ASTHMA

# Response to Comment on "An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness"



**Dhiren F. Patel[1], Amit Gaggar[2,3], J. Edwin Blalock[2], Lisa G. Gregory[1], Clare M. Lloyd[1], Robert J. Snelgrove[1]***

**We provide further evidence to support our assertion that PGP is a potent regulator of epithelial remodeling.**

We read the *Technical Comment* (*1*) concerning our recent Patel *et al.* article (*2*) with great interest but strongly dispute the authors' suggestion that the pathological changes observed in our murine model of allergic airways disease may be attributable to the accumulation of cysteinyl leukotrienes as opposed to the matrikine proline-glycine-proline (PGP). Within our article, we provided a compelling case that accumulation of PGP could drive pathological epithelial remodeling that in turn exacerbates airway resistance. Leukotriene $A_4$ hydrolase ($LTA_4H$)–deficient mice exposed to house dust mite (HDM) demonstrated an accumulation of PGP, supporting our previous studies that unequivocally demonstrated that $LTA_4H$ was the only enzyme of physiological relevance that degrades PGP and that the peptide accumulated in its absence (*3*)—findings corroborated by some of the authors of the *Technical Comment* (*4*). In the context of our allergic airways disease model, this accumulation of PGP resulted in a profound increase in epithelial height and mucus production, despite markers of T helper 2 ($T_H2$) inflammation being substantially reduced [as a consequence of loss of leukotriene $B_4$ ($LTB_4$)]. Subsequently, neutralization of PGP within our model abolished this epithelial phenotype, whereas direct application of acetylated PGP (AcPGP) (a more stable derivative of PGP) onto human bronchial epithelial cells at airway liquid interface directly replicated the in vivo phenotype. We believe that these complementary studies provide a convincing argument that PGP drives the observed epithelial remodeling.

Conversely, the *Technical Comment* postulates that the observed changes are instead attributable to an accumulation of cysteinyl leukotrienes, owing to a shunt from accumulated $LTA_4$ (leukotriene $A_4$) that arises after loss of $LTA_4H$. Despite the fact that we assessed cysteinyl leukotrienes and found no difference between allergen-exposed wild-type and $LTA_4H$ knockout mice, Penno *et al.* suggest that this is because we have not looked early enough after allergen challenge. In mouse models of allergic airways disease, cysteinyl leukotrienes have been implicated in airway eosinophil infiltration and degranulation, $T_H2$ cytokine release, increased airway smooth muscle mass, elevated edema, goblet cell hyperplasia, and augmented subepithelial collagen deposition. Extrapolating the rationale of their argument, if such a profound accumulation of cysteinyl leukotrienes does occur in our HDM-treated $LTA_4H$ knockout mice, then one may imagine that all of these inflammatory and remodeling parameters would be exacerbated, whereas with the exception of the epithelial phenotype, these parameters are either comparable or reduced in the knockout animals. Furthermore, although cysteinyl leukotrienes are able to directly or indirectly promote goblet cell hyperplasia, we believe that this is a subtly distinct phenotype to that we observe in $LTA_4H$ knockout mice where we see a general increase in epithelial height as a consequence of hyperplasia and hypertrophy. Although cysteinyl leukotrienes fail to elicit this exact phenotype, application of AcPGP to bronchial epithelial cells does directly recapitulate these changes.

Supportive of their argument, Penno *et al.* demonstrate that peritoneal cysteinyl leukotrienes accumulate in $LTA_4H$ knockout mice early after intraperitoneal administration of zymosan, although this may not be reflective of what occurs in a pulmonary allergen challenge model. Furthermore, Penno *et al.* correctly highlight that administration of Johnson & Johnson $LTA_4H$ inhibitor JNJ40929837 to patients with asthma in a bronchial allergen challenge model was shown to result in an increase in urine $LTE_4$ (leukotriene $E_4$). However, what they neglect to discuss is the wealth of contradictory literature, including exhaustive preceding preclinical studies by Johnson & Johnson, showing that $LTA_4H$ inhibition does not cause accumulation of cysteinyl leukotrienes but rather anti-inflammatory lipoxins—as exemplified by studies in a murine model of allergic airways disease (*5*). Thus, a buildup of cysteinyl leukotrienes after the loss of $LTA_4H$ function is by no means unequivocal and is likely context dependent. Accordingly, in contrast to our substantial body of data demonstrating that PGP drives the pathological epithelial remodeling observed, assertions that it is instead attributable to cysteinyl leukotrienes seem speculative and are not fully reflective of the literature.

