Figures and figure supplements

ENaC-mediated sodium influx exacerbates NLRP3-dependent inflammation in cystic fibrosis

Thomas Scambler et al
LPS-induced IL-18 secretion in human bronchial epithelial cells is higher in cells with CF-associated mutations and is NLRP3 inflammasome-dependent. Human bronchial epithelial cell (HBEC) lines (BEAS-2B (WT), IB3-1 (ΔF508/W1282X), CuFi1 (ΔF508/D551D), CuFi4 (ΔF508/G551D) (n = 3 independent experiments) were unstimulated or stimulated with Lipopolysaccharide, from *Escherichia coli K12* (LPS Ultrapure), which specifically targets TLR4 (10 ng/mL) for 4 hr before being stimulated for 4 hr with Flagellin (10 ng/mL with Lipofectamine 2000) for NLRC4 inflammasome, TcdB (10 ng/mL) for Pyrin inflammasome or poly(dA:dT) dsDNA (1 μg/mL with Lipofectamine 2000) for AIM2 inflammasome. ELISA assays were used to detect (A) IL-18. To monitor NLRP3 inflammasome activation, HBEC (n = 3 independent experiments) were pre-incubated with MCC950 (15 μM), OxPAPC (30 μg/mL) and YVAD (2 μg/mL) for 1 hr before a stimulation with LPS (10 ng/mL, 4 hr), and ATP (5 mM) for the final 30 min. ELISA assays were used to detect (B) IL-18 and (D) colourimetric assay used to detect caspase-1 activity in protein lysates for LPS/ATP and LPS/ATP/MCC950. (D) Necrosis and pyroptosis are represented as superimposed bar charts. Total necrosis was measured using LDH release assay. For pyroptotic cell death, each sample/condition was repeated in parallel with a caspase-1 inhibitor (YVAD (2 mg/mL, 1 hr)) pre-treatment. The total necrosis level was then taken away from the caspase-1 inhibited sample, or ‘caspase-1 independent’ necrosis, with the remaining LDH level termed ‘caspase-1 dependent necrosis’ or pyroptosis. Cells were then stimulated with LPS (10 ng/mL, 4 hr), and ATP (5 mM) for final 30 min. The assay was performed with HBEC lines (n = 3 independent experiments). (*) Significance for Total Necrosis (α) Significance for Pyroptosis. A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * =< 0.05, ** =< 0.01, *** =< 0.001 and **** =< 0.0001).

DOI: https://doi.org/10.7554/eLife.49248.002
Figure 2. LPS-induced IL-1β/IL-18 secretion in human monocytes is higher in CF and is NLRP3 inflammasome dependent. Primary monocytes from HC (HC, n = 10; CF, n = 10) were unstimulated or stimulated with LPS which specifically targets TLR4 (10 ng/mL) for 4 hr before being stimulated for 8 hr with Flagellin, TcdB, dsDNA or ATP. The secretion of IL-18 (pg/mL) was measured in supernatants using ELISA. (A) IL-18 secretion by monocytes from HC and CF in response to LPS. (B) IL-18 secretion by monocytes from HC and CF in response to LPS. (C) IL-18 secretion by monocytes from HC and CF in response to LPS/ATP, MCC950, YVAD, or OxPAPC. (D) IL-18 secretion by monocytes from HC and CF in response to LPS/ATP, MCC950, YVAD, or OxPAPC. (E) Caspase-1 activity in monocytes from HC and CF in response to LPS, ATP, or MCC950. (F) ASC speck formation in monocytes from HC and CF in response to LPS, ATP, or MCC950. (G) Cell death in monocytes from HC and CF in response to LPS, ATP, or MCC950. 

