

Familial pulmonary alveolar proteinosis caused by mutations in *CSF2RA*

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Primary pulmonary alveolar proteinosis (PAP) is a rare syndrome characterized by accumulation of surfactant in the lungs that is presumed to be mediated by disruption of granulocyte/macrophage colony-stimulating factor (GM-CSF) signaling based on studies in genetically modified mice. The effects of GM-CSF are mediated by heterologous receptors composed of GM-CSF binding (GM-CSF-R α) and nonbinding affinity-enhancing (GM-CSF-R β) subunits. We describe PAP, failure to thrive, and increased GM-CSF levels in two sisters aged 6 and 8 yr with abnormalities of both GM-CSF-R α -encoding alleles (*CSF2RA*). One was a 1.6-Mb deletion in the pseudoautosomal region of one maternal X chromosome encompassing *CSF2RA*. The other, a point mutation in the paternal X chromosome allele encoding a G174R substitution, altered an N-linked glycosylation site within the cytokine binding domain and glycosylation of GM-CSF-R α , severely reducing GM-CSF binding, receptor signaling, and GM-CSF-dependent functions in primary myeloid cells. Transfection of cloned cDNAs faithfully reproduced the signaling defect at physiological GM-CSF concentrations. Interestingly, at high GM-CSF concentrations similar to those observed in the index patient, signaling was partially rescued, thereby providing a molecular explanation for the slow progression of disease in these children. These results establish that GM-CSF signaling is critical for surfactant homeostasis in humans and demonstrate that mutations in *CSF2RA* cause familial PAP.

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Pulmonary surfactant homeostasis is maintained by the balanced production of surfactant by alveolar epithelium and its clearance by pulmonary alveolar macrophages (1). GM-CSF is a critical regulator of surfactant homeostasis in mice (1). GM-CSF, via heterologous cell surface receptors comprised of α and β glycoprotein subunits (GM-CSF-R α and GM-CSF-R β , respectively), activates STAT5 and other signaling pathways (2). N-linked glycosylation of the α subunit is required for GM-CSF binding and receptor signaling (3). In mice, deficiency of GM-CSF (4, 5) or its receptor (6) impairs multiple functions of alveolar macrophages (7), includ-

ing surfactant catabolism (8), which results in progressive surfactant accumulation.

Pulmonary alveolar proteinosis (PAP) is a syndrome characterized by respiratory failure caused by pulmonary surfactant accumulation (9, 10), which can be grouped into distinct categories based on clinical, histopathologic, biochemical, and genetic data (1, 9–12). Primary PAP occurs when GM-CSF signaling is disrupted, for example in individuals with high levels of GM-CSF autoantibodies, which are

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presumed to mediate pathogenesis by neutralizing GM-CSF and reducing surfactant catabolism by alveolar macrophages (10, 13). This form, referred to as autoimmune PAP, comprises 90% of cases (13). Primary PAP has also been associated with reduced detection of GM-CSF-R β on myeloid cells (14, 15), but definitive studies demonstrating heritable deficiency of either GM-CSF or its receptors as the cause of PAP in humans are lacking (16). Secondary PAP occurs as a consequence of an underlying disease presumed to impair surfactant clearance by reducing either the numbers or functions of alveolar macrophages (9). Hereditary disorders of surfactant production, for example, because of mutations in the genes encoding surfactant protein (SP)-B (17), SP-C (18), or ABCA3 (19), exhibit disordered surfactant homeostasis to varying degrees but are distinguished from PAP by their surfactant dysfunction, disruption of alveolar wall architecture, and clinical course (20). In this paper, we describe a family in which two children developed primary PAP in association with loss of GM-CSF responsiveness caused by mutations in the gene encoding GM-CSF-R α .

RESULTS AND DISCUSSION

Clinical presentation and phenotype

The index patient presented at age six with a 2-yr history of progressive tachypnea and failure to thrive (height and weight were third percentile for age [Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>]). Gestation, delivery, birth weight (3.66 kg, 50th percentile), and development were all normal, but weight gain slowed by 6–9 mo as did height by 2–3 yr. There was no history of cough, fever, chest pain, pneumonia or other pulmonary disease, environmental exposure, or drug use. Both parents were well-developed and healthy with no history of lung disease. Examination revealed moderate tachypnea, mild tachycardia, and inspiratory crackles but was otherwise unremarkable. Pulmonary function testing revealed severe restrictive impairment. Oxygen saturation was 88% while breathing room air and decreased while talking or walking a short distance. A diagnosis of PAP was suspected based on chest radiography (Fig. 1 A) and established by histopathologic examination of lung tissue (Fig. 1 B). A serum GM-CSF autoantibody test (21) was negative on two occasions. The patient was transferred to Cincinnati Children's Hospital and underwent whole lung lavage therapy with marked symptomatic and radiographical improvement (Fig. 1 C). MCP-1 and M-CSF levels in the lavage were increased compared with healthy controls (supplemental text, available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>), which is similar to findings in autoimmune PAP patients and GM-CSF KO mice (22, 23).