Penno *et al.* also question several aspects of the methodologies used within our manuscript, which we strongly refute. We used the compound L-arginine-threonine-arginine (RTR) to neutralize PGP within $LTA_4H$ knockout mice to validate that the observed phenotype was PGP-mediated. RTR was created on the basis of the complementary peptide algorithm to act as a selective antagonist of PGP and has been routinely used in multitude of studies to this effect in vitro and in vivo. However, Penno *et al.* argue that experiments with RTR are not conclusive because the compound exhibits polypharmacology. PGP and its derivatives function by mimicking key sequences found in $ELR^+$ CXC chemokines such as interleukin-8 (IL-8). Accordingly, it has been demonstrated that RTR can show some inhibition of IL-8–mediated neutrophil chemotaxis (*6*). However, although RTR was capable of completely inhibiting AcPGP-induced neutrophil chemotaxis when present at close to equimolar concentrations, it was

[1]Inflammation Repair and Development, National Heart and Lung Institute, Imperial College London, London SW7 2AZ, UK. [2]*Division of Pulmonary, Allergy and Critical Care Medicin*e, Program in Protease and Matrix Biology, Gregory Fleming James Cystic Fibrosis Centre and Lung Health Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA. [3]Birmingham VA Medical Center, Birmingham, AL 35233, USA.
*Corresponding author. Email: robert.snelgrove@imperial.ac.uk

only able to achieve a 30% inhibition of IL-8–induced chemotaxis when present at >10,000-fold molar excess to IL-8 (*6*). This indicates a greater specificity for RTR in attenuating PGP peptide–mediated activity and the likely negligible effect of RTR in neutralizing $ELR^+$ CXC chemokines in an in vivo setting. Indeed, concentrations of murine $ELR^+$ CXC chemokines KC and macrophage inflammatory protein 2 (MIP-2) were modestly increased in our allergen-exposed mice but were comparable between wild-type and $LTA_4H$ knockout animals, and thus, one would anticipate that any effect of RTR on these mediators would be comparable between the groups. However, although RTR prevented the epithelial pathology seen in $LTA_4H$ knockout mice where PGP selectively accumulated, it had no effect in wild-type animals where KC and MIP-2 concentrations were comparable to those observed in knockouts. Thus, in combination with other studies, we believe that the RTR experiments further validate our assertion that PGP is indeed driving the observed epithelial remodeling.

The *Technical Comment* also states that "it is assumed that PGP gets converted into acetyl-PGP in vivo", but we make no such assumption and instead explicitly state that only PGP is detected in HDM-challenged $LTA_4H$ knockout mice. As we have previously demonstrated (*3*) [and as has been validated by Röhn's group (*4*)], $LTA_4H$ is unable to degrade AcPGP. We used AcPGP as a surrogate for PGP in vitro for experiments with epithelial cells because epithelial cells have been shown to express and release $LTA_4H$, and thus, we were concerned that PGP degradation by the epithelial cells would confound the interpretation of results. However, in light of the concerns, we now present preliminary data that PGP is able to elicit comparable changes in epithelial cell proliferation as were demonstrated with AcPGP (Fig. 1), and thus, our in vitro findings align exactly with our in vivo studies. In certain animal models or specific subsets of patients, PGP is not degraded as a result of depreciated degradation by $LTA_4H$ or conversion to $LTA_4H$-resistant AcPGP, and it is in these scenarios that PGP and AcPGP exert their pathological effects.

