IL-1 receptor antagonist ameliorates inflammasome-dependent inflammation in murine and human cystic fibrosis

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Dysregulated inflammasome activation contributes to respiratory infections and pathologic airway inflammation. Through basic and translational approaches involving murine models and human genetic epidemiology, we show here the importance of the different inflammasomes in regulating inflammatory responses in mice and humans with cystic fibrosis (CF), a life-threatening disorder of the lungs and digestive system. While both contributing to pathogen clearance, NLRP3 more than NLRC4 contributes to deleterious inflammatory responses in CF and correlates with defective NLRC4-dependent IL-1Ra production. Disease susceptibility in mice and microbial colonization in humans occurs in conditions of genetic deficiency of NLRC4 or IL-1Ra and can be rescued by administration of the recombinant IL-1Ra, anakinra. These results indicate that pathogenic NLRP3 activity in CF could be negatively regulated by IL-1Ra and provide a proof-of-concept evidence that inflammasomes are potential targets to limit the pathological consequences of microbial colonization in CF.

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In patients with cystic fibrosis (CF), a vicious cycle of airways infection, inflammation and tissue damage is responsible for the progressive decline of pulmonary function. The pulmonary innate immune response in CF is dysregulated at several levels, resulting in inefficient bacterial clearance and contributing to lung disease associated with CF. Studies have documented an altered balance of inflammatory/anti-inflammatory cytokines in CF, providing evidence that targeting specific inflammatory/anti-inflammatory pathways is a valid therapeutic strategy in CF.

Dysregulated inflammasome activity is a key mediator of infections and airway inflammation in lung diseases. Indeed, NLRP3 inflammasome activity is involved in host response to acute lung infections, but also during progression of several chronic pulmonary diseases. Assembly of these intracellular danger sensors triggers pyroptosis and secretion of proinflammatory IL-1β and IL-18 that are central to processes mediating lung inflammation. Both bronchial epithelial cells (ECs) and haematopoietic cells are source of IL-1β production in CF (between 2.8 and 32 ng ml⁻¹ in sputum from CF children). IL-1β signalling triggers the activation of pathogenic IL-17A-secreting T cells, thus critically modulating the Th17 regulatory T (Treg) cell balance.

This balance is essential for the efficient control of *Aspergillus fumigatus* colonization and diseases in CF, where the colonization by the fungus is common and may lead to fungal sensitization, bronchitis, allergic bronchopulmonary aspergillosis (ABPA) and FEV1 worsening. Pathogenic Th17 cells accounted for the inherent susceptibility to aspergillosis in CF due to an exuberant inflammatory response that compromises the host’s ability to control the infection. Preventing NLRP3 activation and reducing IL-1β secretion reduced infection severity in chronic granulomatous disease as well as CF. Thus, despite the key role in host protection against the fungus, the inflammasome/IL-1 pathway is tightly regulated to avoid an excessive inflammatory pathology.

Similarly, despite a clear protective role for IL-1R signalling and NLRC4 in the innate immunity against *Pseudomonas aeruginosa*, the most common pathogen in CF, deregulated inflammasome signalling also aggravates *P. aeruginosa* pneumonia in CF and conditions of non-CF. The fact that *P. aeruginosa* has adopted mechanisms to inhibit NLRC4-mediated caspase-1 activation further suggests the importance of this pathway against Gram-negative bacterial pneumonia. Thus, the inflammasome may represent a potential target to limit the pathological consequences of pulmonary infections in CF.

In the present study, we determine the relative contribution of different inflammasomes to infection and inflammation in CF mice, assessed the therapeutic efficacy of the recombinant IL-1R antagonist (IL-1Ra), anakinra, and evaluated whether genetic variations in the inflammasome/IL-1R signalling could contribute to microbial colonization and inflammation in human CF. We found that NLRP3 more than NLRC4 contributes to IL-1β-dependent inflammation in murine and human CF. Pathogenic NLRP3 activity, however, is negatively regulated by IL-1Ra, thus providing a therapeutic angle to ameliorate the pathological consequences of microbial colonization in CF.

**Results**

**Dysregulated inflammasome activity in murine CF.** To evaluate inflammasome activity in murine CF, we infected *Cfrr⁻/⁻* and C57BL/6 mice intranasally with *A. fumigatus* or intratracheally with *P. aeruginosa* and evaluated IL-1β, IL-1α and IL-18 production, caspase-1 cleavage and expression of NLRP3 or NLRC4 for their involvement in response to both *A. fumigatus* and *P. aeruginosa*.

