Out of breath: GM-CSFRα mutations disrupt surfactant homeostasis

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Pulmonary alveolar proteinosis (PAP) is a rare disorder in which surfactant homeostasis in the lung is impaired, causing respiratory distress and, in severe cases, respiratory failure. Most cases of PAP are associated with the formation of autoantibodies against the cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF), which is required for normal surfactant homeostasis and lung function. New studies now identify three patients in whom PAP was caused by mutations in the gene encoding the ligand-binding α chain of the GM-CSF receptor.

The ability to breathe depends on oxygen exchange in the alveoli of the lung. Surfactant, which is composed of phospholipids and surfactant proteins (SPs), is essential to reduce surface tension at the air–fluid interface, thereby preventing pulmonary collapse and facilitating oxygen exchange. Both SPs and surfactant lipids are produced by alveolar type II epithelial cells. Hydrophobic SP-B and SP-C are assembled, along with phospholipids, into intracellular lamellar bodies, whereas hydrophilic SP-A and SP-D are released through secretory vesicles. Breathing triggers the release of the lamellar body contents into a thin aqueous layer covering the alveoli, with the polar heads of the phospholipids facing the fluid, and their hydrophobic tails facing the air. Surfactant homeostasis is maintained by the uptake and recycling of surfactant aggregates by alveolar type II epithelial cells, and by the internalization and intracellular catabolism by alveolar macrophages. This homeostatic turnover is critical, as accumulation of surfactant can impair oxygen uptake by the lung.

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GM-CSF has emerged as an essential factor in maintaining alveolar homeostasis by promoting surfactant catabolism by alveolar macrophages, such that appropriate levels of surfactant are maintained in the alveolar space. GM-CSF, also known as colony-stimulating factor 2 (CSF2), is produced by T cells and macrophages after activation, and can also be produced by other cell types (such as endothelial cells and fibroblasts) after stimulation with TNF-α, interleukin (IL)-1, IL-2, and interferon (IFN)-γ. GM-CSF was originally identified by its ability to induce proliferation and differentiation of myeloid progenitor cells in vitro, but subsequent studies have shown that it can modulate the function of many cell types, including mature monocytes, neutrophils, eosinophils, basophils, DCs, invariant natural killer T (iNKT) cells, endothelial cells, and neuronal cells. GM-CSF also regulates placental function (for a recent review on GM-CSF see [1]). All GM-CSF–responsive cells express the heterodimeric GM-CSF receptor (GM-CSFR), which is composed of the ligand-binding α chain (encoded by the *CSF2RA* gene), and the signal-transducing β chain (encoded by the *CSF2RB* gene); the latter is also shared by the IL-3 and -5 receptors. Surprisingly, disruption of *csf2* (encoding GM-CSF) or *csf2rb* in mice does not impair steady-state hematopoiesis, but triggers a progressive PAP-like lung disease with accumulation of surfactant lipids and proteins in the alveolar space (2, 3); *csf2ra*-deficient mice have not yet been developed. In this issue, two groups of investigators independently report that mutations in the *CSF2RA* gene in humans result in PAP (4, 5).

The biology of PAP

PAP comprises a heterogeneous group of rare disorders in which surfactant lipids and proteins, lymphocytes, and large, foamy alveolar macrophages accumulate within the alveoli, often leading to reduced oxygen uptake (ventilation-perfusion disturbance) and severe restrictive lung disease (6) PAP is usually secondary to the formation of anti–GM-CSF neutralizing autoantibodies (7), but can also be associated with mutations in the genes encoding SP-B or SP-C (8), as well as a variety of conditions that affect the function of alveolar macrophages, including hematologic malignancies, autoimmunity, infections, inhalation of silica or other toxic material, and the use of immunosuppressive drugs (6).

