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A mast cell-ILC2-Th9 pathway promotes lung inflammation in cystic fibrosis

Silvia Moretti1, Giorgia Renga1, Vasilis Oikonomou1, Claudia Galosi1, Marilena Pariano1, Rossana G. Iannitti1, Monica Borghi1, Matteo Puccetti1, Marco De Zuani2, Carlo E. Pucillo2, Giuseppe Paolicelli1, Teresa Zelante1, Jean-Christophe Renauld3, Oxana Bereshchenko4, Paolo Sportoletti5, Vincenzina Lucidi6, Maria Chiara Russo7, Carla Colombo7, Ersilia Fiscarelli8, Cornelia Lass-Flörl9, Fabio Majo6, Gabriella Ricciotti8, Helmut Ellemunter10, Luigi Ratclif11, Vincenzo Nicola Talesa1, Valerio Napolioni1 & Luigina Romani1

T helper 9 (Th9) cells contribute to lung inflammation and allergy as sources of interleukin-9 (IL-9). However, the mechanisms by which IL-9/Th9 mediate immunopathology in the lung are unknown. Here we report an IL-9-driven positive feedback loop that reinforces allergic inflammation. We show that IL-9 increases IL-2 production by mast cells, which leads to expansion of CD25+ type 2 innate lymphoid cells (ILC2) and subsequent activation of Th9 cells. Blocking IL-9 or inhibiting CD117 (c-Kit) signalling counteracts the pathogenic effect of the described IL-9-mast cell-IL-2 signalling axis. Overproduction of IL-9 is observed in expectorates from cystic fibrosis (CF) patients, and a sex-specific variant of IL-9 is predictive of allergic reactions in female patients. Our results suggest that blocking IL-9 may be a therapeutic strategy to ameliorate inflammation associated with microbial colonization in the lung, and offers a plausible explanation for gender differences in clinical outcomes of patients with CF.
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nate lymphoid cells (ILCs) perform a variety of immune functions at barrier surfaces1. Three types of ILCs have been reported, which differ on the basis of the cytokines produced. ILC1 encompass natural killer cells and interferons (IFN-γ)-γ-releasing cells; ILC2 release IL-5, IL-9 and IL-13, and ILC3 release IL-17A and IL-22. ILC2 preferentially localize to the interface between the host and the environment (lung, intestine and skin) and perform a variety of biological functions in mice and humans5. In the lung, ILC2 and their cytokines play pro-inflammatory roles in allergic inflammation2,4,3, but also protective roles in airway epithelial cell repair and control of tissue inflammation linked to pathogens6,7. Thus, ILC2 may affect tissue inflammation linked to pathogens6,7. Thus, ILC2 may affect protective roles in airway epithelial cell repair and control of protective outcomes. Lung ILC2 rapidly produces IL-5 and IL-13 for the efficient control of valid therapeutic strategy in CF (ref. 18). This balance is essential targeting specific inflammatory/anti-inflammatory pathways is a inflammatory cytokines in CF (ref. 17), providing evidence that in infection (from 2.5 ± 0.7 to 3.9 ± 1.0 log colony forming unit (cfu) ± s.d. per lung. C57BL/6 versus Cfr−/− mice, respectively) and allergy (from 9.2 ± 0.7 to 22.4 ± 1.3 ng ml⁻¹ total serum IgE in C57BL/6 and Cfr−/− mice, respectively). A peak production of IL-9 occurred during the first week of the infection in C57BL/6 mice to decline thereafter as opposed to Cfr−/− mice in which levels of IL-9 were sustained throughout the infection (Fig. 1a). Peak production of IL-9 was also observed in Rag1−/−, and less in Rag1−/−/Il9R−/− mice, early even not late in infection (Fig. 1a), a finding suggesting that early IL-9 production is IL-9R-dependent and late is T-cell-dependent. We looked therefore for the presence of IL-9+ ILC2 and Th9 cells in infection by characterizing IL-9-producing Lin− and CD4+ T cells in the lung. ILC2 are marked by expression of the IL-33R as well as the common γ chain (γc) cytokine receptors for IL-2 and IL-7 (ref. 2). Flow cytometry analysis revealed that CD90.2+ ILC2 expressing IL-33R or CD25 were present in the lung of naive C57BL/6 (4.5 and 3.3% for CD25+ and ST2+ cells, respectively) and Cfr−/− mice (5.8 and 4.0% for CD25+ and ST2+ cells, respectively, Fig. 1c). In C57BL/6 mice, and similarly in Rag1−/− mice (Supplementary Fig. 1a), ST2+ ILC2 cells decreased early in infection to return to baseline level 10 days later while CD25+ ILC2 stably decreased (Fig. 1b,c). In contrast, in Cfr−/− mice, both types of ILC2 steeply increased throughout the infection (Fig. 1b) along with the expression of the ILC2 transcription factors, Rora, and Gata3 (Fig. 1d) and the production of ILC2 effector cytokines, IL-5 and IL-13 (Fig. 1e). IL-9-producing CD90.2+ ILC2 were also expanded in Cfr−/− mice but not in C57BL/6 (Fig. 1b,c) and Rag1−/− mice (Supplementary Fig. 1a), as revealed by flow cytometry. In terms of Th9 cell activation, CD4+ IL-9+ T cells appeared in C57BL/6 mice a week after the infection to decline thereafter (Fig. 1h), consistent with the short retention of Th9 at the inflammatory sites22. The expansion was instead sustained in Cfr−/− mice (Fig. 1b) along with the expression of Il9, Pn.1 (purine-rich box 1) and Irf4 (interferon regulatory factor 4) transcription factors (Fig. 1g). These data indicate that IL-9+ ILC2 and Th9 cells are all increased in Cfr−/− mice during A. fumigatus infection.