NLRP3 and NLRC4 activity is non-redundant in lung infections. To unravel the functional activity of either NLRP3 or NLRC4 in these infection models, we assessed Nlrp3⁻/⁻ or Nlrc4⁻/⁻ mice for susceptibility to either infection and inflammasome activity.
Figure 1 | Dysregulated inflammasome activity in murine CF. C57BL/6 and Cftr−/− mice (n = 6 for all groups) were infected intranasally with live A. fumigatus conidia or P. aeruginosa and assessed for IL-1β, IL-18 and IL-1α gene expression and cytokine production in lung homogenates of A. fumigatus-(a) or P. aeruginosa-(b) infected mice at different days post-infection (dpi) by RT–PCR and specific ELISA; (c) Caspase-1 cleavage by immunoblotting with specific antibodies and corresponding pixel density ratio normalized against corresponding β-actin; Nlrp3 and Nlrc4 gene expression (d) by RT–PCR in lung tissues and protein expression (e, f) by lung immunofluorescence staining with anti-NLRP3 antibody followed by anti-rabbit TRICT (e) and (f) anti-NLRC4 antibody and anti-phospho(p)NLRC4 followed by anti-rabbit TRICT and anti-hamster FITC. Cell nuclei were stained blue with DAPI. Representative images were acquired with a high-resolution Microscopy Olympus DP71 with a × 20 objective. Scale bars, 200 μm, inset 50 μm. Note the expression on lung epithelium cells in the inset. Scanning densitometry was done with Image Lab 3.1.1 software. Data are representative (immunoblotting) or pooled from three experiments and presented as mean ± s.d. for all bar graphs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, C57BL/6 versus Cftr−/− mice at different dpi, Two-way ANOVA, Bonferroni post hoc test. For NLRP3 or NLRC4 quantification, see Supplementary Fig. 1.
Figure 2 | Different NLRP3 and NLRC4 expression in lung cells from CF mice. C57BL/6 and Cftr<sup>−/−</sup> mice (n = 6 for all groups) were infected intranasally with live A. fumigatus conidia or P. aeruginosa and assessed for (a) NLRP3 and (b) NLRC4 protein expression in the lungs and lung epithelial and myeloid cells (magnified in the insets) by immunohistochemistry. Cell nuclei were counterstained with haematoxylin. Representative images of two independent experiments were acquired with a × 20 and × 60 (inset, using EVOS FL Color Imaging System) objective. Scale bar, 200 µm. Immunofluorescence staining with (c) NLRP3 followed by anti-rabbit TRICT or (d) pNLRC4 and NLRC4 of epithelia cells and macrophages purified from lungs and neutrophils from the peritoneal cavity of C57BL/6 and Cftr<sup>−/−</sup> uninfected mice exposed in vitro to LPS + ATP, flagellin, A. fumigatus live conidia or P. aeruginosa. Cell nuclei were counterstained blue with DAPI. Images were acquired a high-resolution Microscopy Olympus DP71 using a × 100 objective. Scale bar, 12.5 µm. For number of cells with positive NLRP3 or NLRC4 expression quantification, see Supplementary Fig. 1.
Nlrp3−/− mice showed increased resistance to either infection, as revealed by the reduced fungal or bacterial load (Fig. 4a), IL-1β production (Fig. 4b) and lung inflammatory cell recruitment (Fig. 4c). Only infected with a high number of Aspergillus conidia were Nlrp3−/− mice more susceptible and unable to restrict fungal growth and inflammatory pathology (Supplementary Fig. 5). These data suggest that NLRP3 and NLRC4, despite having overlapping functions, may

In addition, blocking Nlrp3 with siRNA increased resistance to Aspergillus infection in both C57BL/6 and Cfr−/− mice, as revealed by decreased lung colony-forming units (CFUs; Fig. 4e) and pathology as well as decreased positive staining for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL; Fig. 4f), a marker of both apoptotic and pyroptotic cell death30. In contrast, blocking Nlrc4 did not affect the susceptibility of C57BL/6 or Cfr−/− mice (Fig. 4e,f). Similar results were obtained with P. aeruginosa infection (Supplementary Fig. 5). These data suggest that NLRP3 and NLRC4, despite having overlapping functions, may
have a different role in these lung infections whereby NLRP3 may contribute to pathogenic inflammation in the relative absence of NLRC4. As such, these results are consistent with the high or low susceptibility to bacterial or fungal pneumonia observed in mice with a high or low inflammasome activity, such as Il1r8–/– and Il1r1–/– mice, respectively, in which we found that NLRP3 expression was more robust in the former than in the latter (Supplementary Fig. 6). Thus, IL-1RI signalling leading to NLRP3 activation may promote deleterious inflammation in response to A. fumigatus and P. aeruginosa in CF.

**Figure 4** NLRP3 and NLRC4 are non-redundantly activated in lung infections. C57BL/6, Nlrp3−/− and Nlrc4−/− mice (n = 6 for all groups) were infected intranasally with live A. fumigatus conidia or P. aeruginosa and assessed for (a) fungal or bacterial growth at different dpi; (b) IL-1β production in BAL fluids (c) and lung histology at 7 dpi (periodic acid–Schiff staining) (% of neutrophils in the bronchoalveolar lavage are shown in the insets). (d) Survival, (e) fungal growth and (f) lung histology of C57BL/6 and Cftr−/− mice infected with A. fumigatus conidia and treated with specific Nlrp3, Nlrc4 siRNA or scrambled siRNA. Fungal growth (log CFU, mean ± s.d.) and histology were assessed at 7 dpi in (d) and (e) periodic acid–Schiff staining and increased deposition of DNA on lung parenchyma cells on TUNEL staining. Cell nuclei were stained blue with DAPI. Representative images of two independent experiments were acquired using EVOS FL Color Imaging System with a × 20 objective for histology (Scale bar, 100 μm) and a high-resolution Microscopy Olympus DP71 using a × 40 objective for histology (Scale bar, 100 μm) and a high-resolution Microscopy Olympus DP71 using a × 40 objective for TUNEL (Scale bar, 50 μm). Data pooled from three experiments and presented as mean ± s.d. for all bar graphs.

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, C57BL/6 versus Nlrp3−/−, Nlrc4−/− or Cftr−/− mice at different dpi (a,b) or untreated (none) versus siRNA treated mice (e), Two-way (a,b) and One-way ANOVA (e) Bonferroni post hoc test.