Observations in humans and mice support a critical role for perturbed GM-CSFR signaling in the pathophysiology of PAP. In mice, ablation of *csf2* or *csf2rb* resulted in intra-alveolar accumulation of lipoproteinaceous material and foamy macrophages (2, 3). Neither deletion affected the synthesis and secretion of SPs, but both severely perturbed surfactant catabolism (9). Expressing GM-CSF in the lungs (10) or administering recombinant GM-CSF (11) rescued the disease phenotype of $csf2^{-/-}$ mice, whereas bone marrow transplantation

and retrovirus-mediated *csf2rb* gene transfer into hematopoietic stem cells corrected the defect in *csf2rb*$^{-/-}$ mice (3, 12). These studies demonstrate a primary role for alveolar macrophages in GM-CSF–dependent surfactant homeostasis in mice.

In this issue, Suzuki et al. (p. 2703) and Martinez-Moczygemba et al. (p. 2711) describe *CSF2RA* mutations in three patients with primary PAP (4, 5). These studies, along with the previous observations that the majority of patients with PAP have anti–GM-CSF autoantibodies (6), and that abnormalities of GM-CSFRβ may account for some rare congenital forms of the disease (13), unequivocally demonstrate a prominent role for GM-CSF in lung homeostasis in humans.

These new studies raise several interesting questions. From a genetic standpoint, the complicated mechanisms leading to PAP in the affected patients are noteworthy. Unlike the murine *csf2ra* gene, which maps in the telomeric region of mouse chromosome 19, the human *CSF2RA* gene is located in the X-Y pseudoautosomal region (PAR1) (14). This region, comprising ~2.6 Mb of homologous sequence between the X and the Y chromosomes, is essential for a single obligatory recombination event to occur during male meiosis. Genes contained in the PAR1 region of the X chromosome, including *CSF2RA*, escape the phenomenon of X chromosome inactivation. Accordingly, genetic defects in these genes might result in a disease phenotype because of haploinsufficiency or through complex mechanisms that also inactivate the other allele. All three patients reported by Suzuki et al. and Martinez-Moczygemba et al. were females whose genetic defects affected both X chromosomes.

The patient described by Martinez-Moczygemba et al. developed severe respiratory distress and failure to thrive early in life, as well as symptoms associated with Turner syndrome (4). She had a 46,X,i(Xq) karyotype (one copy of the X chromosome was normal and the second had a deletion of the short arm and a duplication of the long arm), in which the apparently normal X chromosome was found to carry an internal deletion in the PAR1 region encompassing exons 5–13 of the *CSF2RA* gene, which prevented the expression of GM-CSFRα on the surface of circulating monocytes. The patient's father and sibling, who were reportedly healthy, had two populations of monocytes, one that expressed GM-CSFRα and one that did not. This observation is difficult to reconcile with the notion that the *CSF2RA* gene escapes X inactivation, as this would predict that all monocytes from individuals heterozygous for null defects should still be able to express the protein through the unaffected allele (although possibly at reduced levels). However, recent data indicate that the levels of GM-CSFRα expression in circulating monocytes may vary substantially—for as yet unknown reasons—even among healthy subjects (15). It is thus possible that the two monocyte populations in the patient's father and sibling represented monocytic cells at different stages of differentiation or activation.

The two siblings reported by Suzuki et al., in contrast, had a normal female 46, XX karyotype, and their monocytes were found to express GM-CSFRα (5). Using a combination of molecular and cytogenetic studies, the authors showed that the maternally derived X chromosome carried a 1.6-Mb deletion in PAR1 that encompassed the *CSF2RA* locus, whereas the paternal *CSF2RA* allele carried a single nucleotide substitution that resulted in an amino acid change (G174R) that abolished an N-glycosylation site. In both articles, functional assays confirmed that the *CSF2RA* genetic lesions disrupted responses to GM-CSF.

