Suzuki et al., http://www.jem.org/cgi/content/full/jem.20080990/DC1

PARTICIPANTS

Patient. The index case was a 6-yr-old girl who was admitted to the hospital in July, 2007 when a chest radiograph revealed bilateral diffuse alveolar infiltrates. She was the product of a normal gestation and delivery and had reached developmental milestones as expected. At birth, she weighed 3.64 kg and was 19.5 cm in length (both at the 50th percentile); however, by 6–9 mo weight gain slowed and by 2–3 yr height acceleration slowed (Fig. S1 A). On admission, both her weight (13.6 kg) and height (103.5 cm) were below the third percentile for her age. She was noted by her mother to avoid swimming underwater at 3–4 yr of age, refusing to hold her breath. By 4–5 yr of age, she had developed dyspnea of insidious onset, first with exercise and then at rest, and finally was noted to "pant" in her sleep. There was no history of cough, fever, chest pain, lymphadenopathy, pneumonia or serious infection, pulmonary or other disease, environmental exposures, or drug use. There was no family history of lung disease in children or unexplained deaths at an early age. On examination, the respiratory rate was 60 per minute, blood pressure was 88/30 mm Hg, and pulse was 106. Pulmonary auscultation revealed mild crackles during deep inspiration, but the remainder of the exam was otherwise unremarkable.

The white blood cell count was 5.9×10^3/ml with normal cytometric indices, the RBC count was 4.9 million/mm^3, the hemoglobin was 13.9 g/deciliter, and the hematocrit was 41.1%. The levels of serum IgG, IgA, and IgM were mildly elevated (995, 150, and 181 g/deciliter, respectively) and IgE was 6.2 g/deciliter. Pulmonary function testing revealed a pattern of severe restriction with a forced vital capacity (FVC) that was 44% of that predicted, forced expiratory volume in one second (FEV1) 44% that of predicted, and a FEV1/FVC ratio 116% of that predicted. Oxygen saturation was 88% while breathing room air and dropped when talking or walking a short distance. A chest radiograph showed diffuse patchy alveolar infiltrates throughout both lung fields (Fig. 1 A, top). Bronchoscopic examination of the airways was unremarkable. Cytological examination of the bronchoalveolar lavage fluid demonstrated the presence of lipoproteinaceous material. A GMS stain was negative and cultures were negative for bacterial, fungal, or microbial pathogens. The lung lavage fluid from the patient contained increased levels of MCP-1 (25,085 pg/ml) and M-CSF (8,313 pg/ml) compared with lung lavage from five healthy controls (23 ± 8.2 and 35.0 ± 3.6 pg/ml, respectively). A high-resolution computed tomogram (HRCT) of the chest showed extensive ground glass opacification and increased interlobular thickening throughout both lung fields, suggesting the diagnosis of PAP (Fig. 1 A, bottom). Histopathological examination of lung parenchyma obtained by open lung biopsy showed alveolar filling with good preservation of alveolar wall integrity and the presence of focal areas of lymphocytosis (Fig. 2 B). Immunohistochemical staining demonstrated the presence of SP-A, mature SP-B, and SP-D within the endoalveolar space and pro–SP-C and ABCA3 within alveolar type II epithelial cells (Fig. 1 B). A serum GM-CSF antibody test was negative on two occasions. The urine did not contain detectable amino acids. No sequence deviations were detected in the coding exons and intron–exon boundaries of the genes encoding SPs B and C and ABCA3 (1, 2).

The patient was transferred to Cincinnati Children's Hospital in October, 2007 and underwent whole lung lavage therapy, which resulted in marked symptomatic improvement (Fig. 1 C). 4 mo later, the oxygen saturation was 98% on room air, and pulmonary function testing revealed an FVC that was 69% of that predicted, an FEV1 of 61% that predicted, and an FEV1/FVC ratio of 116% that predicted. The weight had increased to 16 kg and the height to 107.5 cm. Exercise tolerance had markedly improved and the patient resumed more normal daily activities.

