse underwent a comprehensive clinical evaluation for general appearance, body weights, vital signs, spontaneous activity, muscle strength, movement, changes in fur/coat, eyes, ears, mouth, mucous membranes, signs of respiratory, circulatory, or neurological dysfunction. Fourth, the mice that passed the clinical evaluation and whose weights were within the normal range for the corresponding age/gender were chosen. Fifth, the selected mice were grouped under four equivalent weight classes obtained from the weight range that corresponds to the age/gender of the mice by dividing the weight range into four smaller weight classes (bins). The temporary cage assignment per weight class is termed as "bin." Sixth, once the allocation of animals was completed, the mice were randomly selected from the bin representing each weight class into clean cages based on the study group assignments (saline, PMT intervention groups and study sentinels). Seventh, additional mice were included in each group to serve as replacement mice, in the event of unanticipated death(s), ensuring complete data collection from an adequate number of mice according to a SOP (Table S5). Finally, the cages with male mice were monitored daily for any fighting behavior. The males that were found fighting were reassigned to separate cages according to the CCHMC Vivarium guidelines.

Design, production, characterization, and certification of the lentiviral vector

Lentiviral vector design and construction

The lentiviral vector (SIN-*EFS*-m*Csf2ra*-LV (full name), mGM-Rα-LV (short name)) used in this toxicology study was identical to the vector (hGM-Rα-LV)[31] planned for use in the human clinical trial except for inclusion of a mouse homolog (*Csf2ra*) of the human vector transgene (*CSF2RA*), a difference required because human and mouse GM-CSF are neither functionally interchangeable nor immunologically cross-reactive[32–34]—even though the species-specific homologs regulate their respective myeloid cell targets with striking similarity.[10] The rationale for using the mouse vector instead of the human vector in this study is that it permitted a more full evaluation of the toxicology of gene/PMT therapy because the "therapeutic" molecule (mouse GM-CSF receptor α chain) would be biologically active in mice while the human homolog would not be. Furthermore, availability of mice with homologous ablation of the *Csf2ra* gene (*Csf2ra*^{KO} mice) permitted the evaluation of safety (and efficacy) in a validated model of the human disease caused by biallelic, function-disrupting *CSF2RA* mutations.[8]

The self-inactivating (SIN) lentiviral vector backbone and the transient four-plasmid transfection system used to create mGM-Rα-LV has been described.[52] Both mouse and human vectors do not contain any enhancer elements/enhancer elements, and the self-inactivating design ensures no viral transcriptional regulatory elements are inserted into the host genome upon integration of the vector. The third-generation lentiviral vector backbone contains no intact genes encoding viral structural proteins and thus replication competent lentiviral virus cannot be produced. The vector contains chimeric Rous sarcoma virus-human immunodeficiency virus 5′ long terminal repeat (LTR), and a simian virus 40 polyadenylation signal and origin of replication inserted downstream of the 3′ LTR. A post-transcriptional regulatory element (PRE) from woodchuck hepatitis virus (WPRE) was inserted into the 3′ untranslated region downstream of the transgene expression cassette to increase vector titer and transgene expression. To enhance safety, the WPRE* element included point mutations disrupting all open reading frames of 25 codons or more in length.[30] Expression of the vector transgene is driven from a shortened elongation factor 1α short promoter (EFS).

The pRRL.cPPT.EFS.m*Csf2ra*.pre* vector genome plasmid was created by removing the human *CSF2RA* mRNA coding sequence from pRRL.cPPT.EFS.hCSF2RA^{coop}wpre[31] and inserting the murine *Csf2ra* protein-coding sequence. This was accomplished by incubating the human vector plasmid with the restriction enzymes SgrA1 and Sal1 (New England Biolabs, Ipswich, MA) to remove the *hCSF2RA* cDNA from the vector backbone. The vector transgene, a cDNA fragment including 1,167 bp of the mouse *Csf2ra* mRNA precisely including the entire coding sequence of GM-CSF receptor α chain (GenBank: NM_009970.2), was synthesized by GenScript (Piscataway, NJ); restriction endonuclease cleavage sites (BspE1 and Sal1) were included (at 5′ and 3′ ends, respectively) to facilitate cloning. After cleaving the transgene DNA with BspE1 and Sal1, the Csf2ra protein coding fragment was inserted into the vector backbone using T4 DNA ligase (New England Biolabs).

Vector production

The vector (mGM-Rα-LV) was produced by the CCHMC VPF. The materials and processes used to produce the vector were similar to those used for production of the clinical vector planned for use in the human clinical trial. The raw materials were either residual materials from Good Manufacturing Practice-compliant production of the human vector or the same material but of different grade (i.e., IND grade plasmid vs. research grade plasmid). Manufacturing was performed according to a documented bench protocol. In brief, vector-related plasmids (pEFS.m*Csf2ra*, pΔ8.9-short optimized, pRSV-REV, and pVSVg) were transfected into 293T cells by calcium phosphate transfection, and 6L of vector supernatant were collected, clarified, and processed using Mustang Q Membrane chromatography (Mustang Q XT5, PALL Life Sciences) and tangential flow filtration (TFF) (MIDIKROS D02-S500-05-S TFF system, Spectrum). The final concentrated material was subjected to diafiltration and formulated in X-vivo 10 media (Lonza) with 1% human serum albumin (Baxter)

added to a final concentration of 1% (vol:vol), and transferred to cryovials (250 μL/vial), tested or stored (−80°C) until use.

Vector certification

Aliquots of the vector product were tested for mycoplasma, sterility, endotoxin, infectious titer (IU), and physical titer. Mycoplasma was evaluated by the Translational Trials Development and Support Lab (TTDSL) using PCR-based detection of mycoplasma DNA according to a SOP (Table S5). Sterility was evaluated by Quality Control Laboratory via a 14-day culture method using fluid thioglycolate medium (FTM) and tryptic soy broth (TSB) culture medium to detect aerobic, facultative anaerobic and fungi according to SOP (Table S5). Endotoxin levels were evaluated by TTDSL using the LAL detection assay according to a SOP (Table S5). The infectious titer was expressed as infectious units (IU) per mL and determined in a human embryonic kidney (HEK) cell line (American Type Culture Collection), after transduction with serially diluted virus and measuring the percentage of HEK293 cells expressing CD116+ (R&D Systems) by TPSC study staff in the CCHMC Research Flow Core by using fluorescence-activated cell sorting (FACS) Canto I (BD Biosciences, San Jose, CA). The physical titer was expressed as the quantity of p24 (in ng per mL) and was determined by TTDSL using an ELISA (Alliance, HIV-1 p24 Elisa Kit, ref NEK050B001KT, PerkinElmer) according to a SOP (Table S5). Infectivity was calculated as the ratio of IU/ng of p24. The sequence of the cloned vector plasmid and provirus was verified by DNA sequencing at the DNA Sequencing and Genotyping Core (Table S6).

Production, storage, and certification of *Csf2ra* gene-corrected macrophages

Gene-corrected macrophages (mGM-Rα^+Mϕs) were manufactured, certified, cryopreserved, thawed, formulated, and reevaluated for viability, sterility, purity, and potency using procedures closely similar to those used to prepare the human cell product (hGM-Ra$^+$Mϕs) for clinical use.