Mechanics of GM-CSF signaling

Recent studies have led to the unexpected observation that the high-affinity GM-CSFR complex assumes a hexameric stoichiometry, with a 2:2:2 ratio between GM-CSF and the α and β receptor chains, and that the assembly of two hexamers into a dodecamer is required for receptor activation and signaling (16). This conformation brings two GM-CSFR β chains into close proximity, so that the β chain–bound JAK2 molecules can cross-phosphorylate each other, as well as the β chain itself (Fig. 1). These phosphorylation events trigger the recruitment and phosphorylation of STAT5. Eventually, several GM-CSF target genes are activated, including the gene encoding the transcription factor PU.1, which controls terminal maturation of alveolar macrophages by up-regulating the expression of CD32, mannose receptor, and macrophage CSF receptor (M-CSFR). Accordingly, the expression of PU.1 and of PU.1-dependent proteins is markedly reduced in *csf2*$^{-/-}$ mice, resulting in reduced macrophage adhesion, phagocytosis, and microbicidal activity (17). Similar observations have been reported in alveolar macrophages from patients with PAP (18). Although the expression profile of differentiation markers on neutrophils from patients with autoantibody-mediated PAP is normal, these cells show impaired function in vitro after GM-CSF priming (19).

Leukocytes from the three patients reported by Suzuki et al. and Martinez-Moczygemba et al. failed to up-regulate CD11b in response to GM-CSF. Interestingly, the two siblings in the Suzuki et al. study, who carried the G174R mutation, showed markedly reduced GM-CSF–stimulated STAT5 phosphorylation, which was partially rescued by concentrations of GM-CSF within the range detected in vivo in patients. It is noteworthy that the disease presentation was delayed in these two patients, and one was even thought to be unaffected before this study was performed. These findings raise the possibility that hypomorphic mutations in *CSF2RA* may result in milder clinical forms of the disease and that, in these cases, boosting local concentrations of GM-CSF via aerosol administration might be beneficial. The delayed clinical presentation and milder phenotype observed in the siblings could also be explained by an alternative hypothesis (4). It has been observed that two residues (S585 and Y577) in the GM-CSFR β chain act as a binary switch that triggers distinct downstream events. Low concentrations of the cytokine trigger S585 phosphorylation, resulting in activation of PI3-kinase and cell survival, whereas high concentrations favor phosphorylation of Y577 and JAK2 activation, resulting in