An inherited defect in GM-CSF receptor function was suspected based on the constellation of histopathology showing well-preserved alveolar wall architecture (Fig. 1 B), the absence of GM-CSF autoantibodies (21), and an abnormal CD11b stimulation index test (24). Therefore, all immediate family members were evaluated. Because SP is in serum in PAP and levels reflect lung disease severity (25), serum SP-D

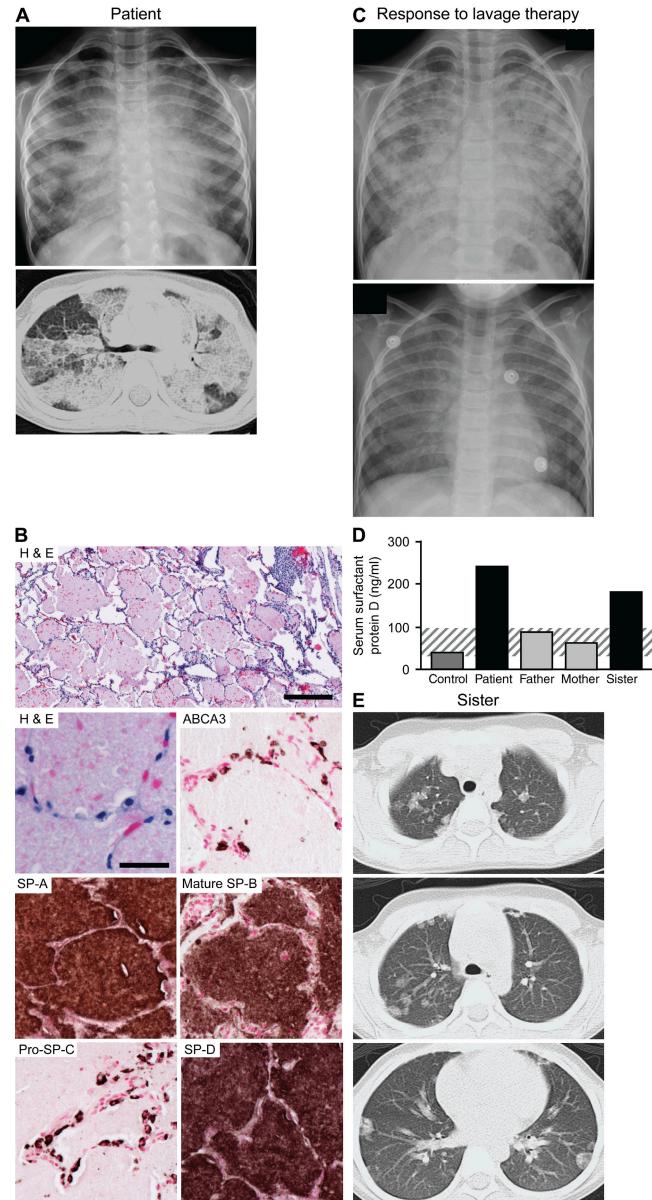


Figure 1. Phenotypic characterization of patients with familial PAP. (A) Chest radiograph (top) and high-resolution computed tomogram of the chest (bottom) of the index patient at presentation. (B) Histopathologic appearance of the open lung biopsy from the index patient after staining with hematoxylin and eosin (H&E) or immunostaining for SP-A, mature SP-B, Pro-SP-C, SP-D, and ABCA3. Note the lymphocytosis in the low-power hematoxylin and eosin-stained section and the intact alveolar wall in the high-powered hematoxylin and eosin-stained section. Images were obtained at a total magnification of 50 \times (top) or 400 \times (all others). Bars: (top) 100 μ m; (all others) 10 μ m. (C) Chest radiograph of the index patient 4 mo after presentation immediately before (top) and 5 d after (bottom) whole lung lavage therapy. (D) Serum SP-D levels in affected siblings, parents, and a control. The mean \pm SD serum SP-D concentration in 67 healthy controls was 63.5 \pm 39 ng/ml (hatched region) and in 12 patients with autoimmune PAP was 174 \pm 83 ng/ml (not indicated). (E) High-resolution computed tomogram showing diffuse patchy ground glass opacities representing mild PAP in the affected sister.

was measured and found to be increased in the patient compared with her parents and controls (Fig. 1 D). Unexpectedly, the patient's 8-yr-old sister, who was previously thought to be healthy, had increased serum SP-D. Subsequent clinical evaluation revealed that the sister had poor growth (height and weight, 10th percentile for age; Fig. S1B), a diffusion capacity for carbon dioxide of 57% that predicted, and mild patchy ground glass opacities throughout both lungs, which are consistent with a diagnosis of PAP (Fig. 1 E).

