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Alveolar macrophages of GM-CSF knockout mice exhibit mixed M1 and M2 phenotypes

Heidi Dalrymple¹, Barbara P Barna¹, Anagha Malur¹, Achut G Malur², Mani S Kavuru¹ and Mary Jane Thomassen^{1*}**Abstract**

Background: Activin A is a pleiotrophic regulatory cytokine, the ablation of which is neonatal lethal. Healthy human alveolar macrophages (AMs) constitutively express activin A, but AMs of patients with pulmonary alveolar proteinosis (PAP) are deficient in activin A. PAP is an autoimmune lung disease characterized by neutralizing autoantibodies to Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). Activin A can be stimulated, however, by GM-CSF treatment of AMs *in vitro*. To further explore pulmonary activin A regulation, we examined AMs in bronchoalveolar lavage (BAL) from wild-type C57BL/6 compared to GM-CSF knockout mice which exhibit a PAP-like histopathology. Both human PAP and mouse GM-CSF knockout AMs are deficient in the transcription factor, peroxisome proliferator activated receptor gamma (PPAR γ).

Results: In sharp contrast to human PAP, activin A mRNA was elevated in mouse GM-CSF knockout AMs, and activin A protein was increased in BAL fluid. Investigation of potential causative factors for activin A upregulation revealed intrinsic overexpression of IFN γ , a potent inducer of the M1 macrophage phenotype, in GM-CSF knockout BAL cells. IFN γ mRNA was not elevated in PAP BAL cells. *In vitro* studies confirmed that IFN γ stimulated activin A in wild-type AMs while antibody to IFN γ reduced activin A in GM-CSF knockout AMs. Both IFN γ and Activin A were also reduced in GM-CSF knockout mice *in vivo* after intratracheal instillation of lentivirus-PPAR γ compared to control lentivirus vector. Examination of other M1 markers in GM-CSF knockout mice indicated intrinsic elevation of the IFN γ -regulated gene, inducible Nitrogen Oxide Synthetase (iNOS), CCL5, and interleukin (IL)-6 compared to wild-type. The M2 markers, IL-10 and CCL2 were also intrinsically elevated.

Conclusions: Data point to IFN γ as the primary upregulator of activin A in GM-CSF knockout mice which in addition, exhibit a unique mix of M1-M2 macrophage phenotypes.

Keywords: Interferon gamma, Activin A, Alveolar macrophages

Background

Activin A, a pleiotrophic cytokine belonging to the transforming growth factor-beta (TGF- β) superfamily, is synthesized by many cell types throughout the body [1,2]. The molecular structure is a disulphide-linked, homodimeric glycoprotein composed of two inhibin β A chains. Activin A was first recognized as an endocrine factor, but is now known to be essential to developmental and repair processes, and total ablation is neonatal lethal [3]. Contrasting regulatory roles have been cited for Activin A in inflammation [4]. Human monocytes

synthesize activin A upon stimulation with classical M1 macrophage activation inducers such as GM-CSF, LPS, and IFN γ [5,6]. Exposure of GM-CSF treated macrophages to anti-Activin A reduces M1 markers and enhances alternative M2 phenotype markers such as IL-10 [7]. Activin A also inhibits monocyte production of IL-1 β and enhances IL-1 receptor antagonist production [8]. Interestingly, in severe asthma, activin A may be elevated in serum, and data from animal models suggests that activin A may suppress T helper 2 (Th2) mediated allergic responses [9]. Collectively these observations suggest multifunctional roles for activin A in inflammatory processes.

Maintenance of lung homeostasis is a complex process dependent upon a network of interacting cells and cytokines. GM-CSF is required for alveolar macrophage

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(AM) function and pulmonary homeostasis [10]. In genetically altered mice homozygous for a disrupted GM-CSF gene (GM-CSF knockout), hematopoiesis is normal but there is accumulation of excess lung surfactant [11,12]. This surfactant pathology mirrors that of human PAP, an autoimmune disease characterized by high levels of autoantibody to GM-CSF [12-14]. Aerosolized GM-CSF resolves the pulmonary pathology of GM-CSF knockout mice, thus demonstrating that surfactant homeostasis can be influenced by local administration of GM-CSF to the respiratory tract [15].