Sister. The sister of the index patient was 8 yr old in February, 2008 when first evaluated after a negative GM-CSF autoantibody test and an abnormal CD11b stimulation index test suggested that an inherited GM-CSF receptor defect may be present in the index case (3). She was the product of a normal gestation and delivery and had reached developmental milestones as expected. At birth, her weight and length were 3.64 kg and 20 cm (both at the 50th percentile) and at the time of evaluation were 22.7 kg and 122 cm, respectively (both at the 10th percentile for age; Fig. S1B). She had been considered by her family to have been healthy all her life with no history of lung disease or major illnesses. On examination, the respiratory rate was 32 per minute, the pulse was 82 per minute, and blood pressure was 104/62 mm Hg and was otherwise unremarkable. Pulmonary function testing demonstrated an FVC that was 88% of that predicted, FEV1 that was 90% of that predicted, a FEV1/FVC ratio that was 92% of that predicted, and a diffusion capacity for carbon dioxide (DLCO) that was 57% of that predicted. Oxygen saturation was 97% while breathing room air. An HRCT of the chest revealed mild patchy ground glass opacities throughout both lungs, which is consistent with the diagnosis of PAP (Fig. 1 E). The severe reduction in DLCO and failure to thrive suggest that the lung abnormalities are more extensive than indicated by the chest HRCT; i.e., scattered ground glass opacifications involving geographically distributed secondary pulmonary lobules. Rather, these results suggest that a thickened alveolar surfactant layer may be present throughout the alveolar surface but is not well visualized by the radiographical technique used.

Father. The father of the index patient was 49 yr old at the time of evaluation in February, 2008. His height was 185.4 cm (85th percentile for his age). He was a lifelong nonsmoker with no history of pulmonary or other major medical illness.

Mother. The mother of the index patient was 48 yr old at the time of evaluation in February, 2008. Her height was 165 cm

(65th percentile for her age). She was a lifelong nonsmoker with no history of pulmonary or other major medical illness.

Patients with autoimmune PAP. 12 individuals with autoimmune PAP are reported as disease controls, all of whom had the typical clinical, physiological, and radiographical features of the disease at the time of evaluation. The detailed case histories and diagnostic criteria of each have been reported previously (3). The group included three pediatric and nine adult patients. The mean ± SE GM-CSF autoantibody titer was 324.3 ± 66 μg/ml and was similar in adult and pediatric cases (365 ± 74 and 202 ± 141 μg/ml, respectively; P = 0.308).

Healthy controls. Volunteers were enrolled in the study as healthy controls. This control group included 67 individuals, 61 of whom were previously reported as part of another study (3). The mean ± SE age was 30 ± 6 yr. All were disease-free healthy individuals without a history of major illness and all were symptom-free at the time of enrollment in the study. None were current smokers.

MATERIALS AND METHODS

Lung histopathology and immunostaining

A formalin-fixed paraffin-embedded open lung biopsy tissue sample was received for evaluation. 5-μm-thick sections were cut on a rotary microtome and loaded onto polylysine-coated slides. Hematoxylin and eosin–stained sections were prepared using routine methods. Immunohistochemistry was performed as described previously (4, 5) using rabbit polyclonal antisera raised against SP-A, SP-B, pro–SP-C, and SP-D, a mouse monoclonal antibody generated to ABCA3 as primary antibodies and biotinylated secondary antibodies (Vector Laboratories), and an avidin-biotin–horseradish peroxidase detection system (ABC reagent; Vector Laboratories). The enzymatic reaction product was enhanced using Ni-DAB and Tris cobalt to give a black precipitate, and the sections were counterstained with Nuclear Fast Red.