Structure and function of the GM-CSF receptor

Flow cytometry indicated that GM-CSF-R α and GM-CSF-R β were present on blood leukocytes in all family members (Fig. 2 A). Western blotting revealed an abnormal electrophoretic pattern for GM-CSF-R α in the patient and her sis-

ter with predominantly lower molecular mass forms compared with the control (Fig. 2 B). The father was heterozygous for normal and lower molecular mass forms. Unexpectedly, the mother exhibited only a normal pattern. To evaluate cell-mediated clearance of GM-CSF, blood mononuclear cells from the patient (Fig. 2 C) and her sister (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>) were incubated with GM-CSF, and both failed to clear GM-CSF in contrast to control and parental leukocytes. The concentration of GM-CSF in lung lavage was markedly increased in the patient compared with healthy controls, in whom it was undetectable (Fig. 2 D). The in vivo concentration of pulmonary GM-CSF in the epithelial lining fluid of the lungs was estimated to be 6.9 and 27 ng/ml (right and left lungs, respectively) using the urea dilution method (26). Pulmonary

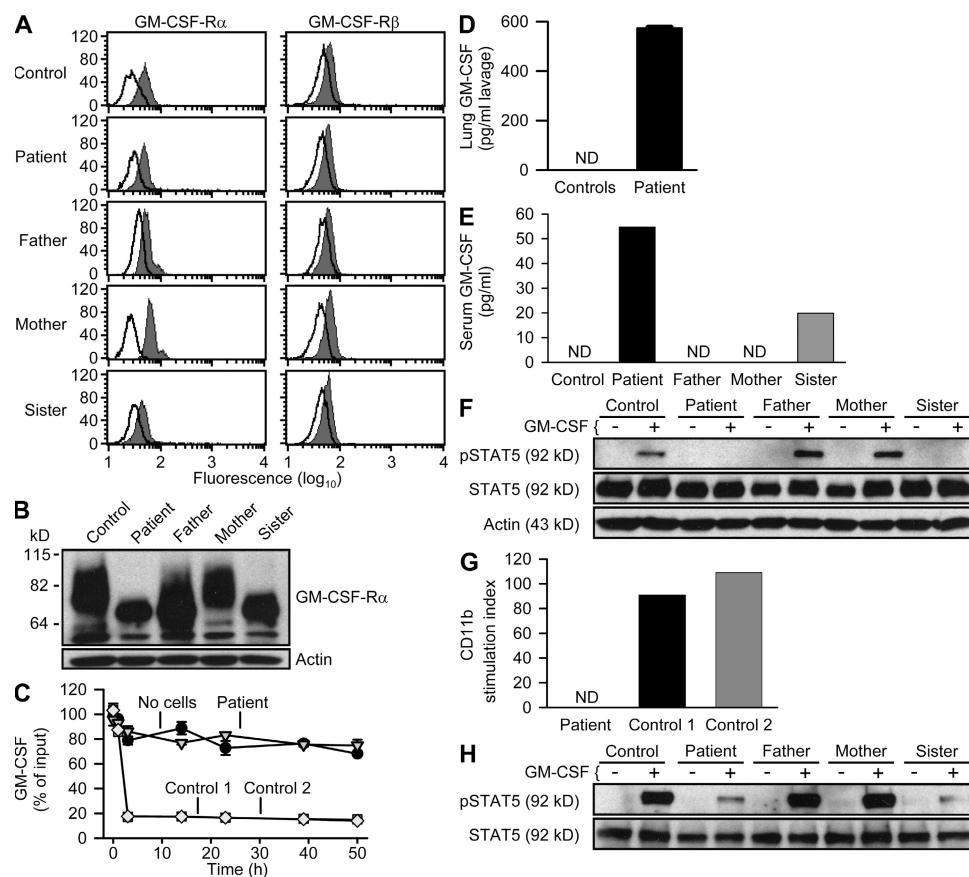


Figure 2. Structural and functional analysis of GM-CSF receptor defects. (A) Flow cytometry demonstrating GM-CSF-R α and GM-CSF-R β on the cell surface of peripheral blood leukocytes in all family members. (B) Western blots of PBMC lysates using antibodies to detect GM-CSF-R α or actin as indicated. (C) GM-CSF clearance assay. Blood leukocytes from the patient were unable to remove exogenous GM-CSF added to culture medium at time zero in contrast to leukocytes from two controls that rapidly bound and cleared GM-CSF. Error bars show the means \pm SE. (D and E) Measurement of GM-CSF concentration by ELISA. GM-CSF was readily detected in lavage from the patient but was not detected (ND) in lung lavage from nine healthy controls (D). GM-CSF was detected in the serum of affected siblings but not their parents or a control (E). (F) Blood leukocytes isolated from the indicated family members were incubated for 15 min in the absence (–) or presence (+) of 10 ng/ml GM-CSF, followed by Western blotting of cell lysates to detect total STAT5 (STAT5), phosphorylated STAT5 (pSTAT5), or actin. (G) Measurement of the GM-CSF-stimulated increase in CD11b levels on leukocytes in whole blood. The CD11b stimulation index (24) was calculated as the mean fluorescence of CD11b on leukocytes incubated with GM-CSF minus that of unstimulated cells and multiplied by 100. (H) Similar to F except that a higher GM-CSF concentration (1,000 ng/ml) was used and cell lysates were immunoprecipitated with anti-STAT5 antibody before Western blotting to detect total STAT5 (STAT5) or phosphorylated STAT5 (pSTAT5).