Hematopoietic stem/progenitor cells

Bone marrow lineage negative (Lin$^-$), Sca-1$^+$, c-kit$^+$ (LSK) hematopoietic stem/progenitor cells were obtained from *Csf2ra*^{KO} mice[30] according to SOPs (Table S5). In brief, bone marrow cells were obtained from 7 to 10-week-old *Csf2ra*^{KO} donor mice (n = 24, 12 males/12 females, body weight = 17.6–27.5 g) by removing and crushing the pelvic iliac crests, tibias, and femurs in IMDM media (Corning) containing 2% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin solution (Gibco). Mononuclear cells were hematopoietic lineage depleted with biotin-conjugated mouse lineage antibodies: mouse monoclonal CD5 (clone 53-7.3; BD Pharmingen), CD8a (clone 53-6.7; BD Pharmingen), CD45R/B220 (clone RA3-6B2; BD Pharmingen), CD11b (clone M1/70; BD Pharmingen), Gr-1 (clone RB6-8C5; BD Pharmingen), and TER-119 (BD Pharmingen) followed by magnetic bead separation (Dynabeads Sheep anti-Rat IgG, Invitrogen). After removing lineage-positive cells, the remaining cells were stained with 7-aminoactinomycin D (7-AAD) (Invitrogen), Streptavidin-FITC (fluorescein isothiocyanate), Sca-1 (clone D7)-PE (R-Phycoerythrin), and CD117/c-kit (clone 2B8)-APC (allophycocyanin) antibodies (BD Biosciences) and Lin$^-$ c-Kit$^+$ Sca-1$^+$ cells were sorted using a FACS Aria II (BD Biosciences) gated on live cells (7-AAD negative).

Transduction, expansion, commitment, and differentiation

TPSC staff manufactured the mGM-Rα^+Mϕs. LSK cells were prestimulated overnight (day 1) in StemSpan (STEMCELL Technologies) medium containing 2% FBS (Corning), 1% penicillin/streptomycin (Thermo Fisher Scientific), murine stem cell factor (mSCF) (50 ng/mL; R&D Systems), murine interleukin-3 (mIL-3) (10 ng/mL; R&D Systems), human Flt-3-ligand (hFlt3-L) (50 ng/mL; PeproTech), and murine GM-CSF (mGM-CSF) (10 ng/mL; R&D Systems). The following day (day 2) LSK cells (1.2×10^6) were transduced on retronectin-coated plates (TaKaRa) with the mGM-Rα-LV vector at a virus concentration of 3.5×10^7 IU/mL/transduction[30] according to a SOP (Table S5) for two 12-h periods. During transduction, LSK cells were incubated in StemSpan medium containing 2% FBS, 1% penicillin/streptomycin, mSCF (100 ng/mL), mIL3 (10 ng/mL), hFlt3-L (50 ng/mL), and mGM-CSF (10 ng/mL).

On culture day 3, the mGM-Rα-LV-transduced *Csf2ra*^{KO} LSK cells were plated for colony-forming unit cell (CFU-C) assays according to a SOP (Table S5) and the remaining cells were cultured using a three-step protocol for progenitor expansion, lineage commitment, and macrophage differentiation to generate the mGM-Rα^+Mϕ cell product according to a SOP (Table S5). For CFU-C assays, cells were plated (1,500 cells/well) in triplicate in complete Methocult GF M3434 (STEMCELL Technologies, Vancouver, Canada) medium on a SmartDish (6-well) plate (STEMCELL Technologies) and incubated in a humidified incubator (37°C, 5% CO_2) for 12–14 days according to a SOP (Table S5). At the end of the incubation period, the colonies were harvested, and genomic DNA was extracted for VCN evaluation according to a SOP (Table S5) to determine initial transduction efficiency. Quantitative real-time PCR (qPCR) analysis to determine VCN per cell was conducted using primers (mouse-ApoB forward: 5'>CGT GGG CTC CAG CAT TCT A; mouse-ApoB reverse: 5'>TCA CCA GTC ATT TCT GCC TTT G; R-U5 forward: 5'>GAA CCC ACT GCT TAA GCC TCA A; R-U5 reverse: 5'>ACA GAC GGG CAC ACA CTA CTT G) and probes (mouse-ApoB: VIC 5'>CCT TGA GCA GTG CCC GAC CAT TC/TAMRA; R-U5 probe: FAM 5'>AAA GCT TGC CTT GAG TGC/TAMRA) that detect the R-U5 region of integrated provirus in Applied Biosystems ABI7900HT Fast Real-Time PCR system (Thermo Fisher Scientific). The R-U5 copies were normalized to the mouse *ApoB* gene copies as described previously.[53] The remaining mGM-Rα-LV-transduced *Csf2ra*^{KO} LSK cells were used to generate the final mGM-Rα^+Mϕ cell product.[6] In brief, mGM-Rα-LV transduced *Csf2ra*^{KO} LSK cells were expanded in progenitor expansion medium containing mSCF (50 ng/mL), hFlt3-L (50 ng/mL), and mGM-CSF (10 ng/mL) for 4 days (day 3–7). This step was followed by macrophage lineage commitment in medium containing mSCF (1 ng/mL), hFlt3-L (1 ng/mL), mGM-CSF (10 ng/mL), and murine macrophage colony-stimulating factor (M-CSF) (5 ng/mL; R&D Systems) for 3 days (day 8–10). Macrophage differentiation was carried out in medium containing mGM-CSF (10 ng/mL) and mM-CSF

(10 ng/mL) for 4 days (day 11–14). DMEM with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin was the base medium for the progenitor expansion, macrophage lineage commitment, and differentiation stages according to a SOP (Table S5). Cells were collected using TrypLE (Gibco) + 0.3% pluronic acid treatment (Gibco) for 20–30 min at 37°C ± 1°C in a tissue culture incubator. TrypLE was neutralized using IMDM (Corning) containing 10% FBS, washed with dPBS (Lonza, Bio Whittaker).

Characterization of the manufactured mGM-Rα$^+$Mϕ cell product

Freshly manufactured mGM-Rα$^+$Mϕs were counted via trypan blue (Gibco) exclusion using a hemacytometer according to a SOP (Table S5) and evaluated for morphologic appearance, complementation of the *Csf2ra* gene defects, macrophage phenotypic markers, GM-CSF receptor signaling, macrophage activation state, and endotoxin contamination. VCN was determined by quantitative PCR detection of transgene sequences in TTDSL according to a SOP (Table S5). CD116 expression was measured during fluorescence microscopy by enumerating CD116+ and DAPI+ cells among 300 cells in five or more random 20× fields and expressing results as a percentage (CD116+ cells ÷ DAPI+ cells × 100). Macrophage phenotypic cell surface markers (F4/80, CD11b, and CD11c) were analyzed by TPSC staff using flow cytometry according to a SOP (Table S5). In brief, mGM-Rα$^+$Mϕs was stained to detect surface expression of F4/80 (clone BM8), CD11c (clone HL3), and CD11b (clone M1/70) (eBioscience). 7-AAD was used to distinguish the live and dead cell populations and phenotype was gated on the viable 7-AAD$^-$ population. The gating strategy was determined using samples stained with appropriate isotype control antibodies. The macrophage phenotype was assessed in the CCHMC Research Flow Core using FACS Canto 1 (Beckman Coulter) and the results were analyzed using FACS Diva software. *Csf2ra* receptor function was assessed by evaluating GM-CSF-stimulated phosphorylation of STAT5 (pSTAT5) according to a SOP (Table S5) by TPSC staff. GM-CSF receptor function in mGM-Rα$^+$Mϕs was tested using a GM-CSF stimulated (10 ng/mL murine GM-CSF for 30 min at 37°C) phosphorylation assay followed by staining using anti-phospho-STAT5 antibody (clone 47/Stat5 [pY694]) (BD Biosciences).[6] STAT5 phosphorylation was assessed in the CCHMC Research Flow Core using FACS Canto 1 (Beckman Coulter) and the results were analyzed using FACS Diva software. Macrophage activation state was assessed by TNF-α ELISA (R&D Systems)[6] according to a SOP (Table S5) by TPSC staff. Proinflammatory cytokine secretion capacity was measured after culturing cells in DMEM containing 10% FBS, 10 ng/mL recombinant murine GM-CSF, and 10 ng/mL recombinant murine M-CSF in the presence or absence of LPS (0.001, 0.01, 0.1, 1.0, 10, and 10 ng/mL) or endotoxin (0.5, 1, 2.5, 5, and 50 Endotoxin units/mL) for 24 h and measuring TNF-α released into the media by ELISA (R&D Systems). Culture medium was tested for the presence of bacteria via gram stain by the Pathology and Laboratory Medicine Microbiology Laboratories according to a SOP (Table S5), endotoxin by endo-SAFE nexgen PTS LAL detection assay in TTSDL according to SOP (Table S5), and mycoplasma using a qPCR method in TTDSL according to SOP (Table S5). Sterility was evaluated by the Translational Core Laboratories aseptic processing laboratory via a 14-day culture method using FTM and TSB culture medium to detect aerobic, facultative anaerobic and fungi according to a SOP (Table S5).