Figure 2 continued on next page.
4 hr with Flagellin (10 ng/mL with Lipofectamine 2000) for NLRC4 inflammasome, or TcdB (10 ng/mL) for Pyrin inflammasome or poly(dA:dT) dsDNA (1 μg/mL with Lipofectamine 2000) for AIM2 inflammasome. ELISA assays were used to detect (A) IL-18 and (B) IL-1β cytokine secretion in supernatants. To monitor NLRP3 inflammasome activation, primary monocytes from HC, CF, SAID and NCFB (HC, n = 10; CF, n = 10; SAID, n = 4; NCFB, n = 4) were pre-incubated with MCC950 (15 μM), OxPAPC (30 μg/mL) and YVAD (2 μg/mL) for 1 hr before a stimulation with LPS (10 ng/mL, 4 hr), and ATP (5 mM) for the final 30 min. ELISA assays were used to detect (C) IL-18 and (D) IL-1β cytokine secretion in supernatants and (E) a colourimetric assay was used to detect caspase-1 activity in protein lysates (HC, n = 10; CF, n = 10; SAID, n = 4; NCFB, n = 4). (F) Flow cytometry was used to detect ASC specks in supernatants of primary monocytes from HC, CF, SAID and NCFB (HC, n = 10; CF, n = 10; SAID, n = 6; NCFB, n = 4) for ±LPS/ATP and (HC, n = 5; CF, n = 5) for MCC950 with LPS/ATP. (G) Necrosis and pyroptosis are represented as superimposed bar charts. Total necrosis was measured using LDH release assay. For pyroptotic cell death, each sample/condition was repeated in parallel with a caspase-1 inhibitor (YVAD (2 mg/mL, 1 hr)) pre-treatment. The total necrosis level was taken away from the caspase-1 inhibited sample, or ‘caspase-1 independent’ necrosis, with the remaining LDH level termed ‘caspase-1 dependent necrosis’ or pyroptosis. Cells were then stimulated with LPS (10 ng/mL, 4 hr), and ATP (5 mM) for final 30 min. The assay was performed with primary monocytes from HC, CF, SAID and NCFB (HC, n = 10; CF, n = 10; SAID, n = 4; NCFB, n = 4). (*) Significance for Total Necrosis (+) Significance for pyroptosis. A 2-way ANOVA statistical test was performed, with Tukey post-hoc correction (p values * =< 0.05, ** =< 0.01, *** =< 0.001 and **** =< 0.0001; error bars ± SEM). Inhibitor treatments in panels a-c were found to significantly reduce cytokine secretion and caspase-1 activity to **p =< 0.01 or less, for CF and SAID groups respectively. Significance values not displayed on the graph.

DOI: https://doi.org/10.7554/eLife.49248.003
Figure 2—figure supplement 1. Primary monocytes from HC, CF, SAID and NCFB (HC n = 9, CF n = 9, SAID n = 4, NCFB n = 4) were unstimulated or stimulated with LPS (10 ng/ml, 4 hr) or LPS (10 ng/ml, 4 hr) and ATP (5 mM) for the final 30 min. ELISAs were used to detect (A) IL-18, and (B) IL-1β cytokine secretion in supernatants. (C) PBMCs were unstimulated or stimulated with LPS (10 ng/ml, 4 hr) or LPS (10 ng/ml, 4 hr) and ATP (5 mM) for the final 30 min. Taqman RT-qPCR was used to measure IFNγ gene expression and (D) Luminex was used to measure IFNγ secretion from peripheral blood mononuclear cells (PBMC) populations from HCs and patients with CF-associated mutations (n = 10).

DOI: https://doi.org/10.7554/eLife.49248.004
Figure 3. Inflammatory serum cytokine signature in CF. (A) ELISA assays were used to detect IL-18 (HC, n = 10, CF, n = 30, SAID, n = 10, NCFB, n = 4), (B) IL-1β (HC, n = 10, CF, n = 30, SAID, n = 10, NCFB, n = 4), (C) IL-1Ra (HC, n = 10, CF, n = 30, SAID, n = 7, NCFB, n = 4) in patient sera. Outliers in SAID group for IL-1β and IL-1Ra correspond to HIDS one and A20 deficiency. (D) Flow cytometry was used to detect ASC specks (HC, n = 10, CF, n = 15, SAID, n = 10, NCFB, n = 4) in patient sera. (E) A colorimetric assay to detect caspase-1 activity in sera of patients with CF, SAID, and NCFB as a percentage of HC (HC, n = 10, CF, n = 15, SAID, n = 4, NCFB, n = 4). Of note, an undetermined amount of detected IL-1Ra is attributed to circulating Anakinra (recombinant IL-1Ra) specifically in the SAID cohort. The Kruskal-Wallis non-parametric test, with Dunn’s multiple comparison test, was performed (p values * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001; error bars ± S.E.M).

DOI: https://doi.org/10.7554/eLife.49248.005
Figure 3—figure supplement 1. Inflammatory serum cytokine signature in CF. ELISA assays were used to detect (A) TNF and (B) IL-6 (HC n = 10, CF n = 30, SAID n = 7, NCFB = 4) in patient serum. The Kruskal-Wallis non-parametric test, with Dunn’s multiple comparison test, was performed (p values * = 0.05, ** = 0.01, *** = 0.001 and **** = 0.0001; error bars ± S.E.M).