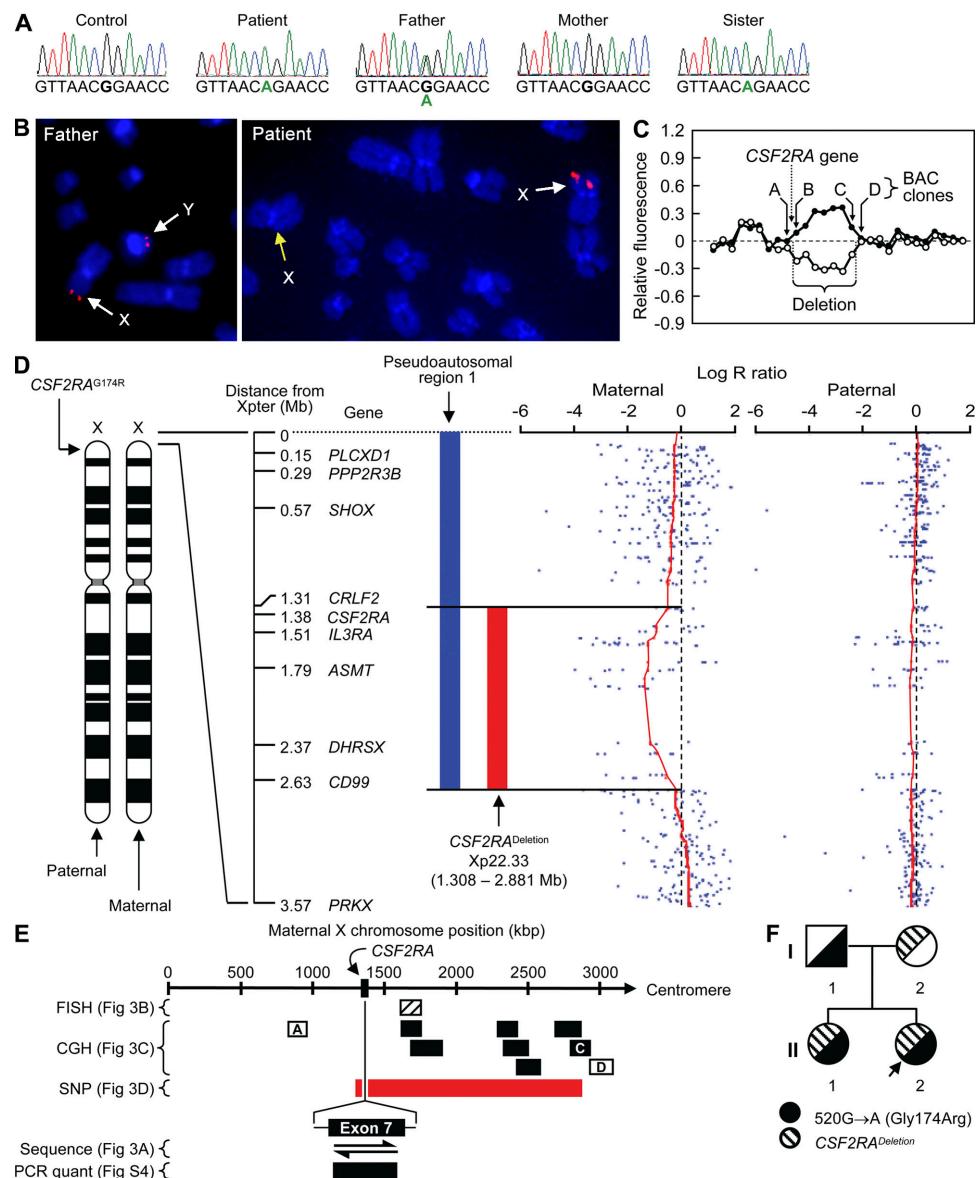


Figure 3. Genetic analysis of *CSF2RA* gene defects. (A) Nucleotide sequence of *CSF2RA* in genomic DNA from each family member, including nt 580–591 of the coding sequence (numbered relative to the initiation codon; from GenBank/EMBL/DDBJ under accession no. NM_006140.3). The index patient and her sister exhibited only a G→A point mutation at nt 586. The father was heterozygous for this substitution and the mother exhibited only the normal sequence. (B) FISH analysis to detect *CSF2RA* sequences in genomic DNA from the father and the patient. The probe (CTD-3047L21), which maps to the pseudoautosomal region (Xp22.33 and Yp11.32), hybridized to both X and Y chromosomes in the father (white arrows) and to one (white arrow), but not the other (yellow arrow), X chromosome in the patient. 8–10 metaphase cells and 25 interphase cells were evaluated for each individual. Similar FISH analyses are shown for each family member in the supplemental material (available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>). Images were obtained at a total magnification of 1000×. (C) CGH analysis for the patient in the region of Xp22.33. The relative fluorescence of fluorescently labeled DNA from the patient (open circles) compared with a same-sex reference DNA (filled circles) after hybridization to various BAC clones on the SignatureSelect V2 chip representing the Xp22.33 region is shown. Reduced hybridization to several BAC clones (clones B and C) is indicated by the lower fluorescence of the patient's DNA compared with the reference DNA for these BAC clones. The telomeric breakpoint is mapped to between clones A and B and the centromeric breakpoint is mapped to between clones C and D. These data indicate an interstitial deletion of ~1.264 Mb at Xp22.33 encompassing ~1,610,183–2,873,864 bp. The dashed line represents a relative fluorescence of zero. (D) High-resolution SNP mapping of the Xp22 region for paternal and maternal X chromosomes. A schematic shows the locations of the point mutation (*CSF2RA*^{G174R}) in the paternal X chromosome and the 1.6-Mb deletion at Xp22.33 in the maternal X chromosome and the genes encompassed. The base 2 ratio of normalized hybridization intensities for patient and reference samples (log R ratio) is shown. Similar SNP analyses for each family member are shown in the supplemental material (available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>). (E) Map showing a portion of the X chromosome summarizing the genetic analysis used to identify the small maternal X chromosomal deletion at Xp22.33 encompassing *CSF2RA*. The probe used for FISH analysis (hatched bar) is the same as clone B on the CGH microarray chip. The locations of selected CGH microarray probes in the region of the *CSF2RA* gene are shown. Those CGH probes showing

GM-CSF was also increased in *CSF2RB*-deficient mice (Fig. S3), confirming the relationship between GM-CSF receptor dysfunction and increased pulmonary GM-CSF levels. Serum GM-CSF was increased in both affected siblings and undetectable in both parents and control, indicating that the abnormality was systemic (Fig. 2 E). GM-CSF receptor function was impaired in both affected siblings, as shown by reduced GM-CSF-stimulated phosphorylation of STAT5 (Fig. 2 F) and failure of GM-CSF to increase CD11b levels on leukocytes from the patient compared with controls (Fig. 2 G).