Cryopreservation and storage of mGM-Rα$^+$Mϕs

The mGM-Rα$^+$Mϕ cell product was cryopreserved according to a SOP (Table S5) in animal component-free, defined cryopreservation medium with 10% DMSO (Cryostor CS10 media [Biolife Solutions]) at cell concentrations of 4–10 million cells/mL in a total of 67 cryovials in vapor phase liquid nitrogen (−135°C to −190°C).

Formulation of the mGM-Rα$^+$Mϕs

Upon confirmation that the frozen mGM-Rα$^+$Mϕs met the predefined release criteria, TPSC staff thawed the cryopreserved mGM-Rα$^+$Mϕs at 37°C ± 1°C only until a little icicle remained in the thawing cryovial. Warm (37°C) DMEM with 10% FBS (1 mL) was then added to the cryovial, and cells were transferred into 40–45 mL of DMEM with 10% FBS to achieve a final DMSO concentration of ≤0.5%. After centrifugation, the supernatant was removed by aspiration, the cells were washed with pharmaceutical grade 0.9% saline (Baxter) and resuspended in preservative-free 0.9% saline. Viable cells were enumerated using trypan blue exclusion in a hemacytometer (Hausser Scientific) according to a SOP (Table S5). The number of cells to be formulated for administration for each sub-group was calculated based on the number of mice to be transplanted, the PMT dose each mouse was to receive, and the cell viability according to a SOP (Table S5).

Characterization of the formulated mGM-Rα$^+$Mϕs

Immediately before administration, the formulated cell product was evaluated for microbial contamination, endotoxin, and sterility. Gram stain and endotoxin assays were done at CCHMC and Charles River Laboratories, respectively, according to standard procedures (Table S5). Sterility was evaluated by 14-day immersion culture in FTM and TSB medium at the CCHMC Translational Core Quality Control Laboratory according to a SOP (Table S5). Restoration of GM-CSF receptor function was evaluated by measuring GM-CSF-stimulated phosphorylation of STAT5 as we have reported.[1,3,6,30] Cell activation state was assessed by measurement of lipopolysaccharide or endotoxin-stimulated TNF-α secretion by ELISA (R&D Systems).[6]

SAD studies

SAD-001a-1 was a 24-h study evaluating one PMT administration in 63 *Csf2ra*^{KO} recipients (31 males, 32 females) 6–10 weeks old and weighing 17.5–27.8 g at enrollment (Figures 1A and 1C). Each received one endotracheal administration of mGM-Rα$^+$Mϕs (500,000 [500K] or 5,000,000 [5M] cells/mouse) or saline alone—PMT-500K, PMT-5M, and saline groups, respectively. Following PMT, mice were monitored for 30 min for alterations in respiration and/or behavior to assess tolerability and cage-side assessments were made at 8, 16, and 24 h to assess safety. Outcome measures included mortality, anaphylaxis, cage-side clinical observations (appearance, movement, and behavior), body weight, hematologic indices (including number of red blood cells [RBCs], hemoglobin [Hb], Hct, mean corpuscular volume [MCV], mean corpuscular

hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], red cell distribution width [RDW], platelets, and white blood cell [WBC] total and differential counts), pulmonary function (airway resistance, lung compliance, and lung elastance), macrophage engraftment, BAL biomarkers (turbidity, GM-CSF, M-CSF, MCP-1, SP-D, IL-1β and IL-6, TNF-α), and lung histopathology.

SAD-001a-2 was similar to SAD-001a-1 except mGM-Rα$^+$Mϕs or saline were administered by PMT to 34 WT recipients (18 males, 16 females) 8 weeks old and weighing 17.2–21.1 g at enrollment (Figures 1A and 1C). The intervention groups, administrations, and tolerability assessment were also similar. Outcome measures included mortality, anaphylaxis, cage-side clinical observations (appearance, movement, and behavior), body weight, hematologic indices (including number of RBCs, Hb, Hct, MCV, MCH, MCHC, RDW, platelets, and WBC total and differential counts), total BAL cell count and differential, macrophage engraftment, BAL biomarkers (turbidity, GM-CSF, M-CSF, MCP-1, SP-D, IL-1β, IL-6, TNF-α), and "wet" lung weight.

SAD-001b-1 was a 14-day study evaluating one administration of cells or saline via PMT in 31 *Csf2ra*^{KO} mice (16 males, 15 females) 7–9 weeks old and weighing 18.5–25.6 g at enrollment (Figures 1A and 1C). The intervention groups, administrations, and tolerability assessment were similar to SAD-001a-1 and SAD-001a-2. Cage-side assessments and body weight were evaluated daily over the 14-day period. Outcome measures included mortality, anaphylaxis, cage-side clinical observations (appearance, movement, strength, behavior, and well-being), body weight, hematologic indices (including number of RBCs, Hb, Hct, MCV, MCH, MCHC, RDW, number of platelets, and number of WBCs and the WBC differential cell count for neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and lung histopathology.

SAD-001b-2 was similar to SAD-001b-1 except mGM-Rα$^+$Mϕs or saline were administered by PMT to 34 WT recipients (17 males, 17 females) 7–9 weeks old and weighing 17.6–24.4 g at enrollment (Figures 1A and 1C). The intervention groups, administrations, and tolerability assessment were similar to SAD-001a-1, SAD-001a-2, and SAD-001b-1. Cage-side assessments occurred daily over the 14-day period. Outcome measures included mortality, anaphylaxis, body weight, daily cage-side clinical observations (appearance, movement, strength, behavior, and well-being), body weight, hematologic indices (including number of RBCs, Hb, Hct, MCV, MCH, MCHC, RDW, number of platelets, and WBC total and differential count), total BAL cell count and differential, macrophage engraftment, BAL biomarkers (GM-CSF, M-CSF, MCP-1, SP-D, IL-1β, IL-6, TNF-α), and "wet" lung weight.