GM-CSF clearance assay

To evaluate receptor-mediated binding of GM-CSF, heparinized blood was obtained by phlebotomy and mononuclear cells were isolated on Ficoll gradients, followed by RBC lysis as previously described (3). Primary mononuclear cells or transfected 293 cells (see Construction of vectors and transfection) were seeded into culture dishes at 1 × 106 cells per dish in DMEM containing 10% bovine calf serum. Human GM-CSF (Leukine; Bayer) was added at a concentration of 1 ng/ml. At subsequent times, GM-CSF was measured in aliquots of the culture medium by ELISA (R&D Systems). The results were expressed as a percentage of initial GM-CSF concentration.

Nucleotide sequence analysis

The nucleotide sequence of *CSF2RA* and *CSF2RB* transcripts and genomic DNA were determined by PCR-based methods in the Genetic Variation and Gene Discovery Core Facility at the Cincinnati Children's Hospital Medical Center. Genomic DNA was also evaluated for the presence of known function-altering mutations in the coding sequences and intron–exon boundaries of the genes encoding SP-B, SP-C, and ABCA3 as previously described (1, 2).

Measurement of gene copy number by PCR amplification

The relative copy number of *CSF2RA* and *CSF2RB* genes among family members and controls was determined using genomic DNA using PCR with the following gene-specific primers: 5′-AGGAG AAGGAGGGAGATCCG-3′, 5′-CACGTG-GCCTCAGTTACACAG-3′ (*CSF2RA*); 5′-ACAG AGCCAGGCATGTGGT-3′, and 5′- CGACAAAACTCTG-GCAGGG-3′ (*CSF2RB*). Amplification conditions were as follows: 94°C for 5 min; 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s (30 cycles); and 72°C for 7 min. PCR-generated products were then evaluated by electrophoresis on 2% agarose gels.

Cytogenetics

Chromosome analysis was performed on cells isolated from peripheral blood according to standard cytogenetic techniques.

Comparative genomic hybridization

Comparative genomic hybridization analysis was performed with genomic DNA isolated from each family member using the SignatureSelect V2 microarray chips (Signature Genomic Laboratories, LLC) and a fluorescence dye reversal assay (6). Microarray chips were processed according to the manufacturer's protocol. Genomic DNA were evaluated with a same-sex reference DNA (patient) or an opposite sex reference DNA (mother and sister) and data analysis was performed using the Genoglyphix software (Signature Genomic Laboratories, LLC).

Fluorescence in situ hybridization

FISH analysis was performed under routine clinical laboratory protocols with the CTD-3047L21 hybridization probe, which identifies the pseudoautosomal region of X and Y chromosomes, Xp22.33 and Yp11.32, respectively. Slides were analyzed with a fluorescent microscope (Carl Zeiss, Inc.) with CytoVision v3.7 software (Applied Imaging). 8–10 metaphases and 25 interphase cells were evaluated in each FISH study.

High-resolution single-nucleotide polymorphism analysis

Blood was obtained from the patient, her sister, and parents, and genomic DNA was isolated using a commercial kit (QIAGEN) according to the manufacturer's instructions. Samples of genomic DNA (750 ng) from each subject were prepared for microarray analysis using the Infinium Assay (Illumina Inc.) according to the manufacturer's protocol. Microarray analysis was performed using the HumanCNV370-duo DNA Analysis BeadChip platform (Illumina Inc.). This chip contains ~370,404 markers, including SNP and copy number variation content. Data were analyzed using the BeadStudio v3.1 analysis software (Illumina Inc.).

Construction of vectors and transfection

PBMC RNA from the index patient and a healthy control was purified and converted to cDNA as described in the Ma-