The slower-than-expected rate of progression of PAP in both affected siblings led us to hypothesize that the GM-CSF-R α abnormality may not completely abolish GM-CSF signaling. This was confirmed by incubation of primary blood mononuclear cells with a higher concentration of GM-CSF (1,000 ng/ml), which resulted in partial receptor signaling as shown by an increase in phosphorylated STAT5 (Fig. 2 H). Thus, although both GM-CSF-R α and β chains were present on leukocytes in all family members, in the two affected siblings, the GM-CSF-R α chain was structurally abnormal, which severely reduced but did not completely abolish GM-CSF binding and GM-CSF-dependent signaling and cellular functions.

Genetic analysis of the GM-CSF receptor

The nucleotide sequence of the *CSF2RA* gene in genomic DNA from both siblings revealed a single G→A point mutation in exon 7 that encoded a nonconservative amino acid change (glycine to arginine) at position 174, G174R (Fig. 3 A). This change alters one of the 11 potential sites of N-glycosylation, which is required for GM-CSF receptor function (3, 27). The father was heterozygous for mutant *CSF2RA*^{G174R} and WT (*CSF2RA*^{WT}) alleles (Fig. 3 A), whereas only the *CSF2RA*^{WT} sequence was detected in the mother (Fig. 3 A). The sequence of *CSF2RA* in leukocyte messenger RNA (mRNA) was similar to the respective DNA sequence for each family member, even when cDNA was overamplified to detect potentially underrepresented mRNA sequences (unpublished data). This absence of a normal sequence for *CSF2RA* exon 7 in both siblings and the absence of the point mutation in the mother suggested that one maternal *CSF2RA* allele may be deleted in the patient, her sister, and their mother. The nucleotide sequence of *CSF2RB* in leukocyte mRNA from all family members was normal (unpublished data).

PCR amplification demonstrated a reduced copy number of *CSF2RA* in genomic DNA from both siblings and their mother compared with the father and a healthy control (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>). In contrast, the genomic copy number

of *CSF2RB* was similar in all family members and a control (Fig. S4). Routine cytogenetic analysis was normal in all family members (Fig. S5), ruling out a large chromosomal abnormality. Fluorescent in situ hybridization (FISH) demonstrated a deletion in the pseudoautosomal region 1 of the X chromosome in the patient (Fig. 3 B), her sister, and mother (Fig. S6) that was not present in the father (Fig. 3 B). Further evaluation using comparative genomic hybridization (CGH) demonstrated an interstitial deletion encompassing the region from ~1,610,183 to 2,873,864 bp on one maternal-derived X chromosome in the patient (Fig. 3 C), her sister, and mother (not depicted). High-resolution single-nucleotide polymorphism (SNP) mapping further defined this small deletion as extending from 1,308,324 to 2,881,011 bp in one maternal X chromosome (Fig. 3 D) and in both siblings (Fig. S7). Importantly, this deletion encompassed the *CSF2RA* allele (Fig. 3 E), thereby explaining the unexpected *CSF2RA* DNA and mRNA sequence results.