Given that ILC1 through IFN-γ (ref. 22) and ILC3 through IL-22 (ref. 23) may affect ILC2 expansion, the differential expansion of ILC2 could reflect the ILCs dynamics in the lung. However, NKP46+NK1.1+ ILC1 cells producing IFN-γ did not expand and ILC1-promoting cytokines IL-15 and IL-18 were not produced in Cfr−/− as opposed to C57BL/6 mice (Supplementary Fig. 2a,b). Similarly, despite expanded in Cfr−/− mice, CCR6+ RORγt+ ILC3 produced IL-17A more than IL-22 (Supplementary Fig. 2c,d). Thus, while confirming the defective production of IFN-γ and IL-22 in Cfr−/− mice18, these findings suggest that the expansion of ILC2 in Cfr−/− mice is not dependent on ILCs dynamics in the lung but rather on the production of IL-22 promoting cytokines. This appeared to be the case, as the levels of cytokines promoting ST2+ ILC2, IL-33 and CD25+ ILC2, IL-2, were constantly elevated in Cfr−/− mice whereas a peak production was only observed at an early time point in C57BL/6 mice (Fig. 1f).

IL-9 contributes to inflammatory pathology in infection. To assess the role of IL-9 in A. fumigatus infection and allergy, we resorted to Il9R−/− mice that, given the crucial role of the IL-9R, a member of the γc receptor family, for the survival of lung ILC2 (ref. 8), also have a decreased ILC2 (ref. 8). Mice were either acutely infected with Aspergillus conidia intranasally or subjected to fungal allergy (ABPA) by repeated sensitization with Aspergillus culture filtrate extracts (5.5 ± 0.7 ng ml⁻¹ versus 11 ± 1.0, total serum IgE in Il9R−/− versus C57BL/6 mice,
respectively). We found that the absence of IL-9R signalling conferred resistance to both infection and allergy, as indicated by the reduced fungal load (Fig. 2a) and decreased inflammatory lung pathology in infection as well as in ABPA (Fig. 2b). The numbers of lung CD25+ and ST2+ ILC2 were decreased in these mice as revealed by immunofluorescence staining (Fig. 2c). Concomitantly, Th9 and, partially, Th2 cells—revealed by Stat6 expression and STAT5 phosphorylation (Supplementary Fig. 1b,c)—were decreased in both infection and allergy (Fig. 2d,e), while Th17 and Treg cells were unaffected and Th1 cells increased (Supplementary Fig. 3a,b). Corroborating these findings, Th9 cell activation was observed upon infection (dpi) in mice (six per group) infected intranasally with live A. fumigatus conidia. (a) Time course of IL-9 production at various days post infection (dpi) in mice (six per group) infected intranasally with live A. fumigatus conidia. (b) Detection of CD90.2+ CD25+, CD90.2+ ST2+ and CD90.2+ IL-9+ lung type 2 ILCs by flow cytometry (numbers refer to percentages of positive cells) and immunofluorescence staining. (c) Absolute number of lung ILC2; (d) ILC2-specific transcript on lineage negative lung cells; (e,f) ILC2 effector and activating cytokines; (g) IIL9 and Th9-cell specific transcripts on lung CD4+ T cells and (h) immunofluorescence staining of lung CD4+ IL-9+ T cells. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×40 objective; scale bar, 100 μm. Mean values ± s.d. cytokines were determined on lung homogenates by ELISA; IIL9 and transcripts assessed by PCR with reverse transcription. 0, uninfected mice. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, knockout versus C57BL/6 mice (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). Gata3, GATA binding protein 3; Irf4, interferon regulatory factor 4; Pui1, purine-rich box 1; Rora, RAR-related orphan receptor alpha.

**Figure 1** | IL-9 production and ILC2-Th9 cells activation in Aspergillus fumigatus infection. (a) Time course of IL-9 production at various days post infection (dpi) in mice (six per group) infected intranasally with live A. fumigatus conidia. (b) Detection of CD90.2+ CD25+, CD90.2+ ST2+ and CD90.2+ IL-9+ lung type 2 ILCs by flow cytometry (numbers refer to percentages of positive cells) and immunofluorescence staining. (c) Absolute number of lung ILC2; (d) ILC2-specific transcript on lineage negative lung cells; (e,f) ILC2 effector and activating cytokines; (g) IIL9 and Th9-cell specific transcripts on lung CD4+ T cells and (h) immunofluorescence staining of lung CD4+ IL-9+ T cells. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×40 objective; scale bar, 100 μm. Mean values ± s.d. cytokines were determined on lung homogenates by ELISA; IIL9 and transcripts assessed by PCR with reverse transcription. 0, uninfected mice. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, knockout versus C57BL/6 mice (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). Gata3, GATA binding protein 3; Irf4, interferon regulatory factor 4; Pui1, purine-rich box 1; Rora, RAR-related orphan receptor alpha.
**Figure 2 | IL-9R signaling contributes to inflammation and allergy.** C57BL/6 and Il9R−/− mice (six per group) were intranasally infected with live Aspergillus fumigatus conidia or subjected to ABPA and assessed for (a) lung fungal growth (log_{10} cfu, mean ± s.d.); (b) lung histology (periodic acid – Schiff staining); (c) expression of CD90.2+CD25+, CD90.2+ST2+ lung ILC2 by immunofluorescence; (d,e) Th-cell specific transcripts and cytokine production. (f) Lung histology (periodic acid–Schiff and, in the inset, Masson’s trichrome staining) and (g) TGF-β production in C57BL/6 or Cftr−/− mice infected as above and treated with IL-9 neutralizing antibody for a week. Days post infection (dpi). (h) Th-cell specific transcripts and IL-9 production of lung CD4+ T cells from naive mice co-cultured with lung lineage negative (Lin−) cells in the presence of A. fumigatus conidia, IL-2 or IL-33. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a x 20 objective; scale bars, 200 μm and a x 40 objective (insets of f, scale bars, 100 μm). Results are mean values ± s.d., ELISA was done on lung homogenates and culture supernatants for cytokines and PCR with reverse transcription on CD4+ lung cells. *P < 0.05, **P < 0.01, ***P < 0.001, Il9R−/−, Cftr−/− versus C57BL/6 or Cftr−/− CD4+ T cells. Naive, uninfected mice. Data represent pooled results or representative images from three experiments, Two-tailed Student’s t-test (a) or Two-way ANOVA (d,e) Bonferroni post test. Gata3, GATA binding protein 3; Irf4, interferon regulatory factor 4; Pu.1, purine-rich box 1.
co-cultivation of CD4$^+$ T cells with Lin$^-$ cells in the presence of IL-2 more than IL-33, an effect magnified in Cfr$^{-/-}$ mice as compared to C57BL/6 mice (Fig. 2h), and requiring the presence of IL-9R on responder CD4$^+$ T cells being significantly negated with Il9R$^{-/-}$ responder cells (Fig. 2h and Supplementary Fig. 4). These results indicate that ILC2, and particularly CD25$^+$ ILC2, may account for the sustained IL-9 production and Th9 activation responsible for pathology in CF.