RAD study

RAD-002 was a 6-month study evaluating repeat administration of PMT in 69 *Csf2ra*^{KO} mice (39 males, 30 females) 6–9 weeks old and weighing 17.0–27.2 g at enrollment (Figures 1B and 1C). *Csf2ra*^{KO} mice were bred in house at CCHMC. Each *Csf2ra*^{KO} mouse received three sequential endotracheal instillations of mGM-Rα$^+$Mϕs (26,000, 105,000, and 500,000 cells/mouse, respectively, formulated in saline) or saline alone at 1-month intervals, PMT and saline groups, respectively (Figure 1B). Following PMT, mice were monitored for a period of 30 min for alterations in respiration and/or behavior to assess tolerability. Cage-side assessments occurred daily over the 6-month period. Hematologic indices and anti-GM-CSF receptor antibody levels were evaluated 1, 2, 2.5, and 6 months following the initial PMT procedure. Other outcome variables were evaluated 6 months after PMT included mortality, anaphylaxis, body weight, daily cage-side clinical observations (appearance, movement, strength, behavior, and well-being), body weight, hematologic indices (including number of RBCs, Hb, Hct, MCV, MCH, MCHC, RDW, number of platelets, and number of WBCs and the WBC differential cell count for neutrophils, lymphocytes, monocytes, eosinophils, and basophils), clinical chemistries, macrophage engraftment, BAL biomarkers (turbidity, GM-CSF, M-CSF, MCP-1, SP-D, IL-1β, IL-6, TNF-α), organ weights, biodistribution of mGM-Rα$^+$Mϕs, histological evaluation of the lungs, liver, and spleen, and collection of organs for histologic evaluation, if needed.

Study procedures

Administration of the interventions

The interventions included sterile normal saline (50 μL) alone or containing mGM-Rα$^+$Mϕs (26,000, 105,000, 500,000, or 5,000,000 cells suspended in 50 μL of sterile normal saline) and were administered by endotracheal instillation as described.[6,8,30] Mice received endotracheal administration of saline or mGM-Rα$^+$Mϕs according to a SOP (Table S5).[54] In brief, mice received controlled light anesthesia by isoflurane inhalation and were suspended on a flat board by a rubber band placed across the upper incisors and placed in a vertical, semi-recumbent (45°) position with the ventral surface and rostrum facing upward. Using a curved blade Kelly forceps, the tongue was gently and partially retracted rostrally. Macrophages were suspended in 0.9% saline (50 μL) and administered using a micro-pipette with the tip positions in the posterior oral cavity. Cells were inhaled into the lungs by subsequent respiratory efforts under direct visualization and mice were returned to their cages for routine care and handling. A similar procedure was used to administer saline without cells to mice that received only saline administration control.

Cage-side observations

All mice in this study were monitored periodically for the occurrence of BEEs and AEs by cage side clinical observations and physical examinations by TPSC staff and detailed findings were recorded in source data records according to a SOP (Table S5). CCHMC Veterinary Services staff also performed routine daily health checks on all study mice. Cage side clinical observation evaluated animal viability, general appearance (fur/coat appearance, body habitus), and behavior (movement, agility, and response to stimuli). Physical examinations evaluated general appearance, body weight, vital signs, changes in fur/coat, eyes, ears, mouth, mucous membranes, signs of respiratory, circulatory, or neurological dysfunction, and muscle strength and tone according to a SOP (Table S5). All mice receiving intervention (gene/PMT therapy

or saline) underwent cage side clinical observations for potential development of abnormal respiration and behavior for a period of 30 min immediately following administration of the intervention. In the 24-h safety study, mice underwent cage side clinical observation approximately every 8 h following administration of the intervention. In the 14-day and 6-month studies, mice underwent cage side clinical observation twice daily (before 10:00 a.m. and after 2:00 p.m.) on weekdays (Monday through Friday) and once daily on weekend days and holidays. Physical examinations were also performed on the day mice were assigned to intervention groups, once weekly (14-day and 6-month studies), and at the end of each study. Animals observed to have ruffled fur, hunched back body habitus, dyspnea, self-mutilation, reduced spontaneous movement and/or reluctance to move upon stimulation were considered to be in poor health and underwent humane euthanasia as prespecified in the study protocol.

Body weight

Body weight was recorded for all mice before assignment to treatment groups, during weekly physical examinations throughout the study, and at end of study (before necropsy) according to a SOP (Table S5).

VCN to evaluate engraftment, persistence, and biodistribution

To evaluate for the potential presence of mGM-Rα^+Mϕs in organs and tissues, PCR amplification was used to quantify the number of vector DNA copies and mouse genomic DNA and expressed as the number of vector genomes per mouse genome—i.e., the VCN per cell according to a SOP (Table S5). DNA was prepared for PCR by extraction from BAL cells or tissue specimens according to a SOP (Table S5). Following cell/tissue lysis and protein removal, DNA was isolated by alcohol precipitation and resuspended in hydration buffer. qPCR amplification was performed by TTDSL staff using primers designed to detect the R-U5 region of the integrated provirus using an Applied Biosystems ABI7900HY Fast Real-Time PCR System (Thermo Fisher Scientific) (Table S5). PCR cycle threshold (Ct) values for detection of vector R-U5 DNA were normalized to the Ct values for detection of mouse ApoB gene, an endogenous control gene to measure genomic DNA concentration.

Engraftment and persistence of transplanted mGM-Rα^+Mϕs in the lungs was determined by measuring VCN in BAL cells in 24-h, 14-day, and 6-month studies. Biodistribution of transplanted mGM-Rα^+Mϕs was determined by measuring VCN in WBCs, bone marrow, brain, gonads, gross lesions/tumor, kidneys, liver, lymph nodes, spleen, and thymus in the 6-month study.

Phlebotomy

Blood was collected from mice by Comprehensive Mouse and Cancer Core staff by retroorbital phlebotomy or inferior vena cava/cardiac puncture according to SOPs (Table S5). In 24-h and 14-day studies, blood was collected by inferior vena cava/cardiac puncture into potassium ethylene diamine tetraacetate acid (EDTA) tubes (BD Biosciences) at the time of scheduled necropsy. In the 6-month study, blood was collected by retroorbital phlebotomy into EDTA tubes 24 h after administration of the second intervention (nominally, 1 month), 24 h and 14 ± 2 days after administration of the third intervention (nominally, 2 months, and 2.5 months, respectively), and by inferior vena cava phlebotomy immediately before necropsy at end of study (nominally, 6 months).

Complete blood count

A complete blood count was performed using a fully automated Hemavet 859 (Drew Scientific) on all blood specimens obtained according to a SOP (Table S5). Hematological indices assessed included Hb, RBC, Hct, MCH, MCV, MCHC, WBC, WBC differential count (i.e., the absolute number of neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and platelet counts. Specimens observed to have a visible clot were re-evaluated.

Clinical chemistry

In the 6-month study, serum clinical chemistry parameters were measured by CCHMC Nephrology Clinical Lab staff using a Siemens Dimension Clinical Chemistry system (Siemens Healthcare Diagnostics) per manufacturer's protocol. Measured parameters included blood urea nitrogen, creatinine, glucose, aspartate amino transferase (AST), alanine amino transferase (ALT), total protein, total bilirubin, and lactate dehydrogenase.