**NLRC4 induces IL-1Ra that dampens NLRP3 activity.** The protective action of NLRC4 in Gram-negative bacterial pneumonia is mostly carried out in cooperation with NLRP3 to drive robust IL-1β signalling31,33. However, the precise mechanism(s) by which NLRC4 mediates its effects in the lung remains to be determined. IL-1Ra is a potent suppressor of inflammasome activity34. Although most intracellular IL-1Ra remains in the cytoplasm of cells, some isoforms may be released from airways ECs in some conditions and may act as extracellular receptor antagonists of IL-1RI (ref. 35). We have evidence that NLRC4 activation in response to Candida albicans resulted in a...
sustained production of IL-1Ra capable of restraining NLRP3 activity. Should this mechanism be operative in the lung, this would suggest that NLRP3 expression is sustained in Nlrc4−/− as well as Il1ra−/− mice and that NLRC4 contributes to IL-1Ra production in either bacterial or fungal pneumonia. We observed a robust Nlrp3 gene (Fig. 5a) and protein (Fig. 5b) expression in Nlrc4−/− or Il1ra−/− (Fig. 6m) infected mice as well as the major contribution of NLRC4 to IL-1Ra production. Indeed, the levels of IL-1Ra were sustained in vitro (Fig. 5c) and through the course of either infection in vivo (Fig. 5d) in C57BL/6 but not Cftr−/− mice and NLRC4, more than IL-1Ra, contributed to this sustained production (Fig. 5d). Accordingly, IL-1Ra was deficient upon blocking TLR5 and NAIP5, both in vitro and in vivo (Supplementary Fig. 7a, b), as well as in A/J mice (Supplementary Fig. 3b) and was associated with increased IL-1β production and inflammatory responses. The opposing findings observed with flagellin (Supplementary Fig. 7a, b), further suggest the protective role of the TLR5/NLRC4 axis in infection. Altogether, these results suggest that NLRC4 and IL-1Ra deficiencies may contribute to NLRP3-mediated pathogenic inflammation in CF and that limiting NLRP3 via IL-1Ra could be of benefit in CF mice.

Anakinra protects from infections and inflammation. These observations prompted us to investigate whether anakinra would ameliorate lung inflammatory pathology in CF. We treated C57BL/6 and Cftr−/− mice infected with either Aspergillus or Pseudomonas with 10 mg kg−1 anakinra, a dose known to mimic human therapeutic dosages and to be pharmacologically active in mice. Anakinra significantly increased survival of Cftr−/− mice to P. aeruginosa infection (Fig. 6a) while reducing bacterial burden (Fig. 6b), neutrophil recruitment and lung damage (Fig. 6c). In addition, anakinra reduced caspase-1 cleavage (Fig. 6d), IL-1β production (Fig. 6e) and lung NLRP3 expression (Fig. 6f). Similar results were obtained in Aspergillus-infected Cftr−/− mice (Fig. 6g–k). Of interest, anakinra greatly increased the resistance to A. fumigatus infection (Fig. 6l), while decreasing NLRP3 expression (Fig. 6m), of the highly susceptible Il1ra−/− mice. Mechanistically, regulation of NLRP3 expression by anakinra occurs at posttranslational level via the ubiquitin proteasome system (Supplementary Fig. 8), a degradation pathway known to regulate NLRP3 half life. Altogether, these results indicate that by impairing neutrophil recruitment, anakinra may ameliorate lung pathology, without adversely affecting pathogen clearance. Of great interest, anakinra treatment was apparently more potent than the inhibition of IL-1Ra by a neutralizing antibody (Supplementary Fig. 9), a finding suggesting that the beneficial effect of anakinra may go beyond inflammasome inhibition.

In this regard, autophagy is a mechanism involved in the intracellular defense against both A. fumigatus and P. aeruginosa and is induced by anakinra. We monitored the effects of anakinra on autophagy induction in response to live conidia or bacteria in lung macrophages purified from C57BL/6 or Cftr−/− mice. Anakinra restored the defective autophagy in CF cells, as seen by LC3 immunofluorescence (Fig. 7a) and immunoblotting (Fig. 7b). Through autophagy, anakinra also increased the microbicidal activity of macrophages (Fig. 7c). Autophagy is activated by ROS and inhibited by chloroquine, a lysosomotropic agent that inhibits the fusion of autophagosome with lysosome and lysosomal protein degradation. Accordingly, the anakinra activity was inhibited by chloroquine and the NADPH oxidase inhibitor diphenyleneiodonium DPI, thus indicating that anakinra activates the autophagy/lysosomal degradation pathway.
pathway. However, the anakinra activity was also inhibited by lactacystin, a known inhibitor of the proteasomal degradation pathway. Thus, the activity of anakinra seems to rely on both the autophagy and the proteasome system, two cooperative and complementary degradation pathways. Consistently, anakinra modified the intracellular routing of live conidia on phagocytes.

We followed the intracellular localization of green fluorescent protein (GFP)-conidia on lung macrophages purified from C57BL/6 and Cfr−/− mice by determining the colocalization and quantifying the degree of overlap with either the lysosomal-associated membrane protein 1 (LAMP1) or the 20S proteasome associated membrane protein (LAMP1) or the 20S proteasome (Figure 7d). Figure 7d shows that phagocytosed GFP-fluorescent protein on lung macrophages of anakinra-treated mice. Assays were done at 7 dpi. (f) Fungal growth (log CFU, mean ± s.d.) and (m) lung histology (periodic acid-Schiff staining and NLRP3 immunofluorescence staining in the inset) of A. fumigatus-infected and anakinra-treated Il1ra−/− mice. Representative images of two independent experiments were acquired using EVOS FL Color Imaging System with a × 40 objective for histology (Scale bar, 100 μm) and a high-resolution Microscopy Olympus DP71 using a × 20 objective for TUNEL and immunofluorescence staining (Scale bar, 200 μm, inset 50 μm). Data pooled from three experiments and presented as mean ± s.d. for all bar graphs.