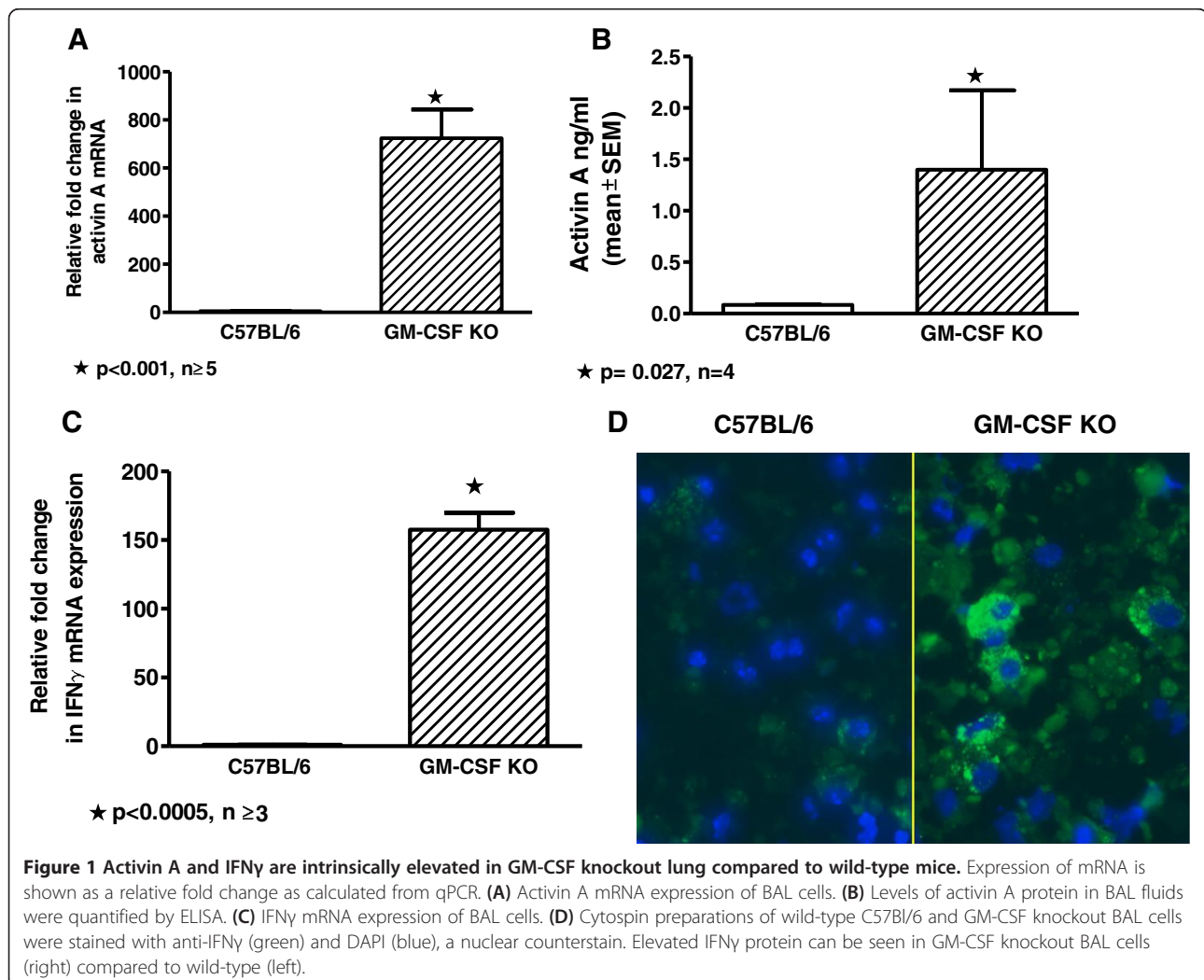
Previously we reported that healthy human AMs synthesize activin A in response to GM-CSF but AMs of patients with PAP are deficient in activin A [16]. In addition, PAP AMs are deficient in the nuclear transcription factor, Peroxisome Proliferator-activated Receptor, (PPAR γ), a regulator of lipid and glucose metabolism that is restored by GM-CSF treatment [17]. PPAR γ has also been shown to be a negative regulator of inflammation [18,19]. Interestingly, alveolar macrophages of GM-CSF