IL-9 activates mast cells to produce IL-2. The sustained production of IL-33 and IL-2 in Cfr$^{-/-}$ mice prompted us to investigate mechanisms behind this production. IL-33 is constitutively expressed at epithelial barrier surfaces where it is rapidly released from cells during tissue injury\textsuperscript{25}. However, tight regulation of IL-33 following its release to dampen ST2-dependent inflammation to fungi has also been described\textsuperscript{26}. This likely occurred in C57BL/6 but not in Cfr$^{-/-}$ mice in which the high levels of epithelial damage observed upon the infection (Fig. 3a) likely accounted for the sustained IL-33-dependent ST2$^+$ ILC2 expansion. For IL-2, predictably high in CF, given the sustained NFAT activity\textsuperscript{27} in addition to CD4$^+$ T cells\textsuperscript{8} and dendritic cells\textsuperscript{28}, MC are known to produce it in the lung\textsuperscript{7}. We looked therefore for MC presence and activity in the lung of C57BL/6 and Cfr$^{-/-}$ mice after the infection. MC were much expanded in Cfr$^{-/-}$ mice, as seen by flow cytometry (Fig. 3b) and toluidine staining (Fig. 3c). MC are distinguished by their granule content whose expression is tissue-dependent\textsuperscript{29}. In the lung, chymase-positive MC numbers positively correlated with better lung function\textsuperscript{30}, whereas chymase- and tryptase-positive MC were expanded in areas of fibrosis in CF lungs and positively correlated with the degree of fibrosis and lung function\textsuperscript{31}. Immunohistochemistry revealed that while tryptase-positive cells could not be detected, chymase-positive MC were present in C57BL/6 mice (insets of Fig. 3c). In contrast, chymase-positive and tryptase-positive MC were observed in Cfr$^{-/-}$ mice (insets of Fig. 3c).

As MC are known to produce cytokines through different receptor mechanisms\textsuperscript{32}, we evaluated cytokine production on magnetically purified c-Kit$^+$ MC (as characterized by morphometry and MC specific transcripts, Fig. 3d) upon stimulation with IgE, IL-9 or IL-33. We found that IL-2 production was induced by IL-33 and, more, by IL-9 and not by IgE, mostly in MC from Cfr$^{-/-}$ mice (Fig. 3e). As IL-9 also induced IL-9 production (Fig. 3e) and IL-9$^+$ MC could also be detected in vivo (Fig. 3b), this suggests that an autocrine loop is needed to trigger the IL-9-dependent IL-2 release by MC. As a matter of fact, IL-2 production (39 ± 6 ng/ml versus 127 ± 22, IL-2 in lung homogenates at 3 dpi in Ibr9R$^{-/-}$ versus C57BL/6 mice, respectively) and IL-2$^+$ MC (Fig. 3f) were greatly reduced in Ibr9R$^{-/-}$ mice, thus contributing to the defective expansion of CD25$^+$ ILC2 in these mice. Of interest, IL-9 stimulation also induced TGF-β in MC from Cfr$^{-/-}$ mice but not IL-6 (Fig. 3e), a finding suggesting that the autocrine IL-9 stimulation appears to be specific for IL-2 and TGF-β (Fig. 3e). These data indicate that MC may contribute to IL-2 production eventually leading to CD25$^+$ ILC2 expansion in Cfr$^{-/-}$ mice. This appears to be the case, as IL-2$^+$ MC, more than IL-2$^+$ CD90.2 or IL-2$^+$ CD4$^+$ T cells, were expanded in vivo, early in infection, particularly in Cfr$^{-/-}$ mice (Fig. 3f).