*P < 0.05, **P < 0.01, ***P < 0.001, anakinra treated versus untreated mice (none), One-way ANOVA (b,h), Two-way ANOVA (e,j) Bonferroni post hoc test and two-sides Student’s t-test. (f) For NLRP3 quantification, see Supplementary Fig. 1.

Figure 6 | Anakinra protects Cfr−/− mice from infections and NLRP3 inflammation. C57BL/6 and Cfr−/− mice (n = 6 for all groups) were infected intranasally with live A. fumigatus conidia or P. aeruginosa and treated with anakinra (100 mg kg−1 per day) throughout the infection. (a,g) Survival, (b,h) fungal growth (log CFU, mean ± s.d.), (c,i) lung histology (periodic acid-Schiff staining) and reduced deposition of DNA on lung parenchyma cells by TUNEL; (d) caspase-1 cleavage by immunoblotting with specific antibodies (scanning densitometry was done with Image Lab 3.1.1 software. Representative of three independent experiments and corresponding pixel density ratio normalized against actin); (e,j) IL-1β levels in lung homogenates; NLRP3 protein expression by immunofluorescence staining (f) and immunoblotting (k) of lungs of anakinra-treated mice. Assays were done at 7 dpi. (i) Fungal growth (log CFU, mean ± s.d.) and (m) lung histology (periodic acid-Schiff staining and NLRP3 immunofluorescence staining in the inset) of A. fumigatus-infected and anakinra-treated Il1ra−/− mice. Representative images of two independent experiments were acquired using EVOS FL Color Imaging System with a × 40 objective for histology (Scale bar, 100 μm) and a high-resolution Microscopy Olympus DP71 using a × 20 objective for TUNEL and immunofluorescence staining (Scale bar, 200 μm, inset 50 μm). Data pooled from three experiments and presented as mean ± s.d. for all bar graphs.
conidia rapidly (at 5 min) colocalized with LAMP1 in either type of macrophages. Colocalization was increased by anakinra. However, at 45 min, anakinra reduced the colocalization with LAMP1, while it increased the colocalization with the 20S proteasome (Fig. 7d). Consistent with the regulation of NLRP3 expression (Supplementary Fig. 8), the proteosomal degradation pathway in RAW cells (Fig. 7e) and in CF-HBE cells (Fig. 7f) was promoted by anakinra. Thus, anakinra not only controls the intracellular routing of pathogens upon phagocytosis through autophagy, but also reduces and NLRP3’s half-life possibly via the proteasome system, and therefore controls both pathogen clearance and inflammation.

Anakinra inhibits inflammasome activation in human CF. To assess whether anakinra would also be able to inhibit NLRP3 activation in human CF bronchial ECs (CF-HBE), we evaluated NLRP3 and NLRC4 protein levels in primary HBE from non-CF patients and CF patients46 after 4 h of exposure to A. fumigatus, P. aeruginosa and/or anakinra. NLRP3, but not NLRC4, expression was increased in the presence of a pathogen in CF-HBE compared to control cells. Anakinra reduced the intensity of NLRP3 staining (Fig. 8a) and, concurrently, IL-1β production (Fig. 8b) without significantly affecting NLRC4 staining. Of interest, the levels of IL-1Ra were significantly lower in CF-HBE (Fig. 8c) and CF expectorates (Fig. 8d) as compared with controls, a finding indicating a defective NLRC4 activity in human CF.

NLRC4 and IL1RN polymorphisms affect microbial colonization.

To assess whether genetic variants in the inflammasome/IL-1RI signalling pathway, and particularly genetic NLRC4 deficiency, are risk factors for specific microbial colonization in CF, we evaluated nine gene variants (four in NLRP3, two in NLRP3, one in IL1B and one in IL1RN) in 284 CF patients (Supplementary Table 1). These variants were successfully genotyped in the samples (genotyping rate 94.3–100%), without displaying any deviation from the Hardy–Weinberg Equilibrium (Supplementary Table 2). Linkage disequilibrium (LD) analyses of the single-nucleotide polymorphisms (SNPs) in NLRC4 and NLRP3 (r² = 0.03), failed to reveal the presence of significant LD blocks (Supplementary Fig. 10). For A. fumigatus colonization, significant associations were found for NLRC4 ACTT haplotype (odds ratio (OR) = 2.930, P = 0.025) and for NLRP3 rs212704 G/G genotype (OR = 0.303, P = 0.030; Table 1). To establish the functional effects of these SNPs we evaluated NLRC4 expression in lung expectorates from genotyped CF patients. The AA genotype at rs212704, the CC genotype at rs455060, the TT genotype at the rs7562653 and the TT genotype at rs385076 show a reduction in NLRC4 expression level compared with other genotypes (Fig. 8e). Therefore, genetic NLRC4 deficiency, but not NLRC4 sufficiency (rs212704), predisposes CF patients to Aspergillus colonization. No significant associations were found between Aspergillus colonization and the other genetic variants (Supplementary Tables 3–6) and no significant gene–gene interaction was detected (Supplementary Table 7). For P. aeruginosa colonization, no significant association was detected either at haplotype and single marker level (allele and genotype) tests (Supplementary Tables 8–11). However, on performing the generalized-multifactor dimensionality reduction analysis, two significant gene–gene interaction models (Table 1), involving NLRC4 and IL1RN gene variants, significantly increased the risk of Pseudomonas colonization (NLRC4 rs212704 × IL1RN VNTR, OR = 3.018, Sign test P = 0.001; NLRP3 rs212704 × NLRP3 rs385076 × IL1RN VNTR, OR = 4.212, Sign test P = 0.001; Fig. 8f). These data indicate that genetic deficiency of NLRC4, either alone or in combination with IL-1RN, could be a risk factor for specific microbial colonization in the lungs of patients with CF.