A previous study by Röhn's group, and made reference to in the *Technical Comment*, suggested that PGP failed to elicit neutrophil chemotaxis in vivo and in vitro and that AcPGP only exhibited very modest activity. Furthermore, it was claimed that neither peptide was able to induce $Ca^{2+}$ influx or generation of reactive oxygen species and consequently had questionable physiological relevance (*4*). We were surprised by these observations, given the weight of literature to the contrary from at least 12 independent international groups published in leading journals over the past 23 years (*7*). These studies have repeatedly demonstrated that AcPGP and/or PGP are capable of directly driving neutrophil chemotaxis or activation (albeit at micromolar concentrations). Furthermore, it has been demonstrated that PGP can potentiate neutrophil chemotaxis to existing chemotactic gradients, whereby neutrophils migrating toward IL-8 or $LTB_4$ in a collagen matrix are capable of generating PGP at their leading edge to promote their directionality and persistence (*8*). In addition, we now present preliminary data demonstrating that PGP peptides, at nanomolar concentrations, are able to drive the release of potent proneutrophilic mediators IL-8 and granulocyte colony-stimulating factor (G-CSF) from airway epithelial cells (Fig. 2), providing a further indirect mechanism by which these peptides can promote neutrophilic responses. Accordingly, multiple studies have demonstrated that PGP and/or AcPGP accumulate in vivo in murine models of disease and that targeting these peptides by blocking their generation, their receptor, or the peptide itself (via RTR or anti-PGP antibody) reduces neutrophil influx and pathology (*7*). A wealth of clinical studies have further validated these assertions, with PGP and AcPGP accumulating in neutrophilic diseases, often correlating with neutrophil numbers, and, in some instances, being directly demonstrated to mediate neutrophilic inflammation (*7*).

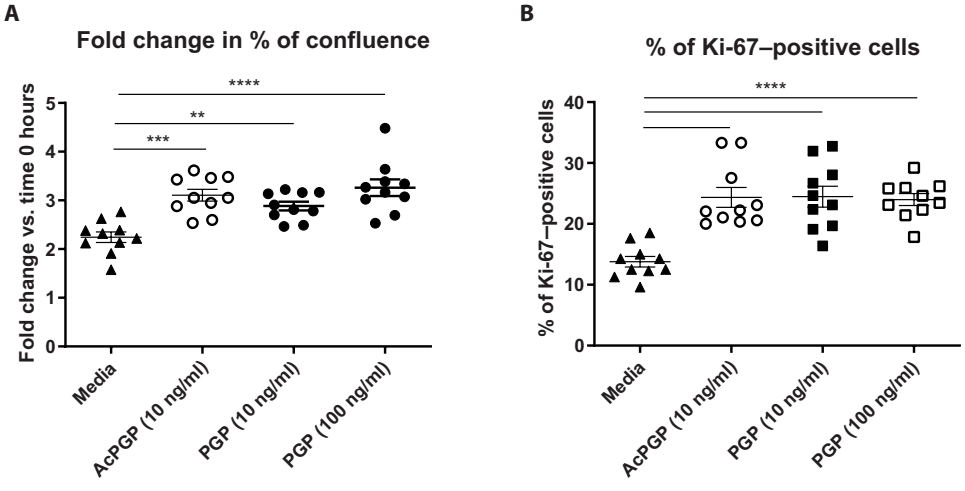


**Fig. 1. PGP is comparable to AcPGP at inducing proliferation of human bronchial epithelial cells.** Undifferentiated normal human bronchial epithelial cells (Lonza) were cultured in media or media supplemented with media alone, AcPGP (10 ng/ml), or PGP (10 or 100 ng/ml) and visualized over a period of 72 hours using a JuLI Stage automated cell imaging system. (**A**) Fold change in cell confluence over a 72-hour period depicted for individual wells in each treatment group. (**B**) After 72 hours, the cells were fixed and stained for Ki-67, with Ki-67–positive cells expressed as a percentage of 4′,6-diamidino-2-phenylindole–positive cells. Figure represents data combined from two independent experiments with five wells per group in each experiment. Results are depicted as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ using analysis of variance (ANOVA) with Bonferroni correction.