To directly probe this, we assessed susceptibility to inflammatory allergy of MC-deficient C57BL/6-Kit$^+$/W$^-$ mice or Cfr$^{-/-}$ mice treated with the tyrosine kinase inhibitor imatinib known to inhibit IL-9-driven mastocytosis in the lung\textsuperscript{12}. Airway mastocytosis was reduced in MC-deficient Kit$^+$/W$^-$ mice (Fig. 4a) along with reduced levels of IgE, IL-2, IL-9 and TGF-β (Fig. 4b). Concomitantly, the number of CD25$^+$ ILC2 were also decreased in the lung but promptly restored upon MC engraftment or exogenous IL-2 administration (Fig. 4a). Thus, MC appear to be able to control CD25$^+$ ILC2 expansion in the lung during the infection via IL-2. Similar results were obtained upon treatment of Cfr$^{-/-}$ mice with imatinib. Both inflammation (Fig. 4c), collagen deposition (insets of Fig. 4c), IL-2, IL-9 and TGF-β production (Fig. 4d) and Th9 cell activation (Fig. 4e) were attenuated. Interestingly, imatinib apparently increased early inflammation in C57BL/6 mice (Supplementary Fig. 5), a finding suggesting that c-Kit$^+$ cells could contribute to pathogen resistance early in infection. As a matter of fact, MC-deficient Kit$^+$/W$^-$ mice displayed increased susceptibility to the infection as compared with C57BL/6 mice, as indicated by the increased fungal load (Supplementary Fig. 6a), neutrophils recruitment (Supplementary Fig. 6b) and a degree of lung inflammation (Supplementary Fig. 6c).

**CFTR deficiency contributes to inflammation.** The above results suggest that a circuit involving IL-33, IL-2 and IL-9 and different types of cells is pathogenically amplified in Cfr$^{-/-}$ mice. The finding that IL-33, IL-2 and IL-9 are also elevated in CF patients\textsuperscript{33,34} prompted us to evaluate the contribution of cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction on the activation of the inflammatory circuit. Given that CFTR dysfunction on both epithelial and myeloid cells impacts on lung inflammation\textsuperscript{35}, we assessed chimeric mice with CFTR unresponsive myeloid or epithelial cells for lung damage and inflammation, production of IL-33, IL-2 and IL-9 and MC/ILC2 activation upon Aspergillus infection. We found that epithelial cell damage and lung inflammation (Fig. 5a), levels of cytokines and IgE (Fig. 5b), MC and ILC2 expansion (Fig. 5c) were all attenuated or decreased in condition of CFTR deficiency in epithelial cells but CFTR sufficient myeloid cells, a finding suggesting that myeloid, and perhaps lymphoid, deficiency could contribute to the activation of the inflammatory circuit in CF. This seems to be the case as the opposite findings were observed in recipient C57BL/6 mice receiving CFTR deficient myeloid cells (Fig. 5a,c) These mice, however, showed an intermediate inflammatory phenotype as compared to Cfr$^{-/-}$ mice, a finding suggesting that, although to a different extent, CFTR deficiency on epithelial and myeloid cells may predispose to lung inflammation in response to microbial and non-microbial stimuli.

The **IL9 rs2069885 SNP correlates with high IgE levels in CF females.** To assess whether IL-9 may contribute to allergy in CF patients, we determined the effect of the non-synonymous IL9 p.Thr117Met (c.350C $\rightarrow$ T, rs2069885) polymorphism, known to be associated with lung function and sensitization\textsuperscript{36,37} on total and Aspergillus-specific IgE levels in CF patients (Supplementary Table 1). Previous association studies demonstrated the existence of sex dimorphism linked to this polymorphism\textsuperscript{36,37}. Therefore, we carried out association testing separately in males and females. The distribution of the total IgE was skewed but after natural logarithmic transformation, the distribution adequately approximated a normal distribution. IL9 rs2069885 genotype distribution did not deviate from Hardy–Weinberg equilibrium ($\chi^2$ test, $P = 0.776$) and it displayed a minor allele frequency of 0.129, comparable to the European population from 1000 Genome Consortium, minor allele frequency $= 0.128$ (ref. 38). IL9 rs2069885 genotype distribution did not differ between males and females ($\chi^2$ test, $P = 0.243$), but a significant rs2069885-sex interaction on total IgE levels was found (general linear model, $P = 0.004$), where female T allele carriers showed high IgE levels (linear regression: females, $\beta = 0.624$, $P = 0.043$; males,
**Figure 3** | IL-9 activates mast cells to produce IL-2. C57BL/6 or Cfr–/– mice (six per group) infected intranasally with live *A. fumigatus* conidia were evaluated at different days after infection (dpi) for (a) deposition of DNA on lung epithelial cells by TUNEL, resulting in bright DNA staining; (b) detection of c-Kit+ FcsR+ and c-Kit+ IL-9+ lung mast cells (MC) by flow cytometry (numbers refer to percentages of positive cells); (c) toluidine blue, relative MC number mm−2 and, in the inset, immunohistochemical staining for chymase- and tryptase-positive MC in lung section. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×100 objective and with EVOS FL Color Imaging System 100 objective and (in the inset) a ×40 objective (immunohistochemical staining). (d) Toluidine blue stain and transcription factors expression (PCR with reverse transcription) of c-Kit+ cells magnetically isolated from lung of uninfected C57BL/6 mice and pulsed with live *A. fumigatus* conidia. (e) Cytokine production (mean values ± s.d., ELISA on culture supernatants) by purified lung c-Kit+ cells, pulsed with *A. fumigatus* and stimulated with IgE, IL-9 and IL-33; (f) detection of c-Kit+ IL-2+, CD90.2+ IL-2+ and CD4+ IL-2+ lung cells by flow cytometry (numbers refer to percentages of positive cells). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; conidia-pulsed versus unpulsed c-Kit+ cells, stimulated versus unstimulated c-Kit+ cells and Cfr–/– versus C57BL/6 c-Kit+ cells (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). Mcpt1, mast cell protease 1; Mcpt6, tryptase beta 2; Rorc, retinoic acid receptor-related orphan receptor C; Rora, RAR-related orphan receptor alpha; Tbet, T box expressed in T cells; Tph1, tryptophan hydroxylase 1.