Discussion

The present study shows that the interplay between NLRP3 and NLRC4 governs host innate immune response and inflammation to colonizing microbes in murine and human CF. As infections have a negative impact on pulmonary functions in CF47, categorizing microbial colonization versus infection may help stratify CF patients for preventive treatment. NLRP3 activation contributed to neutrophil recruitment in both A. fumigatus and P. aeruginosa infections, a finding confirming the contribution of NLRP3, either alone4,20 or in association with other cytoplasmic sensors24, to the inflammatory responses in the lung. However, inflammation and tissue damage were greatly reduced in Nlrp3−/− infected mice and NLRP3-dependent neutrophil infiltration and proinflammatory cytokine responses were associated with disease severity in conditions of unrestrained NLRP3 activity, such as in highly susceptible Il1r8−/−, Il1ra−/− or Nlrp4c−/− mice. NLRP3 activity was instead defective in Il1r1−/− mice, in which the attenuated IL-1β production was concomitant with a reduced disease severity during infections. While it is conceivable that the effect functional activity of NLRP3 in the lung is also contingent, at least for A. fumigatus, on the fungal load (Supplementary Fig. 4), the fungal strain and route of infection15,24, our results indicate that the NLRP3 inflammasome is tightly regulated to avoid its aberrant activation6.

Indeed, NLRP3 was significantly increased in murine and human CF cells and its inhibition ameliorated inflammatory pathology in murine experimental infections and attenuated IL-1β production in human CF cells. The increased NLRP3 inflammatory response could be intrinsic to cells lacking CFTR due to the mitochondrial Ca²⁺ perturbation20. However, we found that NLRP3 activity could also be counteracted by the sustained production of IL-1Ra, to which NLRC4 greatly contributed. Several lines of evidence suggest an important function of NLRC4 for caspase-1 activation in response to intracellular as well as extracellular Gram-negative bacterial infection of the lung17,18,21,27. Activation of NLRC4 can be mediated by cytosolic bacterial flagellin26,27 and/or its cognate recognition by TLR5 (ref. 19), or be flagellin-independent through the type III secretion system18,48. In addition, activation of NLRC4 occurs after the assembly with members of the immune sensors NAIPs family48 that control ligand-dependent oligomerization of NLRC4 and may involve phosphorylation of the serine residue 553 by PKCδ (ref. 25).

Aspergillus, we found that NLRP3 activation occurs through the TLR5/NAIP5/NF-κB-dependent pathway. Because both Tlr5 and Naip5 expressions failed to upregulate in the lungs of CF mice, decreased expression of TLR5 was observed in CF macrophages50, and TLR5 gene variations influenced CF lung function51, these findings may account for the defective NLRP3 activation in CF. However, NLRP3 activation was also sensitive to calcium signalling. How the TLR5/NAIP5 and calcium signalling pathway specifically interact in NLRP3 activation and how it is affected by CFTR deficiency is unknown. However, it is intriguing that a Ca²⁺ flux occurs downstream TLR5 activation52 and tyrosine phosphorylation of PKCδ is calcium dependent53. Whatever the case, the increased [Ca²⁺]i and Ca²⁺-dependent signalling, known to promote NLRP3 activation44, actually restrains NLRP3 activation, a finding further pointing to the different regulation of either inflammasome in Aspergillus infection.

The defective NLRC4 activity observed in murine and human CF is a novel finding that may open new perspectives in the pathogenesis and therapy of inflammatory lung diseases in CF.
Anakinra decreased neutrophil infiltration, ameliorated tissue damage and inflammation against both *A. fumigatus* and *P. aeruginosa* infection, while decreasing NLRP3 activity in both murine and human CF. This occurred through the lysosomal pathway, through which pathogen clearance was promoted, and the ubiquitin/proteasomal degradation pathway through which NLRP3 activity was specifically controlled. Thus, anakinra appears to fulfill the requirement of an ideal immunomodulatory molecule capable of exerting pathogen control with minimum pathology. In addition, a recent report suggests that IL-1β, which is released when ECs become necrotic due to hypoxia, also significantly contributes to neutrophilic infiltration in the lungs of CF mice and this could be ameliorated by the use of anakinra. IL-1β can induce IL-1β and results in an autoinflammatory loop that is also dependent on caspase-1 activation. Therefore, since anakinra will block both the effects of IL-1β and IL-1β and in addition has impact on several key inflammatory mechanisms such as NLRP3 inflammasome activation and autophagy, anakinra might be beneficial in CF by targeting multiple pathogenic mechanisms.