knockout mice are also deficient in PPAR γ [20]. The role of activin A in the lung has not been established. Because of the phenotypic similarities between human PAP and the GM-CSF knockout mouse, this study was undertaken to investigate activin A regulation in the lung. Initially, it was hypothesized that activin A might be impaired in GM-CSF knockout mice based upon previous data from PAP studies [16].

Results

Activin A and IFN γ are intrinsically elevated in GM-CSF knockout mice as compared to wild-type mice

Unlike previous findings of activin A deficiency in human PAP [16], activin A mRNA expression of BAL cells was significantly ($p < 0.005$) elevated in GM-CSF knockout mice compared to wild-type controls (Figure 1A). Quantification of activin A protein in BAL fluids confirmed mRNA findings with significantly ($p < 0.05$) elevated protein levels in GM-CSF knockout compared to wild-type (Figure 1B). GM-CSF knockout expression



of follistatin, an inhibitor of activin A [21], was similar to wild-type mice (data not shown) and thus could not account for the striking elevation of activin A.

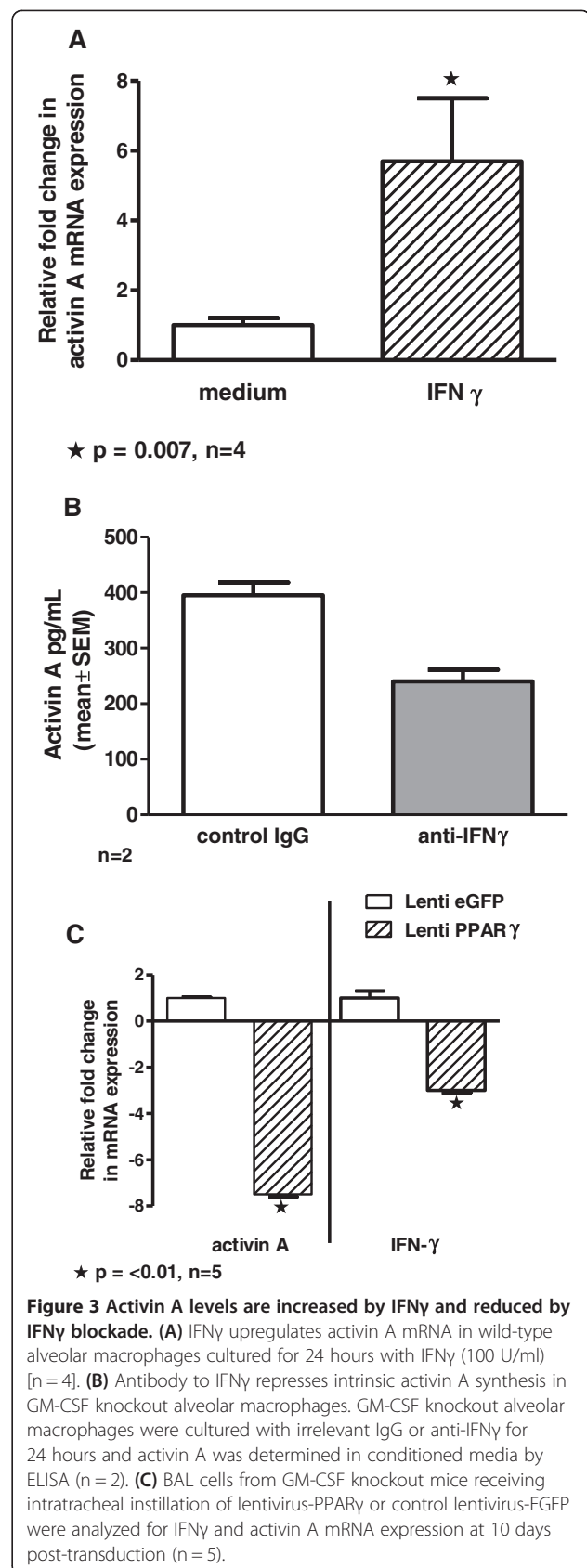
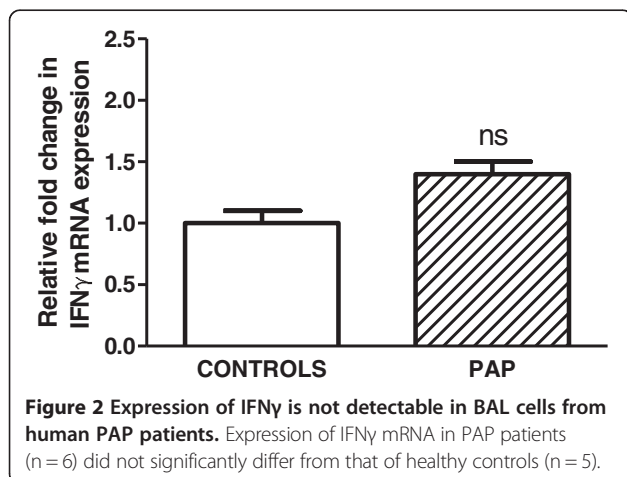
Intrinsic factors that could potentially affect activin A levels were subsequently investigated in GM-CSF knockout mice. Macrophage colony-stimulating factor (M-CSF) has been reported to be upregulated in GM-CSF knockout mice [22]. Examination of M-CSF in the current study, however, indicated no effect on activin A *in vitro* in either wild-type or GM-CSF knockout AMs (data not shown). Elevated IFN γ has been reported in lungs of GM-CSF knockout mice [23] therefore intrinsic levels of IFN γ were examined. IFN γ mRNA expression was significantly ($p < 0.005$) elevated in GM-CSF knockout BAL cells compared to wild-type controls (Figure 1C). Immunocytochemistry of GM-CSF knockout BAL cells confirmed mRNA results and indicated markedly increased expression of intracellular IFN γ protein compared to wild-type (Figure 1D).