Anti-GM-CSF receptor antibody

Plasma anti-GM-CSF receptor α (CD116) antibody levels were measured by TPSC staff in the CCHMC Research Flow Cytometry Core using a novel flow cytometer-based assay to measure antibody captured by CD116-expressing HEK cells according to a SOP (Table S5). In brief, an anti-CD116 antibody "capture cell" line was prepared by transduction of HEK cells with mGM-Rα-LV. An anti-CD116 antibody reference standard was prepared by serial dilution of an anti-CD116 monoclonal antibody (R&D Systems) to achieve an antibody reference standard ranging from 62.5 to 0.001 ng/tube. Capture cells were incubated with mouse plasma or reference standards, washed, and then incubated with secondary "detection" antibody (allophycocyanin-conjugated anti-Rat IgG; 62.5 ng/tube; R&D Systems). After staining, cells were fixed in 2% paraformaldehyde. The mean fluorescence intensity of the stained cells was acquired using a FACS Canto 1 (Beckman Coulter). The concentration of mouse anti-GM-CSF receptor α antibody in plasma specimens was determined from the reference standard curve. As a negative control, CD116-expressing HEK cells were stained with a monoclonal Rat IgG2A isotype control (R&D Systems) instead of the reference standard followed by incubation with the detection antibody and evaluated by flow cytometry. The lower limit of quantification (LLoQ) for the assay was 5 ng/mL plasma, the limit of detection (LOD) was 1.14 ng/ml plasma, and the limit of blank (LOB) was 0 ng/ml plasma. In the 6-month study, anti-GM-CSF receptor α antibody concentration was measured in plasma from blood collected at the times indicated above.

Lung mechanics

Lung mechanics were measured *in vivo* in anesthetized mice using a Flexivent (SCIREQ, Montreal, Canada) according to a SOP (Table S5). In brief, mice were anesthetized, placed in a supine position,

a ventral, midline rostral-caudal incision was made in the neck, followed by a small incision of the ventral inter-cartilaginous tracheal membrane into which a tracheal cannula was inserted. The Flexivent was attached via tubing to the tracheal cannula, and lung mechanics were measured during mechanical ventilation according to a predefined ventilation profile. Lung function parameters measured included airway resistance, compliance, and elastance.

BAL cell and fluid collection

Lung epithelial lining fluid and cells contained therein were collected by TPSC staff by BAL according to a SOP (Table S5) as we reported previously.[14] In brief, mice were anesthetized with pentobarbital, the neck was incised and the trachea cannulated (as described in the previous section), and five 1-mL aliquots of phosphate buffered saline (PBS) containing 0.5 mM EDTA were instilled into the lungs through the cannula using a 1-mL syringe and then immediately recovered by aspiration to collect the BAL. The five BAL aliquots recovered from each mouse were pooled and gently mixed by inversion of the tube to ensure a homogeneous suspension of cells and sediment. In the 24-h (SAD-001a-1, SAD-001a-2), 14-day (SAD-001b-2), and 6-month (RAD-002) studies, BAL was performed at the end of study before necropsy.

BAL turbidity

BAL turbidity was determined by TPSC staff as the optical density of BAL fluid at a wavelength of 600 nm according to a SOP (Table S5). In brief, 1 mL of BAL was placed in a plastic cuvette and the optical density was measured in a DU640B spectrophotometer (Beckman Coulter). Turbidity of the BAL specimens was also obtained after dilution (250 μL of BAL plus 750 μL PBS [pH 7.4]). The optical density at 600 nm was remeasured and the result multiplied by the dilution factor to obtain the specimen turbidity. In the 24-h (SAD-001a-1) and 6-month (RAD-002) studies, BAL turbidity was measured at the end of study before necropsy.

BAL cell counts and differentials

The total number of lung leukocytes recovered by BAL was determined by TPSC staff by enumerating cells in an aliquot of BAL using a hemacytometer and multiplying the result by the ratio of the total volume of BAL. BAL cytology was evaluated after sedimentation (Cytospin, Shandon; 400 revolutions per minute [rpm], 4 min, room temperature [room temp]) onto glass microscope slides and staining with Diff-Quick, (Fisher) according to a SOP (Table S5).[49] The differential count (macrophages, neutrophils, and lymphocytes) of a minimum of 100 BAL cells/slide was determined by an American Board of Pathology-certified pathologist at CCHMC blinded for intervention groups by microscopic examination of Diff-Quick-stained BAL cells. In the 24-h (SAD-001a-2) and 14-day (SAD-001b-2) studies, BAL total cell counts, and BAL cell differential counts (percentage of total) were determined at the end of study before necropsy.

PAP biomarkers and inflammatory cytokines in BAL fluid

Proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and hPAP biomarkers (GM-CSF, M-CSF, and MCP-1) in BAL fluid were measured by CCHMC Research Flow Cytometry Core staff using a Luminex magnetic bead-based multiplex assay platform (Mouse Magnetic Luminex Assay, R&D Systems)[1] using a premixed multianalyte kit (LXSAMSM-06) according to the manufacturer's protocol. In brief, BAL fluid and cells were separated by low-speed centrifugation (room temp, 285 × g, 10 min) by TPSC staff and BAL fluid was stored at −20°C until evaluation. BAL fluid (50 μL) was mixed with the anti-cytokine antibody-coated bead cocktail (50 μL) in a 96-well microplate and incubated with shaking (room temp, 2 h, 800 ± 50 rpm), washed 3 times using a microplate washer (BioTek, Winooski, VT), 50 μL of diluted biotin-antibody cocktail was added to each well, plates were incubated with shaking (room temp, 1 h, 800 ± 50 rpm), washed again, streptavidin-phycoerythrin (50 μL) was added to each well, and plates were incubated (room temp, 30 min, 800 ± 50 rpm). After shaking plates for 5 min, cytokines were measured using a Milliplex Analyzer (MilliporeSigma, Darmstadt, Germany). Cytokine concentrations in specimens were determined from measurements made using recombinant cytokine reference standards and results were expressed in pg/mL. Surfactant protein D was measured by TPSC staff in BAL by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) as described previously[49] according to a SOP (Table S5). The concentration of hPAP biomarkers in epithelial lining fluid was determined by first measuring the urea concentrations in blood and BAL fluid and adjusting the cytokine concentrations measured by ELISA in BAL by multiplying by the ratio of urea concentrations in blood and BAL according to a SOP (Table S5). Proinflammatory cytokines (TNF-α, IL-1β, IL-6) and lung biomarkers of hPAP (SP-D, GM-CSF, M-CSF, and MCP-1) were measured in BAL after PMT at 24 h in SAD-001a-1 and SAD-001a-2, 14 days in SAD-001b-2, and 6 months in the RAD-002 studies. In the latter study, the concentrations of lung biomarkers of hPAP in lung epithelial fluid were calculated by the serum/BAL urea-dilution method.[6]

End of study necropsy and organ weights

At the end of study (and early withdrawal), mice underwent clinical observation and physical examination and were then sacrificed by intraperitoneal administration of sodium pentobarbital (150 mg/kg body weight) followed by exsanguination by inferior vena cava phlebotomy according to a SOP (Table S5).

A complete necropsy was performed as described in the protocol, including examination of body habitus, integument (fur/coat, eyes, ears, mouth, mucous membranes), and the cranial, thoracic, abdominal and pelvic cavities and organs therein, with collection of organs and abnormal masses.