Finally, in line with the functional experimental data, the genetic analysis supports the role of NLR4 in determining the state of microbial colonization of CF patients. In particular, the significant association of the intronic SNP rs212704 in NLR4, leading to defective NLR4 expression, with *A. fumigatus* colonization in CF patients suggests the presence of an 'immunogenetic' background predisposing CF patients to specific microbial colonization. Moreover, consistent with the different activation of NLR4 by *Aspergillus* or *Pseudomonas*, the gene–gene interaction analyses clearly showed that NLR4 SNPs act synergistically with II1RN VNTR60 to increase the susceptibility to *P. aeruginosa* colonization. Notably, the opposite (protective) allele II1RN*2 has been previously associated with higher IL-1RA and lower IL-1β release.

In conclusion, our study shows that the elucidation of pathogenic, inflammasome-dependent mechanisms at the molecular level may help the development of novel strategies for patient stratification and personalized tailored approaches in human CF.

**Methods**

**General experimental approaches.** No samples, mice or data points were excluded from the reported analyses. No randomization procedure was implemented, however, mice were randomly assigned to group allocation at the time of purchase to minimize any potential bias. No blinding was applied on harvesting cells after the treatments.

**Mice.** Six to 8-week female mice were used in all experiments. C57BL/6 mice were purchased from Charles River (Calco, Italy) and genetically engineered homoygote Cfrtsr−/− (Cfrtsr−/−) mice gut-correted on C57BL/6 backgrund were bred under specific pathogen-free conditions at the Animal Facility of San Raffaele Hospital, Milan, Italy. Nlrp3−/−, Nlrc4−/− and Ilrta−/− mice on the C57BL/6 background were bred under specific pathogen-free conditions at the Animal Facility of the University of Perugia, Perugia, Italy. Breeding pairs of Nlrc4−/− were obtained from Genethex (South San Francisco, CA, USA). Breeding pairs of Nlrp3−/− were obtained from Dr Alessandra Mortellaro (Singapore Immunology Network, Agency of Science, Technology and Research, Biopolis, Singapore). Ilrta−/− and Ilrta−/− mice on the C57BL/6 background were bred under specific pathogen-free conditions at the Animal Facility at the Humanitas Hospital, Milan, Italy.

**Infections and treatments.** Viable conidia (95%) of *A. fumigatus* (AF293) were obtained by growth on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI, USA) supplemented with chloramphenicol for 3 days at room temperature. Mice were anesthetized by intraperitoneal injection of 2.5% xylazine (Sigma Chemical Co, St. Louis, MO) before intranasal instillation of a suspension of 2 × 107 conidia per 20 μl saline and/or flagellin (Sigma-Aldrich) at 100 ng per 20 μl saline. For *P. aeruginosa* infection, clinical *P. aeruginosa* strain, isolated from a patient, was obtained from the Diagnostic Unit of Microbiology from the University of Perugia. The bacteria were grown for 3 h to reach exponential phase. Next, the bacteria were pelleted by centrifugation (2,700g, 15 min), washed twice with sterile PBS and the OD of the bacterial suspension was adjusted by spectrophotometry at 600 nm. The intended number of cfu was extrapolated from a standard growth curve. Appropriate dilutions with sterile PBS were made to prepare the inoculum before intranasal instillation of 3 × 105 CFU per mouse. Mice were monitored for fungal growth and histology. Microbial growth was expressed as log10 CFU per mouse.
organ, mean ± s.d. For histology, paraffin-embedded sections (3–4 μm) were stained with Periodic acid-Schiff (PAS). BAL fluid collection was done as described. Differentials were determined by examination of cytospin slides after May Grunwald Giemsa staining (Sigma-Aldrich). Histology sections and cytospin preparations were observed using EVOS FL Color Imaging System (Fisher Scientific) and images were captured using high-sensitivity monochrome Sony ICX285AL CCD camera. Mice were treated intraperitoneally, from the day of infection until the end of the experiment, with anakinra 10 mg kg⁻¹, daily, given the half-life of IL-1Ra of 6–8 h (ref. 62) or with 10 μg per mouse anti-IL1β antibody (RD systems, clone 30311) intranasally at the day of the infection and continuing every other day throughout the experiment.

Cell preparation and culture. RAW264.7 cells (ATCC) were grown in RPMI 1640 medium supplemented with 1% Pen–Strep, 10 μM l-glutamine, 1% HEPES.
(Lonza, Basel Switzerland), 40 mg mL−1 gentamicin (Fisiopharma), 0.1% β-mercaptoethanol ( Gibco, Thermo Scientific) and 10% FCS (Invitrogen, Life Technologies) at 37 °C in 5% CO₂. Lung fluids were isolated from total lung cells by washing and resuspending lung fragments in prewarmed HBSS with 0.1 mg mL−1 DNase I and incubated at 37 °C for 1 h to allow enzymatic digestion. After blocking enzymatic activity with the addition of PBS the residues were magnetically separated with magnetic beads (Miltenyi Biotech). Macrophages were isolated after adherence at 37 °C for 30 min with magnetic beads (Miltenyi Biotech) from the peritoneal cavity of infected mice 8 h after the intraperitoneal injection of 1 ml endotoxin-free 10% thioglycollate solution. Cells were plated in a 8-well culture slide or 12-well culture plate and stimulated for 4 h at 37 °C with 10 ng mL−1 Bagellin, 10 μg mL−1 LPS (Sigma-Aldrich), live Aspergillus conidia or *P. aeruginosa* at the ratio cell: microbes (1:1) or fungal antigens (10 μg mL−1). ATP (Sigma-Aldrich), 2 mM, was added 30 min before the end of the experiment. EDTA 2 mM was added at the same time as the infection. Cells were then fixed with 4% paraformaldehyde and stained with anti-NLRP3 (Cat. Number ab4207, Abcam) followed by goat anti-mouse Alexa Fluor 555 (Clone Poly4053, Biologend) or anti-pNLRC4 (Genetech) followed by polyclonal anti-hamster FITC (Sigma-Aldrich) and Iafp (Cat. Number 06-1125, Millipore) followed by polyclonal anti-rabbit TRITC (Sigma-Aldrich). All primary antibodies were used at the concentration of 0.5 μg mL−1 and secondary TRITC or FITC conjugated antibodies at 1 μg mL−1. Immunostained samples were used to fluorescent microscopy BX51 Olympus with a ×20 objective and the analySIS image processing software (Olympus).