Consistent with a proneutrophilic activity of PGP, we previously observed an increase in PGP concentrations and neutrophil numbers in the lungs of $LTA_4H$ knockout mice after bacterial infection (*9*). Given that neutrophil numbers were reduced by PGP neutralization in this previous study, it is logical to conclude that it was the peptide driving the exacerbated neutrophilic response, although we did not define whether this was mediated via a direct chemotactic potential of the peptide, its ability to potentiate existing chemokine gradients, or even its capacity to promote the release of proneutrophilic mediators from epithelial cells. The increased neutrophilia observed in these bacteria-infected $LTA_4H$ knockout mice was not, as suggested in the *Technical Comment*, attributable to compromised bacterial clearance because pathogen load was comparable to that seen in wild-type animals. Despite the accumulation of PGP in HDM-exposed $LTA_4H$ knockout mice, no increase in neutrophil numbers was observed. As

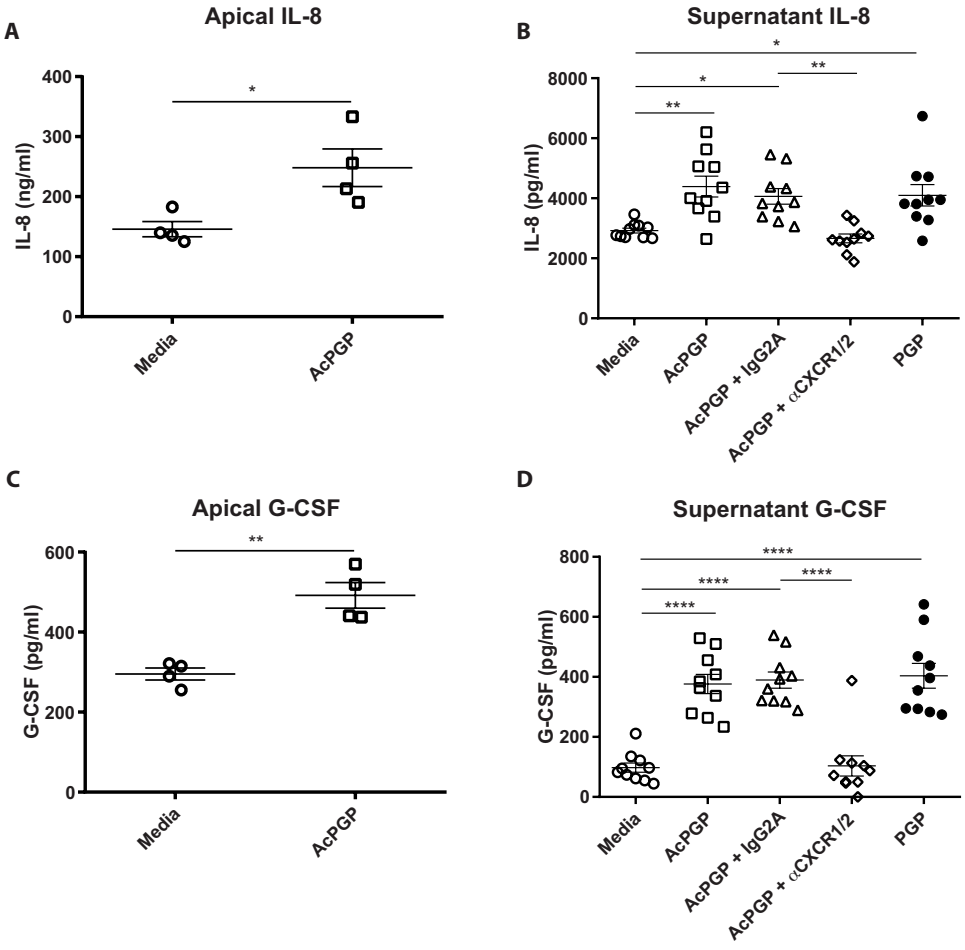


**Fig. 2. AcPGP induces IL-8 and G-CSF release from human bronchial epithelial cells.** (**A** and **C**) Normal human bronchial epithelial cells were cultured at air-liquid interface. Respective wells were treated with media or media supplemented with AcPGP (10 μg/ml). Apical supernatants were collected on day 1 for measurement of IL-8 (A) and G-CSF (C) by enzyme-linked immunosorbent assay (ELISA). (**B** and **D**) Undifferentiated normal human bronchial epithelial cells were cultured in media or media supplemented with AcPGP (10 ng/ml) or PGP (100 ng/ml) for 72 hours. In some groups, AcPGP-treated cells were preincubated with either anti-CXCR1/2 antibodies or IgG2a isotype control antibody. Supernatant was collected for the measurement of IL-8 (B) and G-CSF (D) by ELISA. (A) and (C) present combined data from two independent experiments with two wells per group in each experiment. (B) and (D) represent data combined from two independent experiments with five wells per group in each experiment. Results are depicted as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$, using Mann-Whitney statistical test (A and C) or ANOVA with Bonferroni correction (B and D).

stated within our manuscript, the inflammation observed in our model is profoundly eosinophilic with negligible neutrophil infiltrate, and it is well known that a $T_H2$ environment may be less conducive or even antagonistic to a neutrophilic response [e.g., (*10*)]. Indeed, concentrations of potent, well-heralded neutrophil chemoattractants KC, MIP-2, and $LTB_4$ are all elevated in wild-type mice given HDM in our study, and yet, they show no increase in neutrophil numbers relative to phosphate-buffered saline controls. Accordingly, context, timing, and anatomical location of the PGP are all likely to be critical in defining its biological activity.