\[ \beta = -0.666, \ P = 0.034; \text{ Fig. 6a). In a subset of CF patients (} N=114; 57 males and 57 females), Aspergillus-specific IgE were also significantly higher in IL9 rs2069885-T carriers (general linear model, P = 0.002; Fig. 6b). Notably, in line with the results obtained on total IgE, the association of *IL9* rs2069885-T allele with higher *Aspergillus*-specific IgE levels was observed in females (linear regression, \[ \beta = 1.417, \ P = 0.002 \] more than males (linear regression, \[ \beta = 0.489, \ P = 0.279 \]. We also tested other
**Figure 4 | IL-9 activates mast cells to produce IL-2.** MC-deficient C57BL6-KitW/W-v mice (six per group) were infected intranasally with live *A. fumigatus* conidia, engrafted intravenously with wild-type bone marrow-cultured mast cells (BMMC) or treated intraperitoneally with IL-2 for a week and assessed for (a) c-Kit⁺ FcεR⁺ lung mast cells (MC) and CD90.2⁺ CD25⁺ lung ILC2 by flow cytometry (numbers refer to percentages of positive cells) with relative cell number and (b) IgE and cytokine production. (c) Lung histology (periodic acid-Schiff and Masson's trichrome staining, in the insets); (d) cytokine production and (e) Th9-cell specific transcripts expression in Cftr⁻/⁻ mice infected as above and treated with imatinib intraperitoneally for a week. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with × 20 objective, scale bars, 200 μm and × 40 objective (insets of c, scale bars, 100 μm). Results are mean values ± s.d., ELISA on lung homogenates for cytokines and PCR with reverse transcription on lung CD4⁺ T cells. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, MC-deficient C57BL6-KitW/W-v versus C57BL/6 mice or imatinib-treated versus untreated (none) mice (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). If4 = interferon regulatory factor 4; Pu.1 = purine-rich box 1.
**IL9** tagSNPs (rs2069882, rs31564, rs1859430, rs1799962; Supplementary Tables 2 and 3), encompassing the whole **IL9** gene region, but none associated with IgE levels. Linkage disequilibrium (LD) analyses of the five SNPs in **IL9** failed to reveal the presence of significant LD blocks (Supplementary Fig. 7). Haplotype analysis yielded no significant result.

**Figure 5 | Epithelial and myeloid CFTR deficiency contribute to the inflammatory phenotype.** C57BL/6, Ctr<sup>−/−</sup> and chimeric C57BL/6 and Ctr<sup>−/−</sup> mice (10 per group) received 10<sup>7</sup>–10<sup>8</sup> viable bone marrow cells 4 weeks before the intranasal infection with *A. fumigatus*. Chimeric mice were evaluated 7 days after the infection for (a) lung histology (periodic acid–Schiff and, in the insets, Masson’s trichrome and TUNEL staining); (b) cytokines and IgE levels (mean values ± s.d., ELISA on lung homogenates); (c) detection of c-Kit<sup>+</sup> FcεR<sup>+</sup> mast cells, CD90.2<sup>+</sup>CD25<sup>+</sup> and CD90.2<sup>+</sup>ST2<sup>+</sup> type 2 ILCs by flow cytometry (numbers refer to percentages of positive cells in the lung). Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×20 objective; scale bars, 200 μm and a ×40 objective (insets of panel a, scale bars, 100 μm). *P<0.05, **P<0.01, ***P<0.001, Ctr<sup>−/−</sup> versus C57BL/6, chimeric C57BL/6 versus C57BL/6, chimeric Ctr<sup>−/−</sup> versus Ctr<sup>−/−</sup> mice, Two-way ANOVA, Bonferroni post test.
IL9Rs and definitive validation using a larger new cohort of CF patients, the Powerb IL9R gene expression levels were analysed independently. A difference was noticed between males and females when from CF patients carrying diverse genotypes at rs2069885.

Aspergillus regression, females, bT allele carriers showed opposite effects according to sex (linear expression ratio (general linear model, IL9/IL9R rs2069885 by sex interaction was found when analysing the region Xq/Yq, between males and females. A significant IL9 IL9 and its receptor, which is located on the pseudoautosomal P region, as already reported in several respiratory-related loci, as already reported in several respiratory-related (Supplementary Table 4), although the most common haplotype C–A–A–C–A (frequency 43.2%) turned out to be associated with higher IgE levels in males (linear regression, $\beta = 0.567$, $P = 0.022$).

We next determined whether the reported sex-specific associations could be attributable to a differential expression of IL9 and its receptor, which is located on the pseudoautosomal region Xq/Yq, between males and females. A significant IL9 rs2069885 by sex interaction was found when analysing the IL9/IL9R expression ratio (general linear model, $P = 0.025$) where T allele carriers showed opposite effects according to sex (linear regression, females, $\beta = 0.375$, $P = 0.116$; males, $\beta = -0.426$, $P = 0.375$; Fig. 6c) paralling the finding on specific IgE (Fig. 6a) and on IL9 levels in expectorates (Fig. 6d). No significant difference was noticed between males and females when IL9 or IL9R gene expression levels were analysed independently.

Although these results are to be considered with caution given the small sample size—despite the sufficient power ($\beta = 0.20$, Power = 0.8; Supplementary Table 6)—which demands for a definitive validation using a larger new cohort of CF patients, the present findings confirm the existence of a sex dimorphism at IL9 and IL9R loci, as already reported in several respiratory-related human phenotypes.

