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Reply to D'Alessandro-Gabazza et al.

From the Authors:

We are most grateful for the letter provided by D'Alessandro-Gabazza and colleagues, in which they noted that our work represents a first step in the further development of a novel therapy in idiopathic pulmonary fibrosis (IPF), but they also expressed concerns around the targeting of TAM (Tyro3, Axl, Mer) receptors in this disease. The concerns expressed are well warranted; we share these concerns with the authors and are currently addressing them in our laboratory. Given the importance of alveolar epithelial health in the regeneration of the lung, we have been actively determining the effects of TAM receptors on epithelial cells in human lung tissues. Immunohistochemical analysis showed active Axl immunostaining by epithelial-like cells lining the honeycomb and adjacent to fibroblastic foci in IPF lung samples (1). However, this analysis did not specifically identify what epithelial subtype(s) are expressing this receptor. Mining of data produced by transcriptomic analysis of single epithelial cell RNA-Seq data sets (2–4) revealed that Axl was the only TAM receptor that was highly expressed (i.e., average transcripts per kilobase million expression) by human indeterminate, basal, and club/goblet epithelial cells (Figure 1, top left). However, these epithelial cell types rarely expressed Mertk (Figure 1, top right), Tyro3 (Figure 1, bottom left), and Gas6 (Figure 1, bottom right) transcripts. In addition, alveolar type 2 (AT2) cells express markedly lower Axl transcript compared with the other lung epithelial cell types, and Gas6, Tyro3, and Mertk transcripts were rarely detected in these cells (Figure 1). Our preliminary and unpublished single-cell RNA-Seq analysis of cultured-expanded IPF KRT5⁺ basal and SCGB1A1⁺ club epithelial cells supported

the findings from Xu and colleagues' data sets (2–4) and showed that KRT5⁺ basal cells were positive for Axl and Gas6 and infrequently expressed Tyro3 and Mertk (data not shown). To further assess any potential deleterious effects of targeting TAM receptors on AT2 cells, we performed comparative transcriptomic analysis for *sftpc* (surfactant protein C, a marker for these cells) transcript expression in vehicle- and R428-treated humanized severe combined immunodeficiency (SCID)/Bg mouse lungs and observed that R428 had no effect on *sftpc* transcript expression (Figure 2). Together, these data suggest that other lung epithelial cell types and not AT2 cells consistently express Gas6 and TAM receptors, and that targeting TAM receptors with R428 in humanized SCID/Bg mice did not modulate the transcript levels of the AT2 cell-specific marker *sftpc*. However, given potential differences between transcript and protein expression, future work is warranted to confirm the expression and potential physiological role(s) of TAM receptors on alveolar epithelial cells.

Although we have extensively explored the role of Gas6 in IPF, we have not explored the role of protein S in this disease or in our humanized mouse model of IPF. Protein S is a member of the vitamin K-dependent proteins encoded by the *PROS1* gene. This protein is expressed in multiple tissues including the liver, kidneys, and lungs. In humans, this protein is present in a free form and complexed to C4BP (C4b-binding protein) (5), with the free form being the only form capable of activating TAM receptors. However, in mice, this protein is present exclusively in a free form because of the lack of the β chain in murine C4b that is required to bind to protein S (6). In patients with IPF, several studies have examined changes in circulating free protein S levels, with one study showing a reduction in plasma (7) and another study showing no changes in the concentration of this protein in serum (8) from patients with IPF relative to normal individuals. However, to the best of our knowledge, the levels of this protein in the BAL or lung tissues of patients with IPF have not been reported. Mining of transcriptomic array data sets of IPF diagnostic lung biopsies, end-stage explanted IPF lung, and normal, nonfibrotic lung tissues (Gene Expression Omnibus, series GSE24206) showed that Gas6 but not protein S transcripts were consistently elevated in IPF lung biopsies and explants, relative to normal lung tissues (data not shown). Thus, although protein S has been observed to be protective in a mouse bleomycin injury-driven model of lung fibrosis (7), translating these results to humans is challenging because of differences in the regulation of this protein between human and mice and differing results pertaining to its expression levels in patients with IPF. Future studies utilizing novel genetically modified mice expressing human C4BP and IPF cell-humanized mice are warranted for the better assessment of this protein in lung fibrosis.

The overall impact of TAM receptor inhibition on the function of IPF macrophages is not apparent at the moment. Elegant mouse studies have highlighted the role of the Gas6/protein S/TAM receptor pathway in macrophage-directed efferocytosis, as noted (9). However, we saw no histologic or transcriptomic evidence of enhanced inflammation in either R428-treated humanized SCID mice or bleomycin-challenged Gas6^{-/-} mice (1). As for the assessment of changes in apoptosis, interpretation of these analyses is complicated by our finding that R428 promoted apoptosis in human lung fibroblasts. Nevertheless, we agree that this area requires further investigation, and future studies will address the effect of TAM receptor inhibitors in a newly described NOD *scid*

Supported by funding from Cedars Sinai Medical Center (C.M.H.) and NIH R01 grant HL123899 (C.M.H.).