In the 24-h (SAD-001a-2) and 14-day (SAD-001b-2) studies, lung weights were measured. In the 6-month study (RAD-002), the following tissues were collected: bone marrow, blood, sternum, esophagus, brain, reproductive organs (male: testes, epididymis, prostate, seminal vesicle, and coagulating glands; female: ovaries, cervix, and vagina), stomach, duodenum, jejunum (including Peyer's patches), ileum, cecum, colon, pancreas, kidneys, liver, lung, lymph

node, salivary glands (parotid, sub-mandibular and sub-lingual), spleen, thymus, heart. Any gross tissue lesions/tumors were noted, and the abnormal tissue was collected. Organ weights were obtained for the brain, kidneys, liver, gall bladder, lymph nodes, lungs, spleen, heart, thymus, gonads, and tumor, or grossly abnormal tissue. Organ-to-body weight ratios were calculated.

Tissue collection and processing

For animals prescheduled for histopathologic examination of the lungs, following sacrifice a ventral, midline, sagittal incision was made in the skin of the neck and blunt dissection was performed to expose the trachea. A small (∼1 mm) transverse incision of the ventral tracheal membrane was made between adjacent tracheal rings (avoiding the thoracic inlet to preserve the integrity of the lung and pleura). A cannula was inserted caudally through the trachea incision and ligated with a circumferential suture to ensure a tight seal between the tracheal lumen and catheter. The cannula was connected to a reservoir of fixative (10% neutral buffered formalin) under a hydrostatic pressure of 25 cm. A ventral, midline, sagittal incision was made through the sternum and continued through diaphragm in a ventral to dorsal direction avoiding the lung and visceral pleura (to avoid puncture), the ribs were abducted, and careful blunt dissection was used to separate the lungs and visceral pleura from the chest wall and parietal pleura. The trachea was fully transected above the cannulation site, and the lungs and heart were carefully removed en bloc, submerged in fixative, the trachea was sealed (by circumferential ligature under 25 cm of hydrostatic pressure while removing the canula) and incubated at 4°C to fix the tissues. After 24 h, the lung lobes were separated and removed from the tissue block, cut into 5-mm-thick slices along the long axis of each lobe, washed in cold PBS, dehydrated, embedded in paraffin, and 5-μm sections were prepared on glass slides. Other tissues (thymus, gallbladder, brain, kidneys, lymph nodes, heart, gonads, esophagus, stomach, pancreas, duodenum, jejunum [including Peyer's patches], ileum, cecum, colon, salivary glands and reproductive organs) were fixed in 10% neutral buffered formalin, processed and stored in 70% alcohol for microscopic evaluation as necessary. Paraffin blocks of lungs, liver, spleen and any abnormal tissue were used to prepare 5-μm sections according to a SOP (Table S5), stained with hematoxylin and eosin according to a SOP (Table S5) and examined microscopically. In the 24-h (SAD-001a-1), 14-day (SAD-001b-1), and 6-month (RAD-002) studies, tissues were collected during necropsy and prepared for histological examination.

Microscopy

BAL cells and tissues from the lungs, liver, and spleen of study mice were examined microscopically by an American Board of Pathology-certified pathologist blinded to the intervention groups.

Statistical analysis plan

Overview

An intention-to-analyze approach was taken in the design and execution of this protocol, meaning all mice enrolled in an intervention group were accounted for in the analysis performed. All tolerability, safety, pharmacokinetic, and pharmacodynamic results were summarized using descriptive statistics. Numbers of events, mean, SD, median, first quartile, and third quartile were calculated for the continuous numeric variables. Frequency and percentages were used to summarize categorical variables.

Protocol amendments, dates, and rationale for changes

This statistical analysis plan was based on the initial study protocol as approved by the sponsor (B.C.T.) and study director (P.A.) on February 26, 2018, and on Protocol Amendment 1 (approved on April 6, 2018), Amendment 2 (approved on October 2, 2018), and Amendment 3 (approved on April 25, 2022).

Sample size determination

The total number and numbers of male and female mice per intervention group were as recommended in FDA guidance on the conduct of toxicology studies in rodents including 10 mice including at least 5 males and 5 females in each group.

Hypotheses to be tested

Hypothesis 1. One administration of mGM-Rα^+Mϕs by PMT at a dose of 500,000 cells/mouse in either *Csf2ra*^{KO} or WT recipients will be associated with a similar frequency and profile of AEs or SAEs compared with mice receiving one administration of saline.

Hypothesis 2. Repeated administration of PMT of mGM-Rα^+Mϕs by PMT at sequential one-monthly doses of 26,000, 105,000, and 500,000 cells/administration will result in a similar frequency and profile of AEs or SAEs compared with mice receiving three, one-monthly administrations of saline control.

Assignment of mice to intervention study groups

Both *Csf2ra*^{KO} and WT mice were assigned to intervention groups based on gender, age, and body weight to achieve a balanced representation of each variable in each study group.

Analysis sets

All animals receiving any intervention were evaluated for all outcome measures that could be performed in the same mouse because of technical compatibility. Some outcome measures were technically incompatible (e.g., BAL cytology and lung histology) because the performance of one would interfere with results obtained for the other if performed in the same mouse.

Tolerability analysis set

All animals receiving any intervention were evaluated for tolerability, which included 231 mice (163 *Csf2ra*^{KO}, 68 WT) that received 369 administrations of either saline (152 instillations) or PMT (217 instillations; 36, 36, 93, or 52 instillations of 26,000, 105,000, 500,000, or 5,000,000 cells/mouse, respectively).

Pulmonary function analysis set

All 30 *Csf2ra*^{KO} mice receiving any intervention in the SAD-001a-1 study underwent lung function testing including 10 saline-treated

mice, 10 mice receiving PMT of 500,000 mGM-Rα^{+}Mϕs, and 10 receiving PMT of 5,000,000 mGM-Rα^{+}Mϕs.

General safety analysis set

All animals receiving intrapulmonary instillation of either saline or mGM-Rα^{+}Mϕs were evaluated for all outcome measures of safety except those that were incompatible.

Lung histology analysis set

All animals receiving intrapulmonary instillation of either saline or mGM-Rα^{+}Mϕs not subjected to BAL underwent microscopic examination of lung tissues and those subjected to BAL did not. BAL is incompatible with histopathologic examination of lung tissues due to technical interference. Both outcome measures cannot be performed on the same mouse due to procedural interference that introduces artifacts.

BAL cytology analysis set

All animals receiving any intervention that were not subjected to microscopic examination of lung tissues underwent BAL and microscopic examination of BAL cytology because BAL and microscopic examination of lung histopathology cannot be performed on the same mouse due to procedural incompatibility.

Pharmacokinetics analysis set

All animals receiving intervention and that underwent BAL were evaluated for pulmonary engraftment by measurement of VCN in BAL cells including 30 *Csf2ra*^{KO} mice evaluated at 24-h (SAD-001a-1), 34 WT mice evaluated at 24 h (SAD-001a-2), 34 WT mice evaluated at 14 days (SAD-001b-2), 69 *Csf2ra*^{KO} mice evaluated at 6 months (RAD-002) after administration of intervention. Furthermore, 69 *Csf2ra*^{KO} mice were evaluated at 6 months (RAD-002) after administration for biodistribution of mGM-Rα^{+}Mϕs by measuring VCN in extrapulmonary organs including blood (WBCs), brain, thymus, lymph nodes, liver, spleen, kidneys, ovaries, testes, and bone marrow.