IFN γ is not elevated in human PAP BAL cells

In contrast to results from GM-CSF knockout mice, examination of IFN γ expression in human BAL cells from PAP patients revealed no significant increase compared to healthy controls (Figure 2).

Activin A levels are enhanced by IFN γ and reduced by IFN γ blockade

IFN γ has been shown to upregulate activin A expression in human monocytes [5] but AMs have not been studied. Results from 24-hour *in vitro* cultures of wild-type AMs indicated that IFN γ (100 U/ml) significantly ($p < 0.05$) increased activin A expression (Figure 3A). To determine whether blockade of IFN γ with specific anti-IFN γ antibody would alter intrinsic activin A expression, unstimulated GM-CSF knockout AMs were cultured *in vitro* for 24 hours with irrelevant immunoglobulin (Ig) or anti-IFN γ . ELISA analysis of conditioned media indicated that

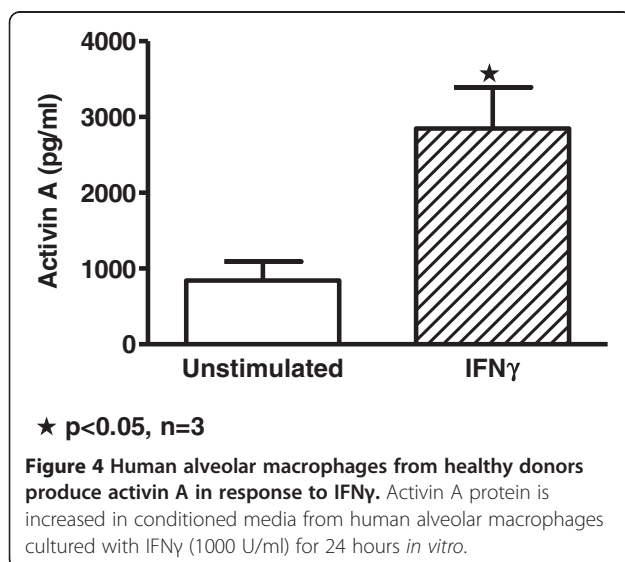


anti-IFN γ reduced activin A protein synthesis compared to irrelevant Ig (Figure 3B) confirming that IFN γ blockade reduced intrinsic activin A production.