These results demonstrate the inheritance of compound heterozygous abnormalities of *CSF2RA* in both siblings (Fig. 3 F). The G174R mutation present on the paternal X chromosome, localized to the extracellular cytokine binding domain of GM-CSF-R α , alters a potential site of glycosylation and impairs GM-CSF binding and receptor signaling. However, it is unclear if the functional impairment is caused by the change in primary amino acid sequence or loss of glycosylation.

Reproduction of the *CSF2RA*^{G174R} abnormality

To reproduce the GM-CSF receptor signaling defect identified in this family, plasmids expressing *CSF2RB* and either *CSF2RA*^{G174R} or *CSF2RA*^{WT} were cotransfected into 293 cells. Western blotting showed receptor proteins of the expected size from *CSF2RA*^{WT} and smaller molecular forms from *CSF2RA*^{G174R} (Fig. 4 A), which is similar to results for primary leukocytes (Fig. 2 B). Treatment of transduced cell lysates with Peptide: N-Glycosidase F, an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose oligosaccharides (3), resulted in a similar electrophoretic pattern for normal and G174R GM-CSF-R α . This confirmed the G174R mutation alters N-linked glycosylation (Fig. 4 A) by disrupting a functional N-linked glycosylation site and that glycosylation is responsible for the smaller molecular forms from this allele. GM-CSF-R β was expressed in *CSF2RB*-transfected cells but was undetectable in nontransfected cells (unpublished data). GM-CSF binding studies demonstrated that cells expressing *CSF2RA*^{WT} and *CSF2RB* rapidly bound GM-CSF and removed it from the media, whereas cells expressing *CSF2RA*^{G174R} and *CSF2RB* or *CSF2RB* alone

balanced hybridization to patient and control DNA are shown as clear boxes (A and D), whereas those showing an unbalanced hybridization representing sequences deleted in the patient are shown in black. High-resolution SNP analysis revealed that the deletion (red bar) extended from 1,308,324 to 2,881,011 bp. Sequence analysis (A) and PCR quantification of *CSF2RA* exon 7 (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>) demonstrated that the deletion included *CSF2RA* exon 7. The chromosomal location of the *CSF2RA* gene (1,347,701–1,388,827 bp) is indicated. (F) Pedigree of the family deduced from sequencing and *CSF2RA* allelic copy number determination experiments. The index case is indicated (arrow).

did not clear GM-CSF (Fig. 4 B). Cotransfection of *CSF2RB* together with *CSF2RA*^{WT}, but not with *CSF2RA*^{G174R}, resulted in expression of functional GM-CSF receptors as demonstrated by GM-CSF-dependent phosphorylation of STAT5 only with the former combination (Fig. 4 C). Incuba-

tion of *CSF2RA*^{G174R}/*CSF2RB*-transduced cells with higher concentrations of GM-CSF in the range of those observed in the index patient gave results similar to those obtained with primary leukocytes and clearly demonstrated a modest dose-dependent increase in STAT5 phosphorylation, which is consistent with a partial rescue of the signaling abnormality (Fig. 4 D).

Clinical relevance of the *CSF2RA*^{G174R} mutation

Our findings demonstrate that familial primary PAP in humans is caused by compound heterozygous abnormalities of *CSF2RA*, including loss of heterozygosity and a function-altering point mutation that severely reduces GM-CSF receptor signaling. These are the first results to demonstrate familial primary PAP in humans and that GM-CSF-R α is critical for surfactant homeostasis. They extend previous findings that PAP is caused by the absence of GM-CSF or GM-CSF-R β in mice (4–6) or is specifically associated with the presence of neutralizing GM-CSF autoantibodies or a reduction in alveolar macrophage numbers or function in humans (9, 10). The allelic frequency of these mutations has not been determined but may be rare given that autoantibodies against GM-CSF account for most individuals with PAP.

Both affected siblings had impaired pulmonary function and failure to thrive, supporting the conclusion that prolonged respiratory insufficiency in children retards growth. Important to this conclusion is the observation that Xp22.33 deletion did not include the *SHOX* (short stature homeobox) gene, the deletion of which impairs growth (28). Further, heterozygous loss of *CSF2RA* in the mother did not impair growth. Early results suggest that whole lung lavage therapy was followed by an increase in growth acceleration in the index patient.