DOI: https://doi.org/10.7554/eLife.49248.006
Figure 4. Dysregulated Na\(^+\) and K\(^+\) in cells with CF-associated mutations can be modulated with ENaC inhibitors. Intracellular Na\(^+\) was detected using an AM ester of sodium indicator SBFI (S-1263) and (B, D) intracellular K\(^+\) was detected using an AM ester of potassium indicator PBFI (P-1266); changes in Figure 4 continued on next page.
Figure 4 continued

Fluorescence were measured by fluorimeter post-stimulation with 5 mM ATP in (A, B) monocytes (HC = 7, CF = 7) (E, F) HBECs (n = 3 independent experiments). Cells were pre-treated with the following: amiloride (100 μM), S18 derived peptide (25 μM, 4 hr) with LPS (10 ng/mL, 4 hr) and ATP (5 mM) for the final 30 min. A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * =< 0.05, ** =< 0.01, *** =< 0.001 and **** =< 0.0001) (*) indicate significance when comparing HC with CF-associated mutants. (●) indicate significance between treatments within the same cell line. (C) Endogenous β-ENaC protein expression was detected using western blot in BEAS-2B HBEC, HC and CF monocytes (C) and densitometry analysis of total β-ENaC (bands A, B, C indicated on blot) was quantified in (D) for CF relative to HC (n = 3 independent experiments). (G) BEAS-2B, IB3-1, CuFi-1 and CuFi-4 HBEC lines and densitometry analysis of total β-ENaC (bands A, B, C indicated on blot) was quantified in (H) (n = 3 independent experiments). Band A represents complex N-Glycosylation, 110 kDa β-ENaC (found when associated as ENaC complex); Band B represents Endo-H sensitive N-Glycosylation, 96 kDa β-ENaC; Band C represents immature non-glycosylated, 66 kDa β-ENaC. The Mann-Whitney non-parametric test was performed (p values * =< 0.05).

DOI: https://doi.org/10.7554/eLife.49248.009
Figure 4—figure supplement 1. Increased sodium influx in cells with CF-associated mutations. (A, C) Intracellular Na\(^+\) was detected using an AM ester of sodium indicator SBFI (S-1263) and (B, D) intracellular K\(^+\) was detected using an AM ester of potassium indicator PBFI (P-1266); changes in fluorescence were measured by fluorimeter post-stimulation in (A, B) monocytes (HC = 7, CF = 7) (C, D) HBECs (n = 3 independent experiments). Cells were pretreated with the following: EIPA (10 mM, 1 hr) and ouabain (100 nM, 24 hr) before a stimulation with LPS (10 ng/mL, 4 hr) and ATP (5 mM) for the final 30 min. A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * = 0.05, ** = 0.01, *** = 0.001 and **** = 0.0001) (*) indicate significance when comparing HC with CF-associated mutatns. (•) indicate significance between treatments within the same cell line. (E) Gene expression of b-ENaC in HC vs CF (n = 1), represented as DCT. The Mann-Whitney non-parametric test was performed (p values * = 0.05).

DOI: https://doi.org/10.7554/eLife.49248.010
Figure 5. Inhibition of amiloride-sensitive sodium channels modulates inflammation in cells with CF-associated mutations. ELISA assays were used to detect IL-18 (A) and (B) IL-1β in monocytes from HC (n = 9 amiloride, n = 10 S18), patients with CF (n = 10), SAID (n = 4) and NCFB (n = 4) and IL-18 (E) HBEC (n = 3, amiloride independent experiments) (F) HBEC (n = 3, S18 independent experiments). (C) Colourimetric assay was used to detect caspase-1 activity in protein lysates (HC n = 11, CF n = 11) and (D) flow cytometry was used to detect ASC specks in supernatant of primary monocytes (HC n = 5, CF n = 5). Cell stimulation was as follows: Amiloride (100 μM or 10 μM, 1 hr) or S18 derived peptide (25 μM, 4 hr) were used as a pre-treatment before a stimulation with LPS (10 ng/mL, 4 hr) and ATP (5 mM) for the final 30 min. (E, F) SCNN1B over-expression in BEAS-2B cells increases pro-inflammatory cytokine secretion. (E) BEAS-2B cells were transiently transfected with 10 μg SCNN1B cDNA (+) or a pcDNA3.1 vector only control (-) for 48 hr then stimulated with LPS (10 ng/mL, 4 hr) and ATP (5 mM) for the final 30 min (n = 3 independent experiments). Cells were lysed and immunoblotted for β-ENaC and β-actin. (F) ELISA assays were used to detect IL-18 in the supernatant fraction. A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001 and **** = ≤ 0.0001) (*) indicate significance, when comparing HC with CF. (•) indicate significance between treatments within the same cell line.