terials and methods section Genetic analysis. The nucleotide sequence for the entire protein coding sequence of *CSF2RA* was generated from cDNA transcripts by PCR amplification using gene-specific primers and subcloned into pSC-A (Agilent Technologies). After confirming the nucleotide sequence, a restriction fragment containing the entire coding region of *CSF2RA* from the control (*CSF2RA*WT) or the index patient (*CSF2RA*G174R) were inserted individually into a plasmid (MIEG3) permitting expression in mammalian cells (7). The cDNA encoding *CSF2RB* was obtained (IART98-E2; Geneservice) and inserted into another plasmid (MSCV2.1) permitting expression in mammalian cells (8). Expression plasmids carrying *CSF2RB*WT and either *CSF2RA*G174R or *CSF2RA*WT were cotransfected into human embryonic kidney epithelial cells (HEK 293 cells; American Type Culture Collection) using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. After 48 h, cells were evaluated for (1) expression of GM-CSF receptor proteins by Western blotting (2), GM-CSF binding, and clearance by culturing with exogenous GM-CSF (see GM-CSF clearance assay) or (3) GM-CSF receptor signaling by evaluation of GM-CSF–dependent phosphorylation of STAT5 (see STAT5).

Western blotting

GM-CSF-Rα.

GM-CSF-Rα was evaluated in blood leukocytes or transfected cells as follows. Leukocytes from 1 ml of blood (evaluated within 1 h of phlebotomy and subjected to RBC lysis) or 1×10^6 transfected 293 cells were lysed in 200 μl RIPA buffer (0.05 M Tris-HCl, pH 8, 0.15 M NaCl [Tris-buffered saline (TBS)], 1% vol/vol nonidet P-40, 0.5% wt/vol sodium deoxycholate, 0.1% wt/vol SDS, 0.004% wt/vol sodium azide) containing 2% vol/vol proteinase inhibitor cocktail, 1% vol/vol phenyl-methyl-sulfonyl-fluoride (PMSF), and 1% vol/volsodium orthovanadate (Santa Cruz Biotechnology, Inc.) as directed by the manufacturer. Samples were kept on ice for 30 min and then centrifuged (9000 *g* at 4°C for 15 min) to remove insoluble debris. Samples were mixed with equal volume of sample buffer and boiled. The lysate was then fractionated on SDS-PAGE gels (4–12% Tris Glycine gel; Invitrogen) under reducing conditions and proteins were transferred to PVDF membranes by electroblotting. Membranes were incubated in blotting solution (TBS, 5% [wt/vol] dry milk, and 0.1% [vol/vol] Tween 20) at 4°C, overnight to block nonspecific binding. Mouse antihuman GM-CSF-Rα antibody (diluted 1:500; Santa Cruz Biotechnology, Inc.) was then added and incubations were continued at room temperature for 2 h. After washing in TBS and 0.1% (vol/vol) Tween 20, membranes were incubated at room temperature for 1 h in blotting solution containing donkey anti–rabbit IgG. After washing in blotting solution, membranes were incubated with ECL-Plus (GE Healthcare) to visualize immunostained proteins as directed by the manufacturer. This procedure was used for measuring actin in the same samples with anti-Actin antibody (diluted 1:1,000; Santa Cruz Biotechnology, Inc.).

STAT5.

GM-CSF receptor signaling was evaluated in blood leukocytes or transfected cells as described for GM-CSF-Rα with the following modifications. 1 ml of blood (evaluated within 1 h of phlebotomy) or 1×10^6 transfected 293 cells (in DMEM containing 10% bovine calf serum) were incubated in the absence or presence of human GM-CSF (Leukine; 1, 10, 100, and 1,000 ng/ml) for 15 min at 37°C. After RBC lysis (whole blood) and washing with PBS (both leukocytes and 293 cells), cell lysates were prepared and evaluated as in GM-CSF-Rα, except that for transfected 293 cell lysates and cells exposed to high GM-CSF concentrations, immunoprecipitation was performed at 4°C overnight using ProteinA/G Agarose (Santa Cruz Biotechnology, Inc.) and anti-STAT5 antibody or control rabbit IgG (Santa Cruz Biotechnology, Inc.). Anti-STAT5 antibody (diluted 1:500; Santa Cruz Biotechnology, Inc.) or anti–phospho-STAT5 (diluted 1:500; Millipore) were used for primary antibodies for Western blotting.