Last, Penno *et al.* refute our claims that $LTA_4H$ inhibitors may have failed in the clinic, owing to PGP accumulation. We have always been relatively circumspect in our assertions in this respect and stated that it "may be a factor" contributing to disappointing results of $LTA_4H$ inhibitors in the clinic. Indeed, we have been keen to downplay this direct association in any interaction with media outlets following the publication of our study. We have stressed that $LTA_4H$ knockout mice may not be reflective of $LTA_4H$ inhibitors where residual enzyme activity may persist to degrade the PGP. Similarly, animal models are not completely reflective of human disease, and in certain settings, accumulation of PGP may be the lesser of two evils relative to the loss of $LTB_4$. However, given that $LTA_4H$ inhibitors potently block PGP degradation and that PGP has been purported to have biological activity upon multiple cells, then it is prudent to pose the question. With regard to the recent Acebilustat trial from Celtaxsys in patients with cystic fibrosis, it was encouraging that a reduction in neutrophil numbers was seen in a phase 1 trial and a modest reduction in exacerbations in a phase 2b study because, ultimately, we all want to see an effective therapeutic. However, to play devil's advocate, the phase 2b trial did not achieve its primary end point of improved lung function, and there was no report on metrics of airway inflammation in the study. The *Technical Comment* also states that because PGP has been shown to accumulate in patients with cystic fibrosis and that Acebilustat had been shown to reduce inflammation, then PGP is not clinically relevant. This statement is not entirely logical because if PGP is already accumulated in patients with cystic fibrosis, then it may already be exerting a pathological/inflammatory effect and perhaps suggests that the $LTA_4H$ pathway is already aberrant.

In summary, we strongly refute the assertions put forth by Penno *et al.*, whose views appear contrary to the abundance of scientific literature from multiple groups and are not supported by the data that we have presented. Our findings and conclusions are based on a much broader view of the scientific literature within this area of research and provide clear evidence for the biological impact of these matrikines in disease.