Because activin A is intrinsically elevated in PPAR γ deficient GM-CSF knockout mice but severely decreased in PPAR γ deficient human PAP patients [16], it appeared unlikely that PPAR γ would exert a direct effect on activin A. Observations made elsewhere [24] also found no evidence of a PPAR γ effect on activin A. We have shown, however, that IFN γ is elevated in macrophage-specific PPAR γ knockout mice and significantly reduced after *in vivo* restoration of PPAR γ via a lentivirus vector [25]. We utilized this approach to determine whether PPAR γ restoration in GM-CSF knockout mice might reduce IFN γ and thereby reduce activin A. Results supported this action. Ten days post intratracheal inoculation of lentivirus reagents into GM-CSF knockout mice, BAL cell mRNA expression of both IFN γ and activin A was significantly reduced in animals receiving lentivirus-PPAR γ compared to controls receiving lentivirus-eGFP ($p < 0.05$) (Figure 3C).

Human alveolar macrophage activin A is increased by IFN γ

While the above studies clearly defined IFN γ -mediated regulation of activin A in murine alveolar macrophages, it was necessary to confirm this pathway in human alveolar macrophages. *In vitro* studies demonstrated that IFN γ significantly enhanced activin A protein production (Figure 4) in healthy human alveolar macrophages. Thus activin A synthesis in both human and murine alveolar macrophages is responsive to IFN γ upregulation even though intrinsic activin A levels differ between human and mouse.



GM-CSF BAL cells show intrinsic elevation of both M1 and M2 macrophage phenotypic markers

We and others reported previously that M-CSF gene expression and protein, a cytokine associated with the M2 macrophage phenotype, was elevated in GM-CSF knockout mice [22,26]. Current data indicate that the M1-associated cytokine, IFN γ (protein and gene expression) is also increased in these mice. Therefore, it was unclear whether GM-CSF knockout BAL cells would express predominantly M1 or M2 profiles. To address this issue, we determined mRNA expression of several M1 and M2 markers in GM-CSF knockout BAL cells. With respect to M1 markers, we examined the IFN γ -regulated target gene, iNOS (Figure 5A), together with CCL5 (Figure 5B), and IL-6 (Figure 5C), and found that all were significantly elevated compared to wild-type mice. The M2 marker, IL-10, has been reported to be suppressed by elevated activin A [7,27], and in PAP, activin A deficiency is accompanied by elevated IL-10 [16,28,29]. Surprisingly, analysis of IL-10 expression in GM-CSF knockout BAL cells revealed significantly elevated levels compared to wild-type mice (Figure 5D). Analysis of another M2-associated marker, CCL2, also indicated significant elevation compared to wild-type mice (Figure 5E). These results suggested that GM-CSF knockout alveolar macrophages might constitute a mixed population of both M1 and M2 phenotypes.

Discussion

The current findings suggest that IFN γ is a major contributory factor to the intrinsic elevation of activin A in AMs. Findings also point out a striking difference in activin A expression in human PAP and GM-CSF knockout mice despite common deficiencies of GM-CSF and PPAR γ (summarized in Table 1). In parallel with activin A, GM-CSF knockout mice displayed over-expression of IFN γ [23], a positive regulator of activin A [5]. In contrast, BAL cells of PAP patients do not exhibit elevated IFN γ and activin A is deficient [16].