**Discussion**

We have shown that IL-9 may expand pro-inflammatory CD25$^{+}$ ILC2/Th9 cell in CF, an activity involving the production of IL-2 by MC. Human MC co-localize near ILC2 in the human lung and could directly promote ILC2 responses in vitro. We found here that, in addition to CD4$^{+}$ T cells, known to contribute to CD25$^{+}$ ILC2 expansion via IL-2 (ref. 8), lung MC may also affect the expansion of CD25$^{+}$ ILC2 thought to contribute to chronic inflammation through multiple mechanisms. Failure to expand CD25$^{+}$ ILC2 occurred indeed in MC-deficient mice or mice treated with imatinib.

MC hyperplasia during chronic allergen challenge is associated with remodeling of airways. In a mouse model of ovalbumin-induced airway inflammation, the influx of MC into lung peaks early after allergen challenge to mature over 14 days into cells expressing lower level of c-Kit, FcεR1 and integrins. MC hyperplasia was observed in the lung of Cfr$^{-/-}$ mice along with the detection of tryptase-positive and chymase-positive MC, known to be expanded in asthmatic patients and in disease areas of CF lung. Tryptases and chymases contribute to infiltration and tissue remodeling through the selective proteolysis of matrix proteins and the activation of protease-activated receptors and matrix metalloproteinases. Consistent with the finding that MC from CF patients are not high in FcεR1 expressing, we found that lung MC from these mice poorly responded to IgE in terms of IL-6 production but released IL-2, in addition to TGF-β, in response to IL-9.

IL-9 is a pleiotropic cytokine that has multiple effects on structural as well as numerous hematopoietic cells, which are central to the pathogenesis of asthma. The important role for the IL-9-MC axis in the pathology associated with chronic allergic inflammation has been already described. IL-9 not only stimulates MC growth and expansion but also stimulates changes in gene expression that might alter responsiveness to other stimuli. In vivo, IL-9 governed allergen-induced MC numbers in the lung, and anti-IL-9 antibody-treatment protected from airway remodeling, decreased expression of the profibrotic mediators TGF-β and improved lung function. The correlation between a reduction in MC numbers and decreased airway remodeling, after IL-9 inhibition, is consistent with reports that MC-deficient mice demonstrate significantly attenuated fibrosis and inflammation after silica, ozone, or bleomycin injury.

We found that not only were IL-9 production and MC expansion significantly increased in CF mice but that the IL-9-MC axis contributed to the expansion of CD25$^{+}$ ILC2 leading to Th9 cell activation that further contributed to the allergic inflammatory pathology. IL-9 promoted IL-2 production by lung MC from Cfr$^{-/-}$ mice, a finding that may explain the increased and persistent expansion of CD25$^{+}$ ILC2 in these mice. CD25$^{+}$ ILC2 were indeed not expanded in condition of IL-9 ablation or MC-deficiency, a finding suggesting that the IL-9/MC/IL-2 axis drives the expansion of CD25$^{+}$ ILC2. In addition, as IL-2 stimulated lung CD25$^{+}$ ILC2 to produce IL-9 (ref. 9), this may have a positive feedback effect on ILCs, since lung ILCs cultured with IL-9 increased the production of type 2 cytokines and up-regulated the anti-apoptotic protein BCL-3, thereby promoting ILC2 survival. Of great interest, Lin$^{-}$ cells from CF lung also promoted Th9 cell activation in vitro, an activity that required IL-9R expression on responding CD4$^{+}$ T cells. Thus, the pathogenic role of IL-9 in promoting allergic inflammation may go beyond CD25$^{+}$ ILC2 expansion to include the activation of Th9 cells. That Th9 cells are a major source of IL-9 in models of allergic inflammation and play an important role in MC accumulation and activation has been reported.

By producing IL-9, Th9 cells may in turn serve as a positive loop amplifying the IL-9/MC/IL2 axis, promoting a deleterious vicious circle in which the production of profibrotic TGF-β by IL-9-stimulated MC plays a plausible important role. The presence of TGF-β dependent signalling in areas of prominent fibrosis in CF has been already documented along with its inhibition of chloride channel activities and the association of the TGF-β genetic variants with more severe lung disease.

![Figure 6](image-url) **Figure 6** | The IL9 rs2069885 polymorphism correlates with high IgE levels in CF females. (a,b) IL9 rs2069885 sex interaction on total or Aspergillus-specific IgE levels and (c) IL9/IL9R expression ratio measured on CF patients. (d) Determination IL-9 (mean ± s.d., ELISA) in expectorates from CF patients carrying diverse genotypes at rs2069885.
myeloid cells may impact on the inflammatory circuit leading to the activation of the inflammatory IL-9/Th9 pathway: namely, among others, on both epithelial damage eventually leading to overproduction of IL-33 and on the MC propensity to respond to IL-9 with IL-2. However, PU.1 translocation into the nucleus has also been shown to be significantly higher in CF monocytes than in controls. It seems that defective CFTR impacts on the mechanisms of communication between innate and adaptive immune response.