This study highlights the usefulness of several novel biomarkers in the diagnostic evaluation of PAP. The combination of a negative GM-CSF autoantibody test (29) and an abnormal CD11b stimulation index (24) predicted the GM-CSF receptor dysfunction, which was confirmed by the lack of GM-CSF binding and defective GM-CSF receptor signaling. GM-CSF levels were increased in lung lavage from the patient and, in serum from the patient and her sister, compared with family members and healthy controls. Because primary PAP is presumed to be caused by a defect in GM-CSF regulation of alveolar macrophage surfactant catabolism (9, 10), an elevated serum GM-CSF level may be useful as a biomarker to distinguish primary PAP caused by GM-CSF autoantibodies (90% of all cases of PAP) (9, 10, 13, 30) from PAP caused by GM-CSF receptor dysfunction (this study; references 14–16) and PAP caused by GM-CSF deficiency (4, 5) (not yet reported in humans). Finally, an increased serum SP-D level predicted the occurrence of PAP in the sister, which was confirmed by subsequent clinical and radiographical evaluation.

The slow progression and differences in severity of the lung disease between affected siblings with the same *CSF2RA* mutations and GM-CSF-R α dysfunction indicates that penetrance is important in familial PAP because of GM-CSF receptor defects. The increased levels of GM-CSF likely reflect impaired clearance but are also consistent with interruption of a

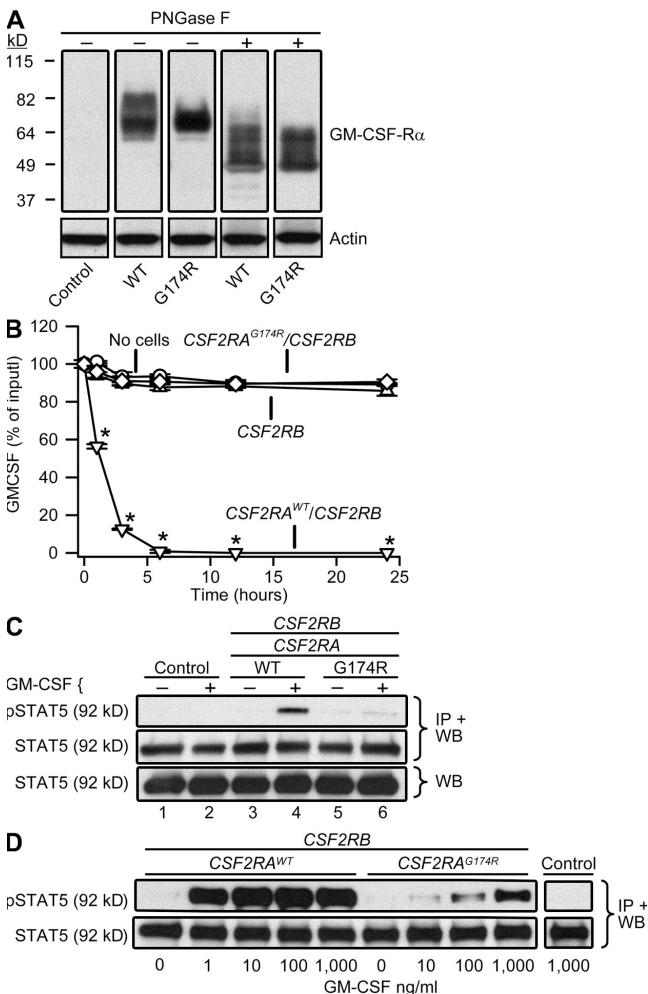


Figure 4. Reproduction of the *CSF2RA*^{G174R} gene defect. (A) Western blots of nontransfected 293 cell lysates (control) or lysates of cells transfected with plasmids expressing *CSF2RB* and either *CSF2RA*^{WT} (WT) or *CSF2RA*^{G174R} (G174R), followed by incubation with (+) or without (−) Peptide: N-Glycosidase F (PNGase F) and detection with antibodies against GM-CSF-R α or actin. (B) Evaluation of cell-mediated binding and removal of exogenous GM-CSF from the culture media. Asterisks indicate significant differences in levels of GM-CSF in media from cells expressing *CSF2RA*^{WT}/*CSF2RB* compared with cells expressing *CSF2RA*^{G174R}/*CSF2RB*, *CSF2RB* alone or media without cells. Error bars show the means \pm SE. (C) Evaluation of GM-CSF-stimulated phosphorylation of STAT5 in 293 cells transfected as described in A and incubated for 15 min in the absence (−) or presence (+) of 10 ng/ml GM-CSF. Lysates were evaluated by Western blotting (WB) to detect total STAT5 (STAT5) or were immunoprecipitated with anti-STAT5 antibody and then evaluated by Western blotting (IP + WB) to detect total STAT5 or phosphorylated STAT5 (pSTAT5). (D) Similar to C, except that increased concentrations of GM-CSF were used for evaluate GM-CSF receptor function.

negative-feedback regulatory loop. Results strongly suggest that the high levels of pulmonary GM-CSF present in epithelial lining fluid result in a reduced but not abrogated receptor signaling and provide a molecular explanation the slow progression of PAP in both affected siblings. It is currently unclear if the level of increased pulmonary GM-CSF correlates with disease severity. They also suggest that aerosolized delivery of GM-CSF to the lungs may be therapeutic because this approach could theoretically achieve pulmonary GM-CSF levels several thousand-fold higher than endogenous levels measured in the patient and 20-fold higher than levels that appeared to completely restore GM-CSF receptor signaling in the transfection experiments. BM transplantation and *CSF2RA* gene transfer represent other potentially therapeutic approaches.