Pharmacodynamics analysis set

All animals receiving any intervention that underwent microscopic examination of lung histology were evaluated for histopathology-based evaluation of biologic effects of the intervention (alveolar sediment score, lymphocytosis score, neutrophilia score). This included a total of 95 mice evaluated at 24 h (33 mice), 14 days (14 mice), or 6 months (31 mice) after the administration of intervention.

All animals receiving any intervention that underwent BAL were evaluated for BAL-based evaluation of the biologic effects of the intervention (BAL turbidity, and SP-D, GM-CSF, MCSF, MCP-1 concentrations; lung weight to body weight ratio (before BAL was done). This included a total of 130 mice evaluated at 24 h (30 mice), 14 days (31 mice), or 6 months (69 mice) after the administration of intervention.

Definitions

The definitions of no observed adverse effect level, NOAEL, lowest observed effect level, AE, non-adverse effect, and biologically significant effect are as originally defined by the United States Environmental Protection Agency,[55] updated by Hays and co-workers,[56] and summarized by Lewis and co-workers.[57]

Biologic effect event. A biochemical, morphological, or physiologic change (in response to a stimulus) in an animal (e.g., mouse) or biologic system (e.g., mGM-Rα^{+}Mϕs) that *is not necessarily considered to have* substantial or noteworthy effects (positive or negative) on the well-being, growth, development, or lifespan of the animal or biological system, or its ability to respond to an additional environmental challenge. This designation does not indicate or imply the change is associated with any statistical significance.

Significant biologic effect event. A biochemical, morphological, or physiologic change (in response to a stimulus) in an animal (i.e., mouse) or biologic system (e.g., mGM-Rα^{+}Mϕs) that *is considered to have* a substantial or noteworthy effect (positive or negative) on the well-being, growth, development, or lifespan of the animal or biological system, or its ability to respond to an additional environmental challenge. This definition does not indicate or imply any the change is associated with any statistical significance. Significant biologic effect events can be a normal or physiologic response to the treatment, for example, reduction in accumulated surfactant burden after PMT of GM-CSF-responsive, functional macrophages.

Non-adverse biological effect event. A biochemical, morphological or physiological change (in response to a stimulus) in an animal (e.g., mouse) or biologic system (e.g., mGM-Rα^{+}Mϕs) that is *not considered to have a detrimental effect* on the well-being, growth, development, performance, lifespan, and/or ability to respond to an additional environmental challenge.

AE. A biochemical, morphological or physiological change (in response to a stimulus) in an animal (e.g., mouse) or biologic system (e.g., mGM-Rα^{+}Mϕs) that either singly or in combination is considered to have a detrimental effect on the well-being, growth, development, performance, lifespan, and/or ability to respond to an additional environmental challenge. This includes events considered to exacerbate a pre-existing condition, for example, pulmonary lymphocytosis or alveolar sediment accumulation. This definition does not indicate or imply a causal relationship to any study-related intervention or procedure.

SAE. A SAE is defined as any AE that results in moribund health status (i.e., markedly reduced movement/impaired reflexes, poor/no oral food and/or water intake), persistent significant disability or incapacity, or results in death.

NOAEL. The highest exposure level at which there are no statistically or biologically significant increases in the frequency or severity of AEs between the exposed population (e.g., PMT-treated mice) and the appropriate control (e.g., saline-treated mice). Some biologic events may be produced at this level, but they are not considered to be adverse or precursors to adverse effects.

Outcome measures

This toxicology study involved evaluation of biologic events of relevance to the third-generation lentiviral vector (mGM-Rα^+-LV) used for *Csf2ra* gene correction, intermediate drug substance (freshly harvested mGM-Rα^+Mϕs prior to cryopreservation), drug substance (cryopreserved mGM-Rα^+Mϕs), or formulated drug product (thawed mGM-Rα^+Mϕs after washing and resuspension in saline).

Planned vector certification of acceptance tests

- Culture sterility: tryptic soy broth
- Culture sterility: thioglycolate media
- DNA sequence (provirus)
- Endotoxin level
- Mycoplasma
- Titer: physical
- Titer: infectious
- Infectious to physical titer ratio

Unplanned BAL assessment measure

- BAL Gram stain

Cell product certification measures

- Activation state (cell product)
- Cell number (cell product)
- Cell viability
- Endotoxin level
- Gram stain
- Morphological appearance (cell product)
- Mycoplasma (cell product)
- GM-CSF receptor signaling
- Macrophage phenotypic marker: F4/80 detection
- Macrophage phenotypic marker: CD11b
- Macrophage phenotypic marker: CD11c
- Transduction efficiency: initial, after transduction (day 3) (intermediate cell product)
- Transduction efficiency: final, after culture (day 14) (final cell product)

Intervention-related outcome measures

- AEs
- Anti-CD116 antibodies
- BAL GM-CSF concentration
- BAL IL-1β concentration
- BAL IL-6 concentration
- BAL macrophage count
- BAL lymphocyte count
- BAL M-CSF concentration
- BAL MCP-1 (CCL-2) concentration
- BAL neutrophil count
- BAL SP-D concentration
- BAL TNF-α concentration
- BAL total leukocyte count
- BAL turbidity
- Biologic effect events
- Body weight
- Bronchospasm assessment
- CD116 expression
- Cage-side observation: ruffled fur
- Cage-side observation: hunched body habitus
- Cage-side observation: abnormal movement
- Cage-side observation: death
- Cage-side observation: moribund appearance (lacking vitality or vigor, at point of death)
- Hematologic indices: WBC count
- Hematologic indices: neutrophil %
- Hematologic indices: lymphocyte %
- Hematologic indices: monocyte %
- Hematologic indices: eosinophil %
- Hematologic indices: basophil %
- Hematologic indices: RBC count
- Hematologic indices: Hb
- Hematologic indices: Hct
- Hematologic indices: MCV
- Hematologic indices: MCH
- Hematologic indices: MCHC
- Hematologic indices: RDW
- Hematologic indices: platelet concentration
- Lung weight to body weight ratio
- Lung histological neutrophilia score
- Lung histological lymphocytosis score
- Lung histological alveolar sediment score
- Organ histologic inflammatory cell accumulation
- Physical exam (PE)-related respiratory distress (rapid/shallow respirations)
- PE-skin lacerations
- PE-ocular stupor
- PE-ocular secretions
- PE-corneal opacifications
- PE-muscle tone reduction
- PE-strength reduction
- PE movement/reflex abnormality
- Serum chemistry: sodium
- Serum chemistry: potassium
- Serum chemistry: chloride
- Serum chemistry: blood urea nitrogen
- Serum chemistry: glucose
- Serum chemistry: creatinine
- Serum chemistry: AST
- Serum chemistry: ALT
- Serum chemistry: total protein
- Serum chemistry: lactate dehydrogenase
- Serum chemistry: total bilirubin
- SAEs
- VCN

Descriptive summary statistics

Numeric data were summarized using descriptive statistics

Observational biologic effect events, AEs, and SAEs

Biological effect events are defined above and were identified by cage-side observation, physical examination, physiologic testing, routine laboratory tests, necropsy, and microscopic examination of tissue specimens.

Determining the relationship of biologic effect events to the intervention

Biologic effect events were examined for a relationship to the intervention by comparison of results for a treatment intervention group (PMT-500K or PMT-5M group in SAD studies or PMT group in RAD study) to results for the appropriate control (corresponding saline groups in SAD and RAD studies). Statistically significant between-group differences were further analyzed by evaluating the results for the outcome variable for gender-related differences independent of the assigned intervention group and among either males or females in the treatment or control groups.