## REFERENCES

1. C. A. Penno, N. Wack, C. Laguerre, F. Hasler, S. Numao, T. A. Röhn, Comment on "An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness". *Sci. Transl. Med.* **11**, eaav4538 (2019).
2. D. F. Patel, T. Peiró, A. Shoemark, S. Akthar, S. A. Walker, A. M. Grabiec, P. L. Jackson, T. Hussell, A. Gaggar, X. Xu, J. L. Trevor, J. Li, C. Steele, G. Tavernier, J. E. Blalock, R. M. Niven, L. G. Gregory, A. Simpson, C. M. Lloyd, R. J. Snelgrove, An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness. *Sci. Transl. Med.* **10**, eaaq0693 (2018).
3. R. J. Snelgrove, P. L. Jackson, M. T. Hardison, B. D. Noerager, A. Kinloch, A. Gaggar, S. Shastry, S. M. Rowe, Y. M. Shim, T. Hussell, J. E. Blalock, A critical role for $LTA_4H$ in limiting chronic pulmonary neutrophilic inflammation. *Science* **330**, 90–94 (2010).

4. S. Numao, F. Hasler, C. Laguerre, H. Srinivas, N. Wack, P. Jäger, A. Schmid, A. Osmont, P. Röthlisberger, J. Houguenade, C. Bergsdorf, J. Dawson, N. Carte, A. Hofmann, C. Markert, L. Hardaker, A. Billich, R. M. Wolf, C. A. Penno, B. Bollbuck, W. Miltz, T. A. Röhn, Feasibility and physiological relevance of designing highly potent aminopeptidase-sparing leukotriene A4 hydrolase inhibitors. *Sci. Rep.* **7**, 13591 (2017).
5. N. L. Rao, J. P. Riley, H. Banie, X. Xue, B. Sun, S. Crawford, K. A. Lundeen, F. Yu, L. Karlsson, A. M. Fourie, P. J. Dunford, Leukotriene $A_4$ hydrolase inhibition attenuates allergic airway inflammation and hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* **181**, 899–907 (2010).
6. A. H. van Houwelingen, N. M. Weathington, V. Verweij, J. E. Blalock, F. P. Nijkamp, G. Folkerts, Induction of lung emphysema is prevented by L-arginine-threonine-arginine. *FASEB J.* **22**, 3403–3408 (2008).
7. D. F. Patel, R. J. Snelgrove, The multifaceted roles of the matrikine Pro-Gly-Pro in pulmonary health and disease. *Eur. Respir. Rev.* **27**, 180017 (2018).
8. P. V. Afonso, C. P. McCann, S. M. Kapnick, C. A. Parent, Discoidin domain receptor 2 regulates neutrophil chemotaxis in 3D collagen matrices. *Blood* **121**, 1644–1650 (2013).
9. S. Akthar, D. F. Patel, R. C. Beale, T. Peiró, X. Xu, A. Gaggar, P. L. Jackson, J. E. Blalock, C. M. Lloyd, R. J. Snelgrove, Matrikines are key regulators in modulating the amplitude of lung inflammation in acute pulmonary infection. *Nat. Commun.* **6**, 8423 (2015).
10. S. S. Cheng, N. W. Lukacs, S. L. Kunkel, Eotaxin/CCL11 is a negative regulator of neutrophil recruitment in a murine model of endotoxemia. *Exp. Mol. Pathol.* **73**, 1–8 (2002).

Submitted 17 December 2018
Accepted 29 May 2019
Published 19 June 2019
10.1126/scitranslmed.aaw0462

**Citation:** D. F. Patel, A. Gaggar, J. E. Blalock, L. G. Gregory, C. M. Lloyd, R. J. Snelgrove, Response to Comment on "An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness". *Sci. Transl. Med.* **11**, eaaw0462 (2019).

Science Translational Medicine

# Response to Comment on "An extracellular matrix fragment drives epithelial remodeling and airway hyperresponsiveness"

Dhiren F. Patel, Amit Gaggar, J. Edwin Blalock, Lisa G. Gregory, Clare M. Lloyd and Robert J. Snelgrove

*Sci Transl Med* **11**, eaaw0462.
DOI: 10.1126/scitranslmed.aaw0462