Author Contributions: Conception and design: M.S.E., D.M.H., and C.M.H.; acquisition of data: M.S.E. and D.M.H.; analysis and interpretation of data: M.S.E., D.M.H., and C.M.H.; drafting the manuscript and intellectual content: M.S.E., D.M.H., and C.M.H.

Originally Published in Press as DOI: 10.1164/rccm.201805-0972LE on July 6, 2018

Figure 1. TAM (Tyro3, Axl, Mer) receptors and Gas6 (growth arrest–specific 6) transcript expression in normal and idiopathic pulmonary fibrosis (IPF) lung epithelial cells. Publicly available single-cell RNA-Seq data sets (2–4) of normal type II alveolar epithelial cells (AT2 cells) and IPF indeterminate, basal, club/goblet epithelial cells were mined for Axl (top left), Mertk (top right), Tyro3 (bottom left), and Gas6 (bottom right) transcript expression. Depicted are a heat map (top) and average TPM expression (bottom) for the transcripts in normal and IPF epithelial cells. TPM = transcripts per kilobase million.

gamma mouse model of pulmonary fibrosis initiated by the introduction of both immune (i.e., CD45⁺ myeloid and lymphoid) and nonimmune cells (i.e., fibroblasts and epithelial cells) (10). In closing, we are grateful for the interest in our study,

and we look forward to providing further clarity regarding both the efficacy and potential adverse challenges associated with TAM inhibition in IPF. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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Figure 2. Transcript expression of SFTPC (surfactant protein C) in a humanized severe combined immunodeficiency (SCID)/Bg model of idiopathic pulmonary fibrosis (IPF). Humanized SCID/Bg mice were injected with IPF fibroblasts and treated orally with R428 (5 mg/kg), BIBF1120 (30 mg/kg), or the appropriate vehicle. Treatments were given from Days 0 to 35 after fibroblast injection, and lungs were analyzed on Day 35 for transcript expression of SFTPC. Data represent means ± SEM, *n* = 4 or 5 per group.

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contextualized within a needed change in the taxonomy of the disease (8). ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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How to Define Early Chronic Obstructive Pulmonary Disease

To the Editor:

We read with interest the recent publication by Martinez and colleagues about early chronic obstructive pulmonary disease (COPD) (1). We fully agree that, for the reasons outlined by the authors, it is imperative to understand the early origins of COPD. To this end, they argue (and we also agree) that it is key to distinguish between early and mild COPD, as we highlighted some years ago (2). However, we respectfully disagree with the proposed operational definition of early COPD (1): “those younger than 50 years with 10 or more pack-years smoking history and any of these abnormalities: 1) early airflow limitation (post-bronchodilator $FEV_1/FVC < \text{lower limit of normal}$), 2) compatible CT abnormalities, 3) rapid decline in FEV_1 (≥ 60 ml/yr) that is accelerated relative to FVC.” We disagree for the following reasons. First, 20–40% of patients with COPD worldwide are never smokers (3), so this definition excludes a significant number of individuals. Second, 50 years of age may be not that early, as between 4% and 12% of individuals in the general population have low lung function before the age of 40 years (4); importantly, these individuals also have a higher prevalence and incidence of comorbidities, as well as an increased risk for mortality (4), so if we want to intervene therapeutically, we probably need to act earlier (school? driving license?) (5). Finally, we now know that about half of adult patients with COPD develop the disease without evidence of accelerated lung function decline (6, 7), so the inclusion of a fast rate of FEV_1 decline as a diagnostic criteria of early COPD also leaves behind a substantial number of individuals. We fully concur with the importance of understanding early COPD, but to really advance the field, we think that this should be

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Erratum: Protection from Lipopolysaccharide-induced Lung Injury by Augmentation of Airway S-Nitrosothiols

The authors of an article published in 2009 (1) have informed the editors that the data presented in Figures 5 and 7 are not reliable because of concerns regarding the work product from the laboratory that performed these measurements. The authors have repeated the experiments measuring lung compliance (Figure 5) and BAL fluid (BALF) (Figure 7) cytokine levels in mice treated with ethyl nitrite (ENO) followed by exposure to aerosolized LPS. This erratum updates a previously published Expression of Concern (2).

The repeated experiments continue to demonstrate that ENO treatment significantly diminishes BALF white blood cell count in LPS-treated mice (0.62×10^5 cells/ml vs 1.00×10^5 cells/ml; $P < 0.05$). However, although ENO treatment still decreased BALF