Elevated IFN γ has been reported previously in the BAL fluids of GM-CSF knockout mice [23]. Our previous studies also found elevated IFN γ expression in macrophage-specific PPAR γ knockout mice [25]. Restoration of PPAR γ via lentivirus vector in these mice greatly diminished IFN γ expression [25]. In the current study, similar results were seen after PPAR γ -lentivirus treatment of GM-CSF knockout mice. Such findings suggest that the PPAR γ deficiency present in GM-CSF knockout mice may contribute to elevated IFN γ . GM-CSF has been shown to be a critical upregulator of PPAR γ [31,34]. The total lack of GM-CSF in knockout mice may maintain an extreme PPAR γ deficiency which is ineffective at repressing inflammatory mediators such as IFN γ . In human PAP, IFN γ levels are not increased

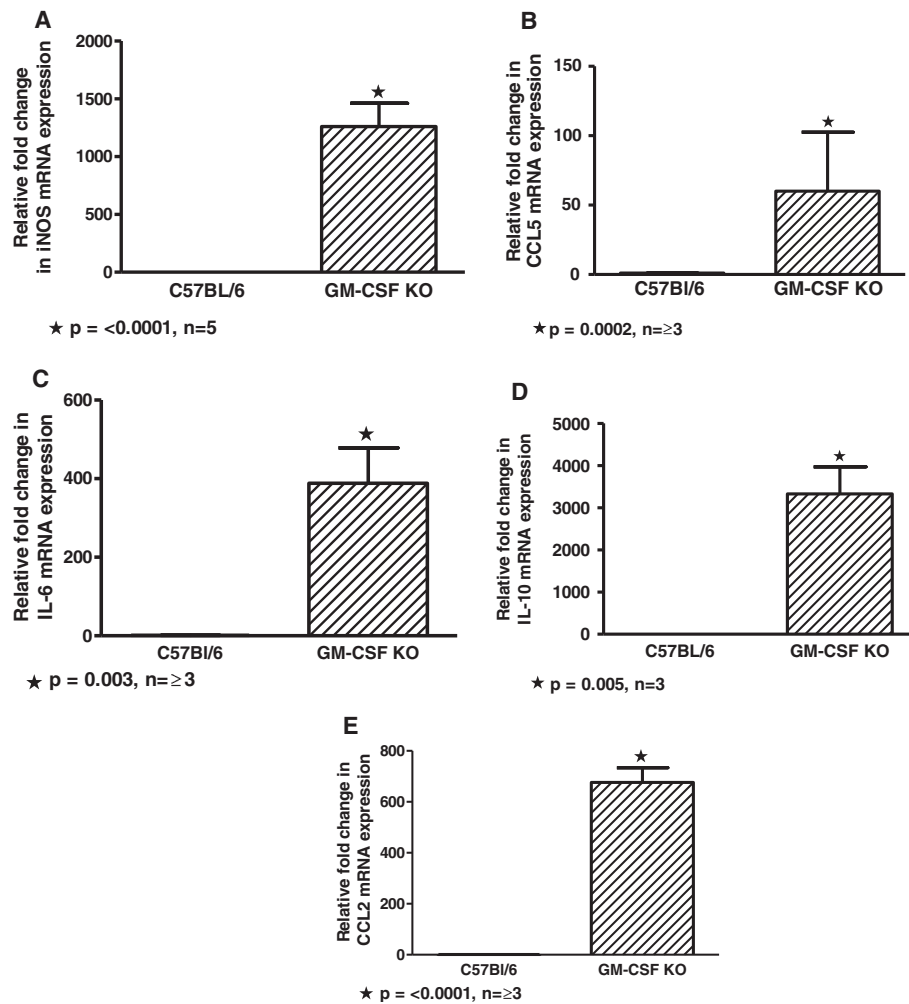


Figure 5 mRNA expression of both M1 and M2 macrophage phenotypic markers is intrinsically elevated in GM-CSF knockout BAL cells compared to wild-type mice. Elevated mRNA expression of M1 markers: (A) iNOS; (B) CCL5, and (C) IL-6. Elevated mRNA expression of M2 markers: (D) IL-10, and (E) CCL2.

despite PPAR γ deficiency, furthermore, GM-CSF is not totally absent [29]. The primary etiology of PAP is considered to be an autoimmune response to GM-CSF in the form of high levels of circulating, neutralizing autoantibody to GM-CSF [13]. It is also possible that additional regulatory mechanisms are present in human lung to help prevent IFN γ buildup in PAP.

