Down-regulation of IL-8 by high-dose vitamin D is specific to hyperinflammatory macrophages and involves mechanisms beyond up-regulation of *DUSP1*

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Supplementary Results

Stimulation of IL-8 production in healthy monocyte-derived macrophages by bacterial virulence factors

To select bacterial virulence factors for stimulation of IL-8 in the experiments described in the main text, we tested various preparations of lipopolysaccharide (LPS), sterile filtrates of *Pseudomonas (P.) aeruginosa* culture, and *P. aeruginosa* flagellin. IL-8 production stimulated by those virulence factors was compared against that stimulated with IL-1β, a potent pro-inflammatory cytokine.

Commercial LPS is available from different microorganisms and in different purification grades. Here we used LPS from *Esherichia (E.) coli* and *P. aeruginosa*. While *E. coli* is not a typical bacterial virulence factor in chronic airway disease, it is commonly used as prototypical LPS. *P. aeruginosa* is the most common bacterial pathogen associated with CF lung disease. In the majority of adult patients, *P. aeruginosa* persists in the form of a multimicrobe biofilm in the airway lumen. It is thought that host cells are exposed to a mix of diffusible virulence factors leaching out of the biofilm of this microorganism (Wu *et al.*, 2005), while contact with live planktonic bacteria is less frequent. This can be mimicked *in vitro* by exposure of host cells to various bacterial virulence factors of the culture of clinical isolates of *P. aeruginosa*, including LPS, flagellin, and sterile filtrates of *P. aeruginosa* culture. The filtrates contain a mix of P. aeruginosa associated virulence factors, as well as pro-oxidants (Roussel *et al.*, 2011).

In the first experiment, healthy monocyte-derived macrophages (MDM) were stimulated in parallel with various LPS (Figure S1A). As expected, *E. coli* LPS caused marked up-regulation of IL-8 (Figure S1A). By comparison, *P. aeruginosa* was slightly less inflammagenic (Figure S1A), although the difference with IL-8 up-regulation by *E. coli* LPS did not reach statistical significance. As per manifacturer's information, due to contamination with lipoproteins, both these LPS stimulate Toll-like Receptors (TLR) 4 and 2. There is also commercially available LPS from *E. coli* with a higher purification grade (ultrapure *E. coli* LPS) that exclusively stimulates TLR4. We next stimulated MDM with this ultrapure LPS. While up-regulation of IL-8 by ultrapure LPS stimulated IL-8 production, the magnitude of this up-regulation was not as potent as with both other LPS preparations (Figure S1A), such that the difference to basal cells no longer reached statistical significance (Figure S1A).

We next assessed healthy MDM responses to flagellin and sterile filtrates of *P. aeruginosa*. A parallel set of cells was stimulated with IL-1β as positive control. The filtrates had to be pre-diluted 1 : 100, as more concentrated filtrates yielded exceedingly high IL-8 (data not shown).

We observed that both *P. aeruginosa* filtrates and flagellin significantly up-regulated IL-8 production in MDM (Figure S1B); this was in contrast to IL-1β, which stimulated markedly less IL-8. Furthermore, *P. aeruginosa* filtrates stimulated production of IL-8 with a trend toward smaller variability of responses, exemplified by a narrower range between minimal and maximal IL-8 (Figure S1B). In addition, up-regulation of IL-8 production by *P. aeruginosa* filtrates was

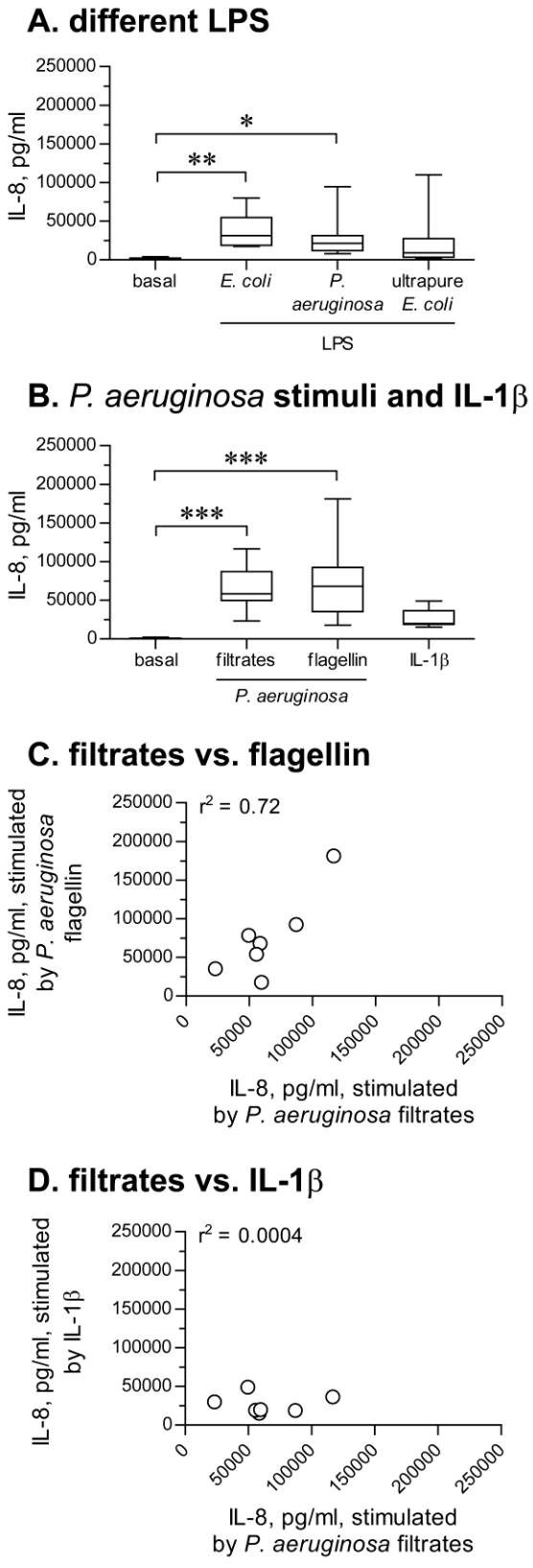
significantly ($p < 0.05$) stronger compared with that by *P. aeruginosa* LPS, while flagellin showed only a trend ($p = 0.056$) toward significant difference over stimulation of IL-8 by *P. aeruginosa* LPS.