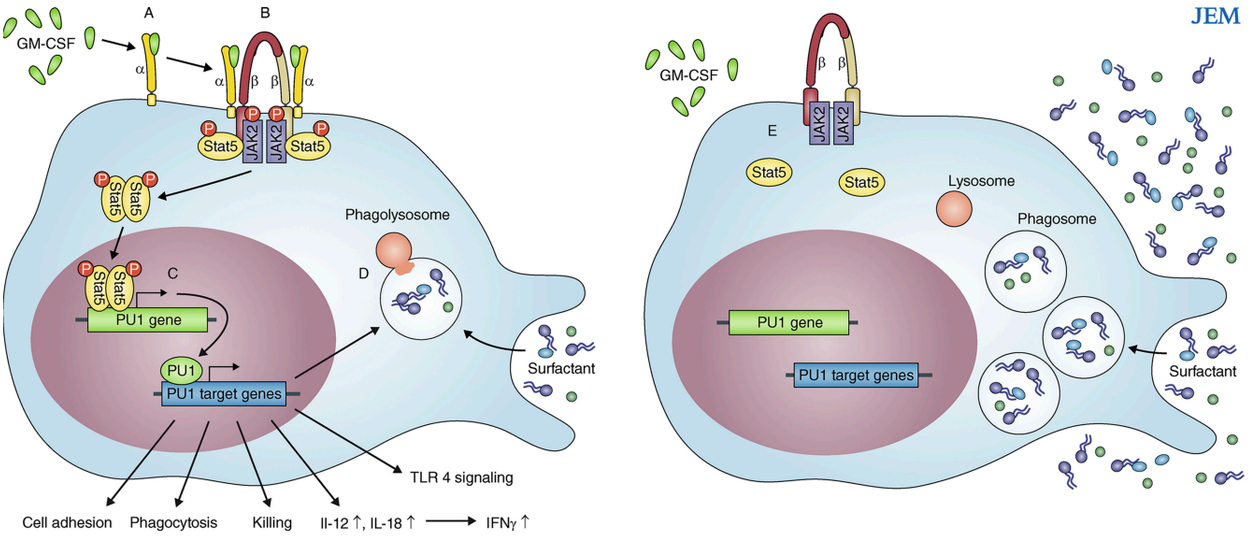

Figure 1. Mutations of GM-CSFRα disrupt alveolar macrophage functions and surfactant homeostasis. (top) (A) GM-CSF binds to GM-CSF receptor α (GM-CSFRα) to form a low-affinity complex, which then interacts with the β complex (two β chains constitutively bound to JAK2) to form the high-affinity hexameric receptor complex (B). Lateral movement and aggregation of two such complexes form the active dodecamer signaling receptor (not depicted), allowing cross-phosphorylation of the JAK2 molecules and the β subunit. Activated JAK2 phosphorylates the transcription factors STAT5, which then dimerizes and migrates to the nucleus where it induces the expression of the PU.1 transcription factor gene (C). This results in the expression of various PU.1-dependent genes and subsequent activation of multiple phagocytic functions, such as adhesion, phagocytosis, killing, signaling through TLR4, and the release of IL-12 and -18, which in turn induce release of IFN-γ by T and NK cells. This pathway also induces surfactant catabolism (D), although the mechanistic details of this pathway remain to be defined. (bottom) GM-CSF signaling is abrogated in phagocytes from patients with primary PAP who fail to express functional GM-CSFRα molecules (E). In these conditions, surfactant is still taken up by the alveolar macrophages, but is not properly catabolized, leading to progressive surfactant accumulation within the phagocyte (producing the typical foamy appearing alveolar macrophages) and in the alveolar space.

cell proliferation, differentiation and activation (20). In the presence of high concentrations of the ligand, it is possible that the G174R mutant α chain could bind GM-CSF with sufficient avidity to promote phosphorylation of the β chain tyrosine residue, and thus enable partial alveolar macrophage function. Furthermore, PU.1 and C/EBP proteins cooperate to induce GM-CSFR β chain expression (21), creating a positive-feedback loop that may also help promote GM-CSF–mediated intracellular signaling in patients with hypomorphic mutations in GM-CSFR components.

On the other hand, functional studies performed in the siblings with the G174R α chain mutation showed clear abnormalities in GM-CSF–mediated signaling. In light of the multimolecular complex of the high-affinity GM-CSFR (16), one might expect that the G174R mutant might interfere with GM-CSF–mediated binding and signaling if expressed at an equimolecular ratio with the wild-type α chain. If so, a mild PAP phenotype might result from heterozygous mutations not because of haploinsufficiency, but rather because of a dominant-negative effect. Although in vitro transfection studies may help solve this issue, it is important to note that the father of the two patients reported by Suzuki et al. carries the G174R mutation and is apparently asymptomatic (5). Although his serum levels of SP-D were in the upper normal range, his leukocytes showed normal uptake of GM-CSF and normal GM-CSF–induced STAT5 phosphorylation. Thus, further studies are needed to determine if heterozygous mutations in the *CSF2RA* gene may lead to a disease phenotype.

The genetic defect in the patient described by Martinez-Moczygemba et al. resulted not only in loss of *CSF2RA*, but also in the complete loss of the *IL3RA* gene (4), which maps next to *CSF2RA* in the PAR1 region and encodes for the IL-3R α chain. In mice, ablation of the *il3* gene does not affect steady-state hematopoiesis or cause lung disease, but reduces mast cell function in response to parasitic infections (22). The course of the disease in the patient with complete loss of *CSF2RA* and *IL3RA* expression was too rapid to ascertain whether the loss of IL-3–mediated signaling might have had any clinical consequence.