The varying characteristics of activated macrophages have led to attempts to categorize activation phenotypes [35-39]. The M1 phenotype is characterized by production of microbial or IFN γ -triggered molecules such as iNOS and IL-12. GM-CSF has been cited as an inducer of M1 phenotypes while M-CSF has been shown to induce the M2 alternative activation phenotype in which IL-10 or TGF β may be produced [7,40]. We have shown that M-CSF is elevated in GM-CSF knockout mice [22] and in human PAP [33] which might suggest the

presence of an M2 macrophage phenotype (see Table 1). Interestingly, PPAR γ , which is deficient in GM-CSF knockout mice, is also a major driver of the M2 phenotype [41]. It has been pointed out however, that macrophage phenotypes were defined by carefully controlled *in vitro* conditions which may be vastly different from the *in vivo* milieu [42]. Thus the juxtaposition of both IFN γ and M-CSF in the lungs of GM-CSF knockout mice could produce the novel combination of macrophage activation phenotypes illustrated by elevated M1 (iNOS, CCL5, IL-6) and M2 (IL-10, CCL2) markers (Table 1). Other IFN γ -inducible pro-inflammatory mediators (chemokines CXCL9, CXCL10, and CXCL11) have been noted in the lungs of GM-CSF knockout mice [23]. Previously, we found that MMP-2, a matrix metalloproteinase associated with M-CSF and alternative M2 activation, is also elevated in GM-CSF knockout BAL cells [33].

Table 1 Summary: comparison of macrophage activation regulatory factors in human pulmonary alveolar proteinosis (PAP) patients and GM-CSF knockout mice

Regulatory factors	Associated macrophage phenotype	PAP patients	GM-CSF knockout mice
GM-CSF	M1	Deficient protein, not mRNA [28]	Absent [11]
Activin A	M1	Deficient [16]	Elevated
IL-6	M1	Not done	Elevated
CCL5	M1	Not done	Elevated
IFN γ	M1	mRNA - not elevated (comparable to healthy controls)	Elevated [23]
INOS	M1	Undetectable in human alveolar macrophages (unpublished observation)	Elevated
M-CSF	M2	Elevated [30]	Elevated [22]
PPAR γ	M2	Deficient [31]	Deficient [31]
CCL2	M2	Elevated [32]	Elevated
IL-10	M2	Elevated [29]	Elevated
MMP2	M2	Elevated [33]	Elevated [33]

Conclusions

The current findings extend our previous studies examining pulmonary mechanisms operative in human PAP and the GM-CSF knockout mouse. It is clear that pathways of activin A regulation may utilize GM-CSF or IFN γ as stimulatory factors. In the GM-CSF knockout mouse, lack of GM-CSF may restrict production of sufficient PPAR γ to control inflammation. The persistent elevation of both M-CSF and IFN γ may influence AMs to express characteristics of both M1 and M2 phenotypes. The current data emphasize the plasticity of alveolar macrophages in assuming a unique activation phenotype when regulatory pathways become dysfunctional.

Methods

Mice

Animal studies were conducted in conformity with Public Health Service (PHS) Policy on humane care and use of laboratory animals and were approved by the institutional animal care committee. The GM-CSF knockout mice were generated by Dr. Glenn Dranoff and have been previously described [11]. Controls consisted of C57BL/6 wild type mice obtained from Jackson Laboratory (Bar Harbor, ME). BAL cells and fluids were obtained from 8-12 week-old GM-CSF knockout mice and age and gender matched wild-type C57BL/6 controls as previously described [43]. Briefly, cytopins of BAL cells were stained with a modified Wright-Giemsa stain for differentials. A minimum of 100 cells was scored for each lavage. Mean (\pm SEM) BAL cells from C57BL/6 mice were composed of $98 \pm 1\%$ macrophages and $2 \pm 1\%$ lymphocytes; GM-CSF knockout BAL cells were composed of $91 \pm 2\%$ macrophages and $5 \pm 1\%$ lymphocytes. For *in vitro* studies, BAL cells were plated at 150,000 cells/well in 48-well plates as previously described [25]. Recombinant murine IFN γ was obtained from

R&D Systems. Neutralizing anti-IFN γ and control antibodies were purchased from BD Biosciences. For all experiments a minimum of 3 sets of pooled BAL cells from 3-5 mice were used except where indicated.