DOI: https://doi.org/10.7554/eLife.49248.011
Figure 5—figure supplement 1. Inhibition of amiloride-sensitive sodium channels modulates inflammation in cells with CF-associated mutations. (A) ELISA assays were used to detect IL-18 in HBECs (n = 3 independent experiments) and (C) IL-18 and (D) IL-1β and (F) TNF in monocytes from HC (n = 10), patients with CF (n = 10), SAID (n = 4) and NCFB (n = 4). Cell stimulation was as follows: (F) Amiloride (100 mM or 10 mM, 1 hr) (A, C, D, E), EIPA (10 mM, 1 hr), were used as a pre-treatment before a stimulation with LPS (10 ng/mL, 4 hr) and ATP (5 mM) for the final 30 min. (H) All the above stimulations were used to detect caspase-1 activity using a colorimetric assay in HBECs and for EIPA in monocytes (G). A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * = 0.05, ** = 0.01, *** = 0.001 and **** = 0.0001) (*) indicate significance when comparing HC with CF-associated mutants. (●) indicate significance between treatments within the same cell line.

DOI: https://doi.org/10.7554/eLife.49248.012
Figure 5—figure supplement 2. Inhibition of amiloride-sensitive sodium channels modulates inflammation in cells with CF-associated mutations. (A, D, G) ELISA assays were used to detect IL-18 and (B, E, H) IL-1β in monocytes from HC (n = 10), patients with CF (n = 10), SAID (n = 4) and NCFB (n = 4) and (C, F) IL-18 in HBECs (n = 3 independent experiments). Cell stimulation was as follows: (A–C) Amiloride (100 mM or 10 mM, 1 hr), (D–F) EIPA (10 mM, 1 hr), (G–I) S18 derived peptide (25 mM, 4 hr) were used as a pre-treatment before a stimulation with LPS (10 ng/mL, 4 hr) and Nigericin (1 μM) for the final 30 min. A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * = 0.05, ** = 0.01, *** = 0.001 and **** = 0.0001) (*) indicate significance when comparing HC with CF-associated mutants. (•) indicate significance between treatments within the same cell line.

DOI: https://doi.org/10.7554/eLife.49248.013
Figure 6. SCNN1B over-expression in BEAS-2B cells increases pro-inflammatory cytokine secretion. BEAS-2B cells were transiently transfected with 10μg SCNN1B cDNA (+) or a pcDNA3.1 vector only control (-) for 48 hr then stimulated with LPS (10 ng/mL, 4 hr) and ATP (5 mM) for the final 30 min (n = 3 independent experiments). Cells were lysed and immunoblotted for β-ENaC and β-actin a). ELISA assays were used to detect IL-18 in the supernatant b) fraction. A 2-way ANOVA with Tukey’s multiple comparison test was performed (p values * = ≤ 0.05, ** = ≤ 0.01, *** = ≤ 0.001 and **** = ≤ 0.0001).

DOI: https://doi.org/10.7554/eLife.49248.014
Figure 7. A schematic diagram of the proposed excessive NLRP3 inflammasome activation observed in individuals and cells with CF-associated mutations. Without functional CFTR, inhibition of ENaC currents is diminished leading to increased intracellular Na+ levels. Dysregulation of ENaC-dependent Na2+ influx leads to increased K+ efflux (via unknown mechanism) and NLRP3 inflammasome activation, with subsequent release of IL-1β and IL-18. In the CF airway, K+ efflux is exacerbated upon K+ channel stimulation by endotoxins, DAMPs or PAMPs, leading to aberrant NLRP3 inflammasome activation and excessive IL-1β and IL-18 secretion. Blocking ENaC currents with S18 peptide restores Na+ and K+ levels which reduces NLRP3-mediated production of IL-1β and IL-18.

DOI: https://doi.org/10.7554/eLife.49248.015