The identification of mild PAP in the older sister provides a unique and important opportunity to observe PAP at a very early stage of disease development. Finally, this case exemplifies the utility of a collaborative approach to the diagnosis and treatment of individuals with very rare diseases involving geographically disparate clinical centers and the benefit derived from integrating basic science, clinical medicine, and translational research.

MATERIALS AND METHODS

Participants. The institutional review board of the Cincinnati Children's Hospital Medical Center approved the study, and all participants gave written informed consent or assent. Detailed case histories and laboratory data for the participants are included in the supplemental text (available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>).

GM-CSF receptor structure and function. GM-CSF receptors were evaluated by flow cytometry using antibodies recognizing GM-CSF-R α (BD) or β (Santa Cruz Biotechnology, Inc.) and by Western blotting (7) with chain-specific antibodies (Santa Cruz Biotechnology, Inc.). The effects of glycosylation on GM-CSF-R α were evaluated by incubating cell lysates with PNGase F (New England Biolabs, Inc.) (3). Actin was measured as a loading control using anti-actin antibodies (Santa Cruz Biotechnology, Inc.). To evaluate receptor-mediated clearance of GM-CSF, Ficoll-purified blood leukocytes or 293 cells (American Type Culture Collection) were cultured in DMEM containing 10% fetal bovine serum and human GM-CSF (Leukine; Bayer) at 1 ng/ml. At subsequent times, GM-CSF was measured in the culture medium by ELISA (R&D Systems) (24). To evaluate GM-CSF receptor signaling, heparinized blood was incubated with or without 10 ng/ml of human GM-CSF Leukine for 15 min. After lysing RBC, leukocytes were evaluated by Western blotting (24) using anti-human STAT5 (Santa Cruz Biotechnology, Inc.) and anti-phospho-STAT5 (Millipore) antibodies. The CD11b stimulation index was measured as previously described (24).

ELISA. SP-D, GM-CSF, MCP-1 (monocyte chemoattractant protein-1), M-CSF, and urea were measured using commercial ELISA kits (BioVendor, R&D Systems, BD, R&D Systems, and BioAssay Systems, respectively) (24). The concentration of cytokines in epithelial lining fluid of the lungs was estimated by multiplying the concentration in lavage fluid by the ratio of urea concentrations in serum and lavage fluid (26).

Genetic analysis. Genomic DNA or total mRNA was purified from blood leukocytes with commercially available kits (QIAGEN). The PCR was used to generate products spanning exons and flanking intronic sequences (genomic DNA) or the coding sequences and 5' and 3' flanking untranslated sequences (mRNA) of the *CSF2RA* and *CSF2RB* genes, which were then analyzed by direct sequencing. The resulting sequences were compared

with published sequences (from GenBank/EMBL/DDBJ under accession nos. NM_006140.3 and BC070085, respectively). The relative copy number of *CSF2RA* and *CSF2RB* genes among family members and a healthy control was measured by amplification of genomic DNA using PCR with gene-specific oligonucleotides followed by analysis of products by electrophoresis on 2% agarose gels. Experimental methods for measuring gene copy number, evaluating the genes encoding SP-B, SP-C, and ABCA3 (19, 31) for function-altering mutations, cytogenetic analysis, CGH analysis, FISH, and high-resolution SNP array analysis (32) are described in the supplemental Materials and methods (available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>).

Reproduction of the GM-CSF receptor defect. The methods used to clone and express mRNA transcripts encoding GM-CSF-R α and β chains in cultured cells and evaluate protein glycosylation are described in the supplemental Materials and methods.

Statistical analysis. Numeric data are presented as means \pm SE. Statistical comparisons were made with Student's *t* test for two-group comparisons and with one-way analysis of variance with posthoc analysis according to the Holm-Sidak method for multiple-group comparisons. P-values of <0.05 were considered to indicate statistical significance.

Online supplemental material. Fig. S1 shows normalized growth curves for the index patient and her sister. Fig. S2 shows GM-CSF binding and clearance by blood leukocytes from affected and unaffected family members and control. Fig. S3 shows increased pulmonary GM-CSF levels in GM-CSF receptor β chain (*Csf2rb*) KO mice. Fig. S4 shows evaluation of *CSF2RA* and *CSF2FB* gene copy number by PCR amplification. Fig. S5 shows karyotypes of high-resolution GTG-banded chromosomes. Fig. S6 shows evaluation of heterozygosity at the *CSF2RA* locus by FISH. Fig. S7 shows high-resolution SNP analysis. The supplemental text contains additional details regarding the case histories and clinical laboratory and other data cited throughout the text. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20080990/DC1>.

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