The potential contribution of IL-9 to CF pathogenesis in humans is unknown. Elevated levels of IL-9 were observed in the expectorates from CF patients, likely accounting for the expansion of TGF-β-producing MC in diseased lung areas. It is of interest that in human asthmatic lung tissue, MC were the main IL-9R expressing population. It seems therefore that the IL-9/MC/IL-2 axis may have a pathogenic role in CF patients and that its targeting could lead to a reduction in chronic inflammation and improved lung function of these patients. Studies have indeed highlighted the importance of Th9 cells in allergic lung inflammation by promoting epithelial alterations, goblet-cell hyperplasia, mucus production and infiltration of MC and eosinophils. Considering that the costimulatory signal OX40 is required for Th9 activation, it is intriguing that OX40 ligand was critical in driving Th2-allergic responses to *A. fumigatus* in peripheral CD4+ T cells isolated from CF patients with ABPA (ref. 63). It is clear that the inflammatory response in the lung involves different Th cell types whose specific role in CF remain unclear. Intriguingly, there are conditions where promoting IL-9 might be therapeutically beneficial and ILC2 and MC could be exploited for pathogen immunity and tissue repair. In this regard, the fact that imatinib apparently exacerbated signs of inflammation during infection points to a some beneficial role c-Kit+ cells may have in the control of *Aspergillus* infection. MC indeed exhibited conidocidal activity (Supplementary Fig. 6d), a finding suggesting that MC could serve as tissue sentinels modulating antifungal immune responses, as suggested.

In conclusion, IL-9 and MC may have an important role in the pathogenesis of lung disease and inflammation in CF. Considering the inherent resistance to steroids of MC in asthmatic patients, a better understanding of cellular and molecular pathways leading to inflammation and impaired lung functions may inspire new treatment avenues in patients with CF. Our study would suggest that imatinib, known to inhibit lung fibrosis, could be therapeutically exploited in CF patients with an exalted IL-9/Th9 responses. In addition, it is of great interest that the IL9 rs2069885 polymorphism, linked to high IgE levels, was associated with females more than males with CF, a finding offering an explanation for the, as yet unexplained, ‘gender gap’ in mortality between females and males in CF and fostering gender medicine in CF.

**Methods**

**General experimental approaches.** Mice were randomized and 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.** C57BL/6 (wild-type, WT), *Rag1-/-* and MC-deficient C57BL/6-*KitW/W-s* mice, 6–8 week old, of both sexes, were purchased from Charles River (Calco, Italy). Genetically engineered homozygous *Cftr-/-* mice were bred at the CF core animal facility at San Raffaele Hospital, Milan, Italy. *Bgr-/-* and *Rag1-/-* mice were from the Ludwig Institute for Cancer Research, Brussels.

**Fungal infection allergy and treatments.** Anaesthetized (by inhalation of 3% isoflurane (Forane Abbot)) mice were infected with the intranasal instillation of 2 × 107 resting conidia/20 μl saline. For allergic broncho-pulmonary aspergillosis, *A. fumigatus* culture filtrate extract in incomplete Freund’s adjuvant

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**Figure 7 | Proposed model for the role of IL-9 in promoting a mast cells/ILC2/Th9 fibrotic pathway in CF.** IL-9, produced by IL-33-expanded ILC2, activates MC for IL-2 production leading to the expansion of CD25+ ILC2 that promote Th9 cell activation. The resulting increased production of IL-9 further amplifies the inflammatory loop by promoting ILC2 survival and type 2 cytokines production and by activating MC for the production of fibrotic TGF-β. IL-9 ablation or MC inhibition (imatinib) are potential drugable pathways through which inflammation and allergy could be restrained in CF. EC, epithelial cells. αIL-9, IL-9 neutralizing antibody.
recombinant IL-2 and pulsed with *A. fumigatus* conidia. Three days later, IL-9 levels in culture supernatants were analysed by ELISA and Th9 transcription factors by real-time PCR. To separate Lin- and CD4+ T cells, we used the Transwell culture system (Costar, 0.4 μm pore size; Corning) with CD4+ T cells in the lower wells and Lin- in the upper wells. For c-Kit+ cells culture, 5 × 104 cells were cultured overnight in RPMI medium and pulsed with *A. fumigatus* conidia with or without 10 μg ml−1 IL-10, 100 ng ml−1 IL-13 and 300 ng ml−1 IL-9.

**Flow cytometry.** Flow cytometry on enriched Lin− cells was performed with a combination of the following fluorescence-conjugated mAbs (all from Miltenyi Biotech unless specified otherwise): APC-conjugated anti-NKp46 (29.A1.4.9), anti-CD90.2 (30-H12), anti-CD117 (ReA278), anti-FcRα (MAR-1), anti-CD4 (GG7.1); PE-conjugated anti-NK1.1 (PK136), anti-CD25 (7D4), anti-T1-ST2 (DII9, from Biolegend), anti-CD117 (C111), anti-IL-9 (RMV94A) and anti-IL-2 (JES6-5H4). For intracellular staining, phorbol 12-myristate 13-acetate (PMA)- and ionomycin-stimulated cells were added of brefeldin, and then permeabilized with the CytoFix/Cytoperm kit (BD Biosciences) for intracytoplasmic detection of IL-9 and IL-2. Flow cytometry was done at 4°C on cells first exposed to Fc receptor mAb (2.4.G2). Cells were analysed with a BD LSRFortessa flow cytometer equipped with BD FACSDivA 7.0 software.

**ELISA and real-time PCR.** The levels of cytokines and IgE in lung homogenates, culture supernatants or excreta were determined by ELISA kits (R&D Systems) following manufacturer’s instructions. Real-time PCR with reverse transcription was performed using CFX96 Touch Real-Time PCR Detection System and SYBR Green chemistry (Bio-Rad) on total RNA reverse transcribed with the cDNA Synthesis Kit (Bio-Rad). The PCR primers were as listed in Supplementary Table 5. In all reactions, 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. The messenger RNA data were expressed as relative gene messenger RNA in treated versus untreated groups or cells.