Human subjects

The protocol was approved by the East Carolina University Institutional Review Board and written informed consent was obtained from all patients and control subjects. Healthy control subjects had no history of lung disease and were not on medication. PAP subjects were recruited from patients undergoing routine clinical evaluation. The diagnosis of idiopathic PAP was confirmed by histopathological examination of material from open lung or transbronchial biopsies as previously described [29]. Alveolar macrophages were derived from bronchoalveolar lavage (BAL) obtained by fiberoptic bronchoscopy as previously described [29]. Differential cell counts were obtained from cytopins stained with a modified Wright's stain. For PAP patients, the mean BAL cell percentages (means \pm SEMs) were: alveolar macrophages, $83 \pm 9\%$, and lymphocytes, $10 \pm 5\%$. Healthy control values were: alveolar macrophages, $93 \pm 2\%$ and lymphocytes, $7 \pm 2\%$. For *in vitro* culture, BAL cells were plated into 24-well plates (300,000 alveolar macrophages per well) or chamber slides (60,000 cells/well) as previously described [16].

RNA purification and analysis

Total RNA was extracted from BAL cells or cultured alveolar macrophages and analyzed by Q-PCR as previously described [25]. RNA specimens were analyzed in duplicate using primer-probe sets for activin A, IL-10, iNOS, CCL2, CCL5, IL-6, IFN γ and GAPDH as previously described [25]. Data were normalized to GAPDH and expressed as fold change in mRNA expression compared to controls values as previously described [44].

Lentivirus plasmid and transduction

The self-inactivating lentivirus expression vector used here has been described previously [45]. Construction of the lentivirus-PPAR γ (lenti-PPAR γ) and control lentivirus construct has also been described in detail [20,25]. Control consisted of a lentivirus vector expressing Enhanced Green Fluorescent Protein (eGFP) (lenti-EGFP). Animals received 50 μ g of lentivirus vector in 50 μ l PBS or PBS alone (sham) by intratracheal instillation. After 10 days, five animals per group were lavaged, BAL differential counts were obtained and RNA was extracted.

Activin A and follistatin protein assays

Activin A or follistatin proteins (pg/ml) in BAL fluids or conditioned media from cultured alveolar macrophages were quantified by ELISA according to the manufacturer's instructions (Serotec, Raleigh, NC; R&D Systems, Minneapolis, MN).

Immunocytochemistry

Immunocytochemistry for IFN γ was carried out on cytospin samples from freshly isolated BAL cells using rat anti-mouse IFN γ (Santa Cruz Biotechnology, 1:100) followed by goat anti-rat IgG (Invitrogen) as described [25]. Slides were counter-stained with DAPI (Invitrogen) to allow nuclear localization.

Statistics

Data were analyzed by student's t-test using Prism software (GraphPad). Values from treated cells were compared to untreated. Significance was defined as $p \leq 0.05$.

Abbreviations

AM: Alveolar macrophage; GM-CSF: Granulocyte macrophage colony stimulating factor; PAP: Pulmonary alveolar proteinosis; BAL: Bronchoalveolar lavage; PPAR γ : Peroxisome proliferator activated receptor; IFN γ : Interferon; iNOS: Inducible nitric oxide synthetase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HD contributed to acquisition of the data, analysis and interpretation of data, drafting of the manuscript and final approval of the version to be published; BPB contributed to the design, analysis and interpretation of data, drafting of the manuscript and final approval of the version to be published; AM contributed to the conception and design, acquisition of the data, analysis and interpretation of data and final approval of the version to be published; AGM contributed to acquisition of the data and final approval of the version to be published; MSK contributed to the acquisition of data and final approval of the version to be published; MJT contributed to the conception and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript and final approval of the version to be published. All authors read and approved the final manuscript.

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