Evaluation of CSF2RA glycosylation

Expression plasmids carrying either *CSF2RA*G174R or *CSF2RA*WT were transfected into 1×10^6 HEK 293 cells as described in Construction of vectors and transfection. After 48 h, cells were lysed in 200 μl RIPA buffer containing proteinase inhibitor cocktail, PMSF, sodium orthovanadate (Santa Cruz Biotechnology, Inc.). Samples were kept on ice for 30 min and then centrifuged at 9,000 *g* at 4°C for 15 min to remove insoluble debris. Nine μl of lysate was treated with PNGase F (New England Biolabs, Inc.) at 37°C for 30 min according to the manufacturer's protocol and then evaluated by Western blotting as in GM-CS-Rα.

Measurement of cytokine concentration in lung epithelial lining fluid recovered by bronchoalveolar lavage

The volume of epithelial lining fluid (ELF) recovered in lung lavage fluid was estimated using the urea dilution method described by Rennard et al. (9). In brief, the concentration of urea in the lung lavage fluid and serum were measured by ELISA. The volume of ELF (in milliliters) was calculated as the volume of lavage fluid in milliliters multiplied by the concentration of urea in lavage fluid in milligrams per milliliters divided by the concentration of urea in plasma in milligrams per milliliters. The concentration of cytokines in ELF was estimated by multiplying their concentration in lavage fluid (measured by ELISA) by the ratio of the lavage fluid in milliliters to ELF volume in milliliters.

REFERENCES

1. Nogee, L.M. 2004. Alterations in SP-B and SP-C expression in neonatal lung disease. *Annu. Rev. Physiol.* 66:601–623.
2. Shulenin, S., L.M. Nogee, T. Annilo, S.E. Wert, J.A. Whitsett, and M. Dean. 2004. ABCA3 gene mutations in newborns with fatal surfactant deficiency. *N. Engl. J. Med.* 350:1296–1303.
3. Uchida, K., D.C. Beck, T. Yamamoto, P.Y. Berclaz, S. Abe, M.K. Staudt, B.C. Carey, M.D. Filippi, S.E. Wert, L.A. Denson, et al. 2007. GM-CSF autoantibodies and neutrophil dysfunction in pulmonary alveolar proteinosis. *N. Engl. J. Med.* 356:567–579.

4. Nogee, L.M., S.E. Wert, S.A. Proffit, W.M. Hull, and J.A. Whitsett. 2000. Allelic heterogeneity in hereditary surfactant protein B (SP-B) deficiency. *Am. J. Respir. Crit. Care Med.* 161:973–981.
5. Stahlman, M.T., V. Besnard, S.E. Wert, T.E. Weaver, S. Dingle, Y. Xu, K. von Zychlin, S.J. Olson, and J.A. Whitsett. 2007. Expression of ABCA3 in developing lung and other tissues. *J. Histochem. Cytochem.* 55:71–83.
6. Bejjani, B.A., and L.G. Shaffer. 2006. Application of array-based comparative genomic hybridization to clinical diagnostics. *J. Mol. Diagn.* 8:528–533.
7. Williams, D.A., W. Tao, F. Yang, C. Kim, Y. Gu, P. Mansfield, J.E. Levine, B. Petryniak, C.W. Derrow, C. Harris, et al. 2000. Dominant negative mutation of the hematopoieticspecific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood.* 96:1646–1654.
8. Hansen, W.K., W.A. Deutsch, A. Yacoub, Y. Xu, D.A. Williams, and M.R. Kelley. 1998. Creation of a fully functional human chimeric DNA repair protein. Combining O6- methylguanine DNA methyltransferase (MGMT) and AP endonuclease (APE/redox effector factor 1 (Ref 1)) DNA repair proteins. *J. Biol. Chem.* 273:756–762.
9. Rennard, S.I., G. Basset, D. Lecossier, K.M. O'Donnell, P. Pinkston, P.G. Martin, and R.G. Crystal. 1986. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *J. Appl. Physiol.* 60:532–538.