Discriminating factors supporting the interpretation that a relationship exists between a biologic effect event and treatment intervention (PMT) include: (1) an obvious dose-response, (2) it is not due to finding(s) in one or more animals that could be considered outlier(s), (3) the outcome measure under evaluation is inherently precise, (4) the result is outside the normal range of biologic variation, i.e., it is not within range of historical control values or other reference values, (5) there is biological plausibility—i.e., a result consistent with class effects, mode of action, or what is otherwise known or expected of the intervention (mGM-Rα^+Mϕs administered by PMT).

Determining biologic effect events as non-adverse or adverse

Biological effect events were evaluated to determine if they were adverse (or beneficial). Discriminating factors supporting the interpretation as adverse include: (1) there is alteration in the well-being, growth, development, performance, lifespan of the recipient mouse, and/or its ability to respond to an additional environmental challenge, or the event is (2) not an adaptive response, (3) not transient, (4) severity is not limited or is above a threshold of concern, (5) not isolated or independent (changes in other parameters usually associated with the effect of concern were also observed), (6) a precursor or part of a continuum of changes known to progress with time to an established AE (e.g., moribund behavior leading to death), (7) not secondary to another adverse effect, (8) not a consequence of the experimental model.

Determining the severity of events

The severity of a biologic effect event or AE was determined by criteria specific to each outcome measure. *Cage-side observations*: BEEs for this outcome measure focused to abnormalities of the animal's coat (i.e., ruffled fur), movement (i.e., slow/no movement in response to an auditory stimulus created by tapping the cage), or body habitus (i.e., hunched back). Cage-side observations were made twice daily on weekdays and once daily on weekends and holidays in both SAD studies and the RAD study. In the 6-month-long RAD study, a DHS was calculated for each mouse on each day of its participation in the study. The severity of the DHS was determined according to the scale of 0–4 as follows. BEEs associated with a DHS of 0 or 1 were judged to be non-adverse with "0" referring to no abnormal BEE observed during a specific cage-side observation. A DHS score of 1 refers to only one BEE identified during a specific cage-side observation. BEEs associated with a DHS of 2–4 were judged to be adverse and to have a severity increasing in proportion to the DHS as mild, moderate and severe, respectively. "Mild" refers to two BEEs identified during a specific cage-side observation. "Moderate" refers to three BEEs identified during specific cage-side observation or moribund habitus identified during a specific cage-side observation. "Severe" refers to moribund appearance or spontaneous death identified during a specific cage-side observation. The DHS for each day was determined as the highest value determined based on all cage-side observations made on that day. *Physical examinations*: BEEs identified during physical examinations were identified as adverse and the severity determined by the Study Investigator and confirmed by the Study Sponsor. *Laboratory measurements*: a laboratory test result was identified as a biological event if it was different from the range of values observed in saline-treated control mice, and the severity was determined by how far outside the control range the value was observed to be. For *liver function tests* (aspartate aminotransferase, alanine transaminase, bilirubin), increased values were judged to be mild, moderate, or severe AEs if they were increased and $<3\times$, $<5\times$, or $\geq 5\times$ the upper limit of the range in control mice respectively. For *hematologic indices* (WBC, RBC, Hb, Hct, MCV, MCH, MCHC, RDW, number of platelets, and the WBC differential cell count for neutrophils, lymphocytes, monocytes, eosinophils, and basophils), increased values were judged to be mild, moderate, or severe AEs if they were increased by <110%, <130%, or $\geq$130% of the upper limit of the range in control mice, respectively.

Tolerability analysis

Tolerability of administering mGM-Rα^+Mϕs by PMT was assessed through cage-side observation for signs of AEs for 30 min immediately after each administration of intervention. Comparisons were made between results for each SAD treatment group (PMT-500K or PMT-5M) or the RAD treatment group (PMT) and the corresponding appropriate saline intervention control group.

Safety analysis

Analyses of safety-related data were performed by comparing the measurements of outcome variables made in mice receiving the treatment intervention (PMT-500K or PMT-5M group in SAD studies or PMT group in RAD study) to those made in mice receiving the control intervention (saline) in corresponding control groups. The biologic effect events were evaluated in two steps, first for between-group differences to determine if a relationship to the treatment intervention exists, then by determining if the event was adverse, and finally by determining the severity of the event. An important determination was the identification of the highest level of exposure to mGM-Rα^+ Mϕs (administered by PMT) at which there are no statistically or biologically significant increase in the frequency or severity of AEs between mice exposed to PMT or to pulmonary instillation of saline—the no adverse effect level or NOAEL.

Pharmacokinetic analyses

The presence and relative number of mGM-Rα^{+}Mϕs in various tissues was determined by measuring the VCN relative to genomic mouse DNA in PMT-treated mice and compared to saline-treated mice.

Pharmacodynamic analyses

The biologic effects of PMT were determined by measuring outcomes relevant to the clinical manifestations of hPAP in the lungs including lung weight to body weight ratio, histologically determined alveolar sediment score, BAL turbidity, BAL SP-D, BAL GM-CSF, BAL M-CSF, BAL MCP-1 (also known as CCL2). Biological effect events were identified by comparison of the results from PMT-treated mice with those of saline-treated mice.

Statistical methods

Numeric data were evaluated for normality using the Shapiro-Wilk test, for equal variance using the F test, and were presented as mean ± SD (parametric data) or median and interquartile range (nonparametric data). Statistical comparisons were done using Student's t test (parametric data of equal variance), Student's t test with Welch's correction (parametric data of unequal variance), Mann-Whitney test (nonparametric data) or Fisher's exact test (categorical data) as appropriate. p values of ≤ 0.05 were considered to indicate statistical significance. No adjustment was made for multiple testing. Analyses were done by TPSC staff using Microsoft Excel (v.16.61) and GraphPad Prism software (v.9.1). Oversight was provided by the University of South Florida Health Informatics Institute. Individual animal listings and group mean data listings are included in the tables section of the report. Analysis of safety parameters were performed on animals surviving to the end of study.

DATA AND CODE AVAILABILITY

Study data, study protocols, and specialized materials will be made available upon request to qualified investigators for academic, noncommercial purposes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101213.

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AUTHOR CONTRIBUTIONS

Study design and management, P.A. and B.C.T.; FDA document preparation, submission, and communications, B.C.C., P.A., and B.C.T.; statistical design and oversight, J.K.; development of SOPs, P.A., B.C.C., M.W., J.A.C., L.R., C.L., M.A., E.H., D.L.-D., S.W., A.K., and B.C.T.; design and development of novel assays. P.A., B.C.C., and B.C.T.; conduct of study-related experiments and testing, P.A., K.S., M.W., Y.M., D.B., D.J.K.Y., C.C., J.S., T.S., M.I., and M.C.; histopathologic and cytologic analysis, K.A.W.-B.; data analysis and interpretation, P.A., B.C.C., K.A.W.-B., M.W., C.C., J.S., H.S., B.C.T., and J.K.; preparation and FDA submission of the study report, B.C.C., C.C., J.S., P.A., A.S., and B.C.T.; data management, B.C.C.; first draft of the manuscript, P.A. and B.C.T. All authors approved the final manuscript.

DECLARATION OF INTERESTS

B.C.T. has equity in Altius Therapeutics, a biopharmaceutical company with a license from Cincinnati Children's Hospital Medical

Center to develop PMT as a therapeutic platform beyond the planned clinical use as therapy of hPAP.

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