![Image](https://via.placeholder.com/150)

**Figure 8 | Anakinra inhibits inflammasome activation in human CF.** (a) NLRP3 or NLRC4 staining of human bronchial epithelial (HBE) cells homozygous for ΔF508 mutation and control cells exposed to *P. aeruginosa* or *A. fumigatus* conidia at cells: microbes ratio of 1:1, and/or 10 μg mL−1 of anakinra. Images were acquired using the Olympus BX51 fluorescence microscope with a ×40 objective. Scale bar, 12.5 μm. DAPI was used to detect nuclei. Representative images of two independent experiments from three patients. Histograms indicate per cent of human bronchial epithelial cells with positive NLRP3 or NLRC4 expression. (b) IL-1b or (c) IL-1Ra in the supernatants of HBE cells from control or two CF patients exposed as above and (d) IL-1Ra levels in expectorates from control or CF patients (ELISA). (e) NLR4 expression by RT-PCR of CF patients carrying diverse genotypes at rs212704, rs455060, rs7562653 and rs3850767. (f) Best three-factor model. Dark grey and light grey boxes correspond to the high- and low-risk genotype combinations, respectively. The left and right bars within each box correspond to *Pseudomonas* + and *Pseudomonas*, respectively. The top number above each bar is the sum of scores for the corresponding group of individuals. The heights of the bars are proportional to the sum of scores in each group. Data pooled from two experiments and presented as mean ± s.d. for all bar graphs. *P < 0.05, **P < 0.01, ***P < 0.001, treated versus untreated (none) (a,b) or control versus CF patient (c,d). Two-way ANOVA (a-c) Bonferroni post hoc test and two-sides Student’s t-test (d).
Human bronchial epithelial cells. HBE cells, homozygous for the ΔF508 mutation and its isogenic wild type were obtained from lung transplants (CF patients) or lung resections (non-CF patients) from and cultured as described. Cells were maintained at 37°C in a humidified incubator in an atmosphere containing 5% CO₂, and the experiments were done 5 days after plating. Cells were exposed to *A. fumigatus* conidia or *P. aeruginosa* at cells:microbes ratio of 2:1, and treated with 10 μg ml⁻¹ anakinra or vehicle. Cells were incubated for 4 h at 37°C in 5% CO₂ (as indicated by preliminary experiments). Cultures growing on culture slides were fixed for 20 min in PBS containing 4% paraformaldehyde then incubated with human anti-NLRP3 at 4°C in PBS containing 3% normal bovine serum albumin, washed and incubated with anti-mouse-TRITC secondary antibody (Sigma-Aldrich). Images were acquired using the Olympus BX51 fluorescence microscope with a × 40 objective and the analySIS image processing software (Olympus). DAPI was used to detect nuclei.
Reverse transcriptase-PCR and ELISA. The levels of cytokines in lung homogenates and supernatants were determined by ELISAs (R&D Systems). Real-time RT–PCR was performed using the BioRad CFX96 System and SYBR Green chemistry (BioRad). Cells were lysed and total RNA was reverse transcribed.

Amplification efficiencies were validated and normalized against GAPDH. The thermal profile for SYBR Green real-time PCR was at 95°C for 3 min, followed by 40 cycles of denaturation for 30 s at 95°C and an annealing/extension step of 30 s at 60°C. Each data point was examined for integrity by analysis of the amplification plot. The messenger RNA (mRNA)–normalized data were expressed as relative gene mRNA in treated compared with untreated experimental groups or cells.

Western blotting. Blots of cell lysates were incubated with antibodies against the following proteins: Caspase-1p10 (M-20 Cat. Number sc-514, Santa Cruz Biotechnology), NLRP3 (Cat. Number ab4207, Abcam), NLRC4 (Cat. Number 06-1125, Millipore) and pNLRC4 (Genetech), LC3b-I or LC3b-II (Abcam) followed by IgG–HRP-conjugated secondary antibody (Sigma–Aldrich) after separation in 12% Tris/glycine SDS gel and transfer to a nitrocellulose membrane. Normalization was performed probing the membrane with mouse-anti-β-actin antibody (Sigma–Aldrich). Chemiluminescence detection was performed with LiteAblotPlus chemiluminescence substrate (Euroclone S.p.A), using the ChemiDocTM XRS+ Imaging system (Bio-Rad), and quantification was obtained by densitometry image analysis using Image Lab 3.1.1 software (Bio-Rad). Images have been cropped for presentation. Full size images are presented in Supplementary Figs 12-14.