Since *P. aeruginosa* filtrates contain ligands to TLR5 (Roussel *et al.*, 2011), it is not surprising that there was a good agreement in IL-8 production stimulated by the filtrates vs. by flagellin. Specifically, MDM producing high IL-8 levels when stimulated with the filtrates also produced high IL-8 upon stimulation with flagellin (Figure S1C). In contrast, there was no such agreement in IL-8 production stimulated by *P. aeruginosa* filtrates vs. by IL-1β (Figure S1D) or LPS (data not shown). This indicates that *P. aeruginosa* filtrates stimulate IL-8 in MDM largely through TLR5.

While we interpret cautiously the comparisons between different virulence factors due to different preparations or dilutions used (e.g., *P. aeruginosa* filtrates were diluted and not used at a specific concentration), these experiments appear to indicate that MDM tend to most strongly respond to a mix of bacterial virulence factors, such as less pure LPS or *P. aeruginosa* filtrates. Since in subsequent experiments we wanted to use a potent bacterial virulence factor relevant to CF, we chose *P. aeruginosa* filtrates.

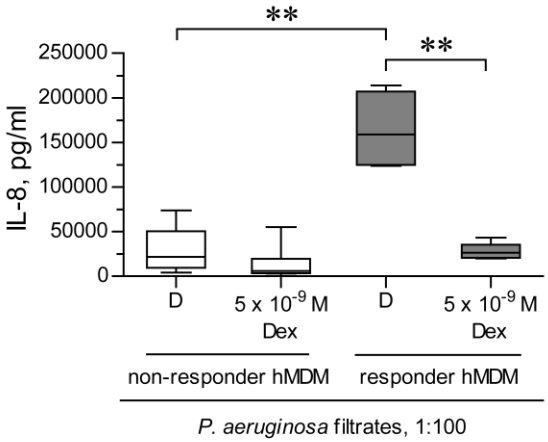
Supplementary Figures

Figure S1



Legend to Figure S1. Up-regulation of IL-8 production by bacterial virulence factors.
(**A**): Healthy monocyte-derived macrophages were stimulated for 24 hours with 0.25 µg/ml lipopolysaccharide (LPS) from *E. coli* or *P. aeruginosa*. Ultrapure *E. coli* LPS preparation that specifically stimulates Toll-like Receptor 4 was also used for stimulation at similar concentration. IL-8 levels in cell supernatants were quantified by ELISA. * $p < 0.05$; ** $p < 0.01$. Data are presented as box-and-whisker plots (medians, interquartile ranges, and min-max values) of 7 cultures from different donors tested in parallel with these stimuli. (**B**): Healthy monocyte-derived macrophages were stimulated for 24 hours with diluted (1 : 100) sterile filtrates of *P. aeruginosa* culture or 0.5 µg/ml of *P. aeruginosa* flagellin. As a positive control for IL-8 production, cells were stimulated for 24 hours with the pro-inflammatory cytokine IL-1β (10 ng/ml). IL-8 levels in cell supernatants were quantified by ELISA. *** $p < 0.001$. Data are presented as box-and-whisker plots (medians, interquartile ranges, and min-max values) of 7 cultures from different donors tested in parallel with these stimuli. (**C**): Association between IL-8 up-regulation by sterile filtrates of *P. aeruginosa* culture and that by *P. aeruginosa* flagellin. (**D**): Association between IL-8 up-regulation by sterile filtrates of *P. aeruginosa* culture and that by IL-1β.

Figure S2



Legend to Figure S2. Responsiveness of "non-responder" and "responder" cells to low-dose dexamethasone

Healthy "non-responder" and "responder" monocyte-derived macrophages (hMDM) were pre-treated with diluent (D) or 5 x 10^{-9} M dexamethasone (Dex), and stimulated for 24 hours with diluted (1:100) sterile filtrates of *P. aeruginosa* culture in the presence of D or Dex. Definition of "non-responder" and "responder" hMDM was as in Figure 6 of main text. IL-8 levels in cell supernatants were quantified by ELISA. ** $p < 0.01$. Data are presented as box-and-whisker plots (medians, interquartile ranges, and min-max values).

Supplementary references

Roussel L, Martel G, Berube J, Rousseau S (2011). P. aeruginosa drives CXCL8 synthesis via redundant toll-like receptors and NADPH oxidase in CFTRF508 airway epithelial cells. *J Cyst Fibros* 10**:** 107-113.

Wu Q, Lu Z, Verghese MW, Randell SH (2005). Airway epithelial cell tolerance to Pseudomonas aeruginosa. *Respir Res* 6**:** 26.