Beyond surfactant

The demonstration of *CSF2RA* mutations in patients with primary PAP raises important questions regarding the role of this signaling pathway beyond surfactant homeostasis. Opportunistic infections have been documented in >10% of PAP patients, and uncontrolled infections are the second most important cause of death after respiratory insufficiency (23). Increased susceptibility to infections with *Pneumocystis jiroveci*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, adenovirus, and group B streptococcus has been reported in *csf2*$^{-/-}$

mice (24). This increased susceptibility reflects severe defects in the GM-CSF–dependent, PU.1-induced expression of IL-18 and IL-12, cytokines that are needed to prime T and NK lymphocytes to secrete IFN-γ (25). GM-CSF also regulates the macrophage response to bacterial lipopolysaccharide through PU.1-dependent activation of the TLR4 pathway (26). Finally, GM-CSF promotes neutrophil phagocytosis, adhesion, respiratory burst, and bactericidal activity (19). Analysis of additional patients with genetic defects in GM-CSF–mediated signaling is needed to determine whether increased susceptibility to infections is an important feature of PAP. If so, therapeutic measures based on whole-lung lavage (the mainstay treatment for PAP) and aerosol-mediated administration of GM-CSF (in patients with hypomorphic mutations of the GM-CSFR components) may not be sufficient to prevent long-term complications, and additional or more aggressive forms of treatment may be needed. These may include antimicrobial prophylaxis or possibly hematopoietic cell transplantation, which was attempted without success in the patient described by Martinez-Moczygemba et al. (4). In this context, however, it is interesting to note that irradiation or use of radiomimetic drugs induces the production of GM-CSF by stromal cells, and that lethally irradiated *csf2rb*$^{-/-}$ mice showed poor survival after transplantation with purified wild-type hematopoietic stem cells, indicating an essential role for GM-CSF signaling in nonhematopoietic cells (27).

It remains to be determined whether the increased susceptibility to pulmonary infections observed in *csf2*$^{-/-}$ mice and in patients with PAP reflects an intrinsic functional defect of alveolar macrophages, with a possible role for these cells in the associated lung damage, or whether disease is primarily caused by a generalized impairment of macrophage and neutrophil function. Although the former hypothesis is supported by evidence that the disease phenotype in *csf2*$^{-/-}$ mice can be rescued by inducing expression of PU.1 in alveolar macrophages (17), several in vitro studies have provided evidence for a generalized impairment of the ability of macrophages and neutrophils to mediate antimicrobial functions (for review see [6]).

Overall, the two independent observations of *CSF2RA* mutations leading to abnormal GM-CSFRα chain expression and function further support the importance of GM-CSF–mediated signaling in surfactant homeostasis, and the phenotypic differences observed by the two groups demonstrate the complexity of this process. These novel clinical findings will hopefully prompt further basic and clinical studies that will lead to a better understanding not only of PAP and its consequences but also of other pathological and physiological processes involving the GM-CSF–surfactant axis.

Importantly, the observation that PAP may be inherited as a Mendelian trait, and that mutations of the *CSF2RA* gene result in a lung-specific phenotype, make PAP an unusual presentation of a primary immune deficiency disorder (PID), and thus further broadens the clinical definition of PIDs (28). Finally, the fact that PAP may be caused either by an autoimmune process (with production of anti–GM-CSF antibodies) or by abnormalities of the *CSF2RA* gene provides yet another example of how PIDs may be phenocopied by autoimmune processes that abrogate a specific immune pathway. This observation is similar to reports in patients with increased susceptibility to mycobacterial diseases resulting from genetic defects in the IL-12/IL-23–IFN-γ pathway (29) or the production of autoantibodies to IFN-γ (30–32).

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