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Human studies

Patient data. A prospective multicenter longitudinal genetic association study involving 284 patients of Caucasian origin who had a proven diagnosis of CF (CFTR genotyping, sweat testing and clinical phenotype) was performed (Supplementary Table 1). Clinical records from each patient were reviewed and clinical data including age, gender, lung function testing, measures of nutrition, microbiological findings and vital status were abstracted. A. fumigatus or P. aeruginosa positivity was defined as the presence of persistent positive Aspergillus cultures, but negative galactomannan and no immunological responses or persistent, for at least 6 months, Pseudomonas, respectively.

SNPs selection and genotyping. Patients provided a blood specimen for DNA isolation performed using the QIAamp DNA Mini (Qiagen, Milan, Italy) following microbiological findings and vital status were abstracted. Clinical data including age, gender, lung function testing, measures of nutrition, microbiological findings and vital status were abstracted. A. fumigatus or P. aeruginosa positivity was defined as the presence of persistent positive Aspergillus cultures, but negative galactomannan and no immunological responses or persistent, for at least 6 months, Pseudomonas, respectively.

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Table 1 | Haplotype (a) and genotype (b)* association study between NLRC4 and Aspergillus infection; (c) best models assessed by the GMDR for one to five-way combinations, to test gene–gene interactions in determining Pseudomonas infection.

| (a) | NLRC4 SNPs | Asp – | Asp + | OR | P value |
|---|---|---|---|---|---|
| rs212704 | rs455060 | rs7562653 | rs385076 |
| H1 | A | T | C | C | 122 (40.8%) | 34 (47.6%) | Reference |
| H2 | G | C | T | T | 72 (24.1%) | 20 (28.9%) | 0.875 | 0.729 |
| H3 | G | T | C | C | 39 (13.1%) | 8 (10.8%) | 0.812 | 0.644 |
| H4 | G | C | C | T | 32 (10.7%) | 1 (1.4%) | 0.169 | 0.177 |
| H5 | G | C | C | C | 11 (3.8%) | 1 (1.4%) | 0.387 | 0.452 |
| H6 | A | T | T | C | 8 (2.7%) | — | 0.000 | 0.268 |
| H7 | A | C | T | T | 5 (1.7%) | 4 (5.3%) | 2.930 | 0.025 |
| H8 | A | T | T | T | 4 (1.4%) | 1 (1.4%) | 0.854 | 0.995 |
| H9 | A | T | C | C | 1 (0.4%) | 1 (1.4%) | 4.879 | 0.234 |
| H10 | G | C | T | C | — | 1 (1.4%) | 9.989e+013 | 0.149 |

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ILIRN were also studied. SNP genotyping was performed by KASPar assays (KBiosciences, Hertfordshire, UK) according to manufacturer’s instructions using Applied Biosystems 7500 Fast qPCR system (Life Technologies, Milan, Italy). The ILIRN VNTR polymorphism (86-bp repeat in intron 2) was analysed as previously described26. The PCR products were analysed by 2% agarose gel electrophoresis. Genotyping sets comprised randomly selected replicates of previously typed samples and two negative controls (water). Concordant genotyping was obtained for ≥99%.

Statistical analysis. Data are expressed as mean ± s.d. Horizontal bars indicate the means. Statistical significance was calculated by one- or two-way ANOVA (Bonferroni’s post hoc test) for multiple comparisons and by a two-tailed Student’s t-test for single comparison. Since the distribution of levels tested by Kolmogorov–Smirnov normality test turned out to be non-significant. We considered all P values ≤0.05 significant. The data reported are either representative from three representative experiments (histology, immunofluorescence, TUNEL and western blotting) or pooled otherwise. The in vivo groups consisted of 4–6 mice per group. Data were analysed by GraphPad Prism 4.03 program (GraphPad Software). No statistical method was used to predetermine sample size. For human data, Hardy–Weinberg equilibrium (HWE) was tested using Haploview v4.2. LD analysis was performed using Haploview, and defining LD blocks based on the solid spine of LD algorithm27. Case–control single marker and haplotype association tests were performed using UNPHASED28 under an additive model and adjusting for sex and age at sampling. Gene–gene interaction analyses were performed with the Generalized-Multifactor Dimensionality Reduction method (version 0.9)29. This method uses the same data-reduction strategy as the original MDR method30, but score-based statistics using maximum-likelihood estimates are introduced to classify classifier cells into two treatment groups31. As in MDR, the best n-locus model is selected based on the highest training accuracy, and the maximum balanced testing accuracy and cross validation consistency are used to determine the overall best epistasis model. Balanced testing accuracy is calculated using the formula (sensitivity + specificity)/2 to yield an unbiased estimate for unbalanced case–control studies. Last, the x2 and sign tests determined whether the factors are significantly associated with the phenotype of interest. In particular, a significant x2-test reveals that the SNP or interaction between SNPs is associated with phenotype tested, while a significant sign test suggests that the best model with one or more SNPs is significantly better than the null model. Two-tail P values are reported. Bonferroni’s correction for multiple testing was not performed since uncorrected hypotheses are reported. Bonferroni’s correction for multiple testing was not performed since we are assessing specific questions on genes involved in functional pathways related to Pseudomonas and Aspergillus infection and we are not searching for associations without a priori hypotheses31.

Study approval. Murine experiments were performed according to the Italian Approved Animal Welfare Authorization 360/2015-PR and Legislative decree 26/2014 regarding the animal licence obtained by the Italian Ministry of Health lasting for five years (2015–2020). Infections were performed under avertin anaesthesia, and all efforts were made to minimize suffering. Human studies approval was obtained from institutional review boards at each site and written informed consent was obtained from the participants, or, in case of minors, from parents or guardian.

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