**Human study.** A cohort of 347 patients of Caucasian origin with a proven diagnosis of CF (CFTR genotyping, sweat testing and clinical phenotype) was enrolled in a prospective multicenter longitudinal genetic association study. See Supplementary Table 1 for clinical data including age, gender, lung function testing, measures of nutrition, microbiological findings and vital status of the patients’ cohort.

**SNPs selection and genotyping.** DNA was isolated from blood with the QiAamp DNA Mini (Qiagen, Milan, Italy) system and stored at −20°C. IL9 SNPs were selected based on literature review and their ability to tag surrounding variants in the HapMap-CEU population of the International HapMap project, NCBI build 36 assembly Hapmap phase III (http://www.hapmap.org). Haplotype-based tagging SNPs were selected by assessing LD blocks from the genes of interest with a correlation coefficient r2 of at least 0.80 and a minor allele frequency higher than 5% in the HapMap-CEU population. Five IL9 SNPs complied with the selection criteria: rs2069885, rs2069882, rs313564, rs1859430 and rs1799962. The applied Biosystems 7500 Fast qPCR system (Life Technologies) was used for SNP genotyping by KASPar assays (KBiosciences, Hertfordshire, UK). Each genotyping set was 50% selected independently of any genotyping or negative controls. Agreement between original and duplicate samples was ≥99% for all SNPs; laboratory personnel were blind to the sample status.

**Statistical analysis.** Data are expressed as mean ± s.d. Horizontal bars indicate the means. Statistical significance was calculated by two-way ANOVA (Bonferroni’s post hoc test) for multiple comparisons and by a two-tailed Student’s t-test for single comparison. The distribution of levels tested by Kolmogorov–Smirnov test turned to be non-normal. *P* values of 0.05 were considered significant. The data reported are either representative from two or three experiments (FACS data, histology, immunofluorescence and TUNEL assay) or pooled otherwise. The *in vivo* groups consisted of 6 mice per group. Data were analysed by GraphPad Prism 4.03 programme (GraphPad Software). No statistical methods were used to predetermine sample size. Genetic association was carried out considering additive and dominant models by linear regression implemented in Plink v1.07 (ref. 70), adjusting for age at sampling. Haplotype-based association tests were performed by general linear model using Plink v1.07 (ref. 70). LD analysis was performed using Haploview, and defining LD blocks based on the solid spine of LD algorithm. Analyses were conducted stratifying the study population according to sex since previous evidence highlighted the existence of sex dimorphism at IL9 locus. IL9 rs2069885 by sex interaction was tested by *t*-test for single comparison. The distribution of levels tested by Kolmogorov– Smirnov test turned to be non-normal. *P* values of 0.05 were considered significant. The data reported are either representative from two or three experiments (FACS data, histology, immunofluorescence and TUNEL assay) or pooled otherwise. The *in vivo* groups consisted of 6 mice per group. Data were analysed by GraphPad Prism 4.03 programme (GraphPad Software). No statistical methods were used to predetermine sample size. Genetic association was carried out considering additive and dominant models by linear regression implemented in Plink v1.07 (ref. 70), adjusting for age at sampling. Haplotype-based association tests were performed by general linear model using Plink v1.07 (ref. 70). LD analysis was performed using Haploview, and defining LD blocks based on the solid spine of LD algorithm. Analyses were conducted stratifying the study population according to sex since previous evidence highlighted the existence of sex dimorphism at IL9 locus. IL9 rs2069885 by sex interaction was tested by general linear model using PLINK v1.07. Two-tail *P* values are reported. Bonferroni’s correction for multiple testing was not performed since we are assessing specific questions on a candidate gene and we are not searching for associations without a priori hypotheses. Power calculation using QUANTO v1.2.4 was performed to determine whether the sample study had sufficient power to detect a significant...
association between IL9 rs2069885 and total IgE levels. Considering sample size (N = 237), allele frequency (13%), dominant genetic model, mean level of IgE and its standard deviation (131.7 ± 248.2 U ml⁻¹) and z = 0.05 two-sided, we could establish that our sample have sufficient power (β = 0.20, Power = 0.8) to detect an association explaining more than 3% of total IgE levels variance (Supplementary Table 6).

**Study approval.** All animal experiments were approved by local government authorities and were in agreement with the Italian Approved Animal Welfare Authorization 360/2015-PR and Legislative decree 26/2014 obtained from the Italian Ministry of Health lasting and lasting for five years (2015–2020). Infections were performed under anesthesia and all efforts were made to minimize suffering. Human studies approval was obtained from institutional review boards the Bambino Gesù Children’s Hospital (Rome, Italy), Ospedale Maggiore Policlinico, University of Milan, (Milan, Italy), Innsbruck Medical University, (Innsbruck, Austria) and Servizio di Supporto Fibrosi Cistica, (Cerignola, Foggia, Italy). Written informed consent was obtained from the participant, or, in case of minors, from parents or guardian.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions
G.R. and V.O. performed in vivo murine experiments. C.G. and V.N. performed human genotyping. M.P., M.P. (Puccetti), R.G.I. and M.B. performed immunohistochemistry, immunofluorescence and histopathology; M.D.Z., G.P., T.Z. and C.E.P. contributed to mast cell experiments; J.-C.R. provided scientific advice; O.B. and P.S. provided reagents and contributed to experimental design; V.L., C.C., M.C.R., E.F., C.L.-F., F.M., G.R., H.E. and L.R. (Ratclif) provided the clinical samples. S.M., V.N.T., V.N. and L.R. designed the experiments, analysed data and wrote the paper.

Additional information
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