Profibrotic function of pulmonary group 2 innate lymphoid cells is controlled by regnase-1

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Regnase-1 controls the proliferation and activation of ILC2, which thereby attenuates lung fibrosis in mice. In humans, lower regnase-1 level correlates with more abundant ILC2 number, which potentially associates with the prognosis of IPF patients. https://bit.ly/3c3GhKo

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ABSTRACT Regnase-1 is an RNase critical for post-transcriptional control of pulmonary immune homeostasis in mice by degrading immune-related mRNAs. However, little is known about the cell types Regnase-1 controls in the lung, and its relevance to human pulmonary diseases.

Regnase-1-dependent changes in lung immune cell types were examined by a competitive bone marrow transfer mouse model, and group 2 innate lymphoid cells (ILC2s) were identified. Then the associations between Regnase-1 in ILC2s and human diseases were investigated by transcriptome analysis and a bleomycin-induced pulmonary fibrosis mouse model. The clinical significance of Regnase-1 in ILC2s was further assessed using patient-derived cells.

Regnase-1-deficiency resulted in the spontaneous proliferation and activation of ILC2s in the lung. Intriguingly, genes associated with pulmonary fibrosis were highly upregulated in *Regnase-1*-deficient ILC2s compared with wild-type, and supplementation of *Regnase-1*-deficient ILC2s augmented bleomycin-induced pulmonary fibrosis in mice. Regnase-1 suppresses mRNAs encoding transcription factors *Gata3* and *Egr1*, which are potent to regulate fibrosis-associated genes. Clinically, Regnase-1 protein levels in ILC2 negatively correlated with the ILC2 population in bronchoalveolar lavage fluid. Furthermore, idiopathic pulmonary fibrosis (IPF) patients with ILC2s >1500 cells·mL^{-1} peripheral blood exhibited poorer prognosis than patients with lower numbers, implying the contribution of Regnase-1 in ILC2s for the progression of IPF.

Collectively, Regnase-1 was identified as a critical post-transcriptional regulator of the profibrotic function of ILC2s both in mouse and human, suggesting that Regnase-1 may be a novel therapeutic target for IPF.

Introduction

A variety of immune cell types reside in the lung, and they coordinate to eliminate pathogens or enhance recovery from injury [1]. Immune cells are regulated through transcriptional and post-transcriptional mechanisms [2, 3]. Regnase-1, also known as ZC3H12A or MCPIP1, is vital for post-transcriptional immune regulation by acting as an RNase degrading mRNA such as *Il6* or *Icos* through the recognition of stem–loop structures in its 3′ untranslated regions (UTR) [4–6]. Regnase-1 deficiency in mice results in the aberrant activation of immune cells and development of systemic inflammatory diseases [4]. Particularly, *Regnase-1*-deficient mice show a massive infiltration of various immune cells in the lungs [4, 7, 8]. However, cells intrinsically regulated by Regnase-1 remain unclear except for a study showing its role in the activation of type 2 helper T cells (Th2) cells and allergic inflammation *via Gata3* regulation [7]. In humans, decreased Regase-1 expression potentially contributes to the pathogenesis of psoriasis [9] and enhanced antitumor T cell activity [10]. However, little is known about the clinical significance of Regnase-1 in the lungs.

Dysregulation of the immune system causes a number of pulmonary disorders such as asthma and pneumonia [1]. In addition, the immune system contributes to the progression of pulmonary fibrotic diseases including idiopathic pulmonary fibrosis (IPF) [11]. Particularly, type 2 immunity plays an important role in IPF through the production of cytokines including interleukin (IL)-4, IL-5 and IL-13 [12, 13]. These cytokines act on the fibroblasts to enhance collagen production and promote fibrosis. However, immune cell types responsible for the progression of fibrosis are yet to be clarified.

Group 2 innate lymphoid cells (ILC2s) were reported to be major sources of IL-5 and IL-13 in the lungs [14, 15]. Owing to this cytokine-producing capacity, ILC2s largely contribute to the development of bronchial asthma [16, 17]. In addition, recent studies implied that ILC2s are involved in the pathogenesis of pulmonary fibrosis [17–20]. However, the role of ILC2s in pulmonary fibrosis and their regulatory mechanisms are largely unknown.

In this study, we investigated cell types intrinsically regulated by Regnase-1 in the lung, and discovered ILC2s. Lack of Regnase-1 in ILC2s worsened experimental pulmonary fibrosis, consistent with the control of genes related to pulmonary fibrosis by Regnase-1 in ILC2s. Furthermore, the human Regnase-1 protein levels inversely correlated with the number of ILC2s in the bronchoalveolar lavage (BAL), and the increased number of blood ILC2s was an independent factor for poor prognosis in IPF patients.

Methods

Mice and cell lines

Regnase-1$^{-/-}$, *Rag2*$^{-/-}$, *Il2rg*$^{-/-}$ and CD45.1 congenic mice were previously described [4, 5, 21, 22]. *Regnase-1*$^{-/-}$ mice and CD45.1 congenic mice were maintained on a C57BL/6 background. *Regnase-1*$^{-/-}$ mice crossed with *Rag2*$^{-/-}$ (*Regnase-1*$^{-/-}$*Rag2*$^{-/-}$) and *Rag2*$^{-/-}$ mice crossed with *Il2rg*$^{-/-}$ (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$) mice were generated and maintained on a Balb/c background. All mice were grown under specific pathogen-free environments, and mice at ages between 7 and 12 weeks were subjected to the analysis. The sex of the mice is summarised in supplementary table S1. The animal experiments were performed with permission from the Kyoto University animal experimentation committee (120002). *Regnase-1*-deficient and parental Jurkat cells have been described previously [23].

Clinical study design

We performed an observational cohort study which included consecutive patients who were diagnosed with IPF at Kyoto University Hospital from May 2013 to February 2018. The diagnosis of IPF was made according to the international guideline [24] and confirmed to meet the current criteria [25]. Patients who had complications of acute infection, acute exacerbation of IPF, pneumothorax or active malignancies at the time of sample collection were excluded from the study. The day of sample collection was set as the baseline. For the analyses of ILC2s in the BAL, the patients who underwent BAL screening as a systemic evaluation under the suspicion of sarcoidosis and were proven to have no abnormality in the lung were included as control subjects. The study was approved by the Kyoto University Hospital institutional review board (G0296 and G1059).

Further details of experimental methods, clinical data collection and statistical analyses are described in the supplementary material.

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Results

Regnase-1 deficiency drastically increases lung ILC2s in a cell-intrinsic manner

To identify the lung immune cell populations that are cell-intrinsically controlled by Regnase-1, we took advantage of the competitive bone marrow (BM) transfer model (supplementary methods and supplementary figure S1a). The proportion of CD45.2 *Regnase-1*$^{-/-}$ ILC2s (defined as lineage^{-}CD45^{+}T1/ST2^{+}Sca-1^{+}KLRG1^{+} lymphocytes; figure 1a), but not ILC1 or ILC3, was significantly higher than that of CD45.1 wild-type (WT) cells (figure 1b). As the expression pattern of surface markers of ILC2s potentially varies [26], the expression of ILC2 markers on lineage^{-}CD45^{+} cells was compared using t-distributed stochastic neighbour embedding [27, 28]. A cell cluster exhibiting high-expression levels of T1/ST2, Sca-1 and KLRG1 was identified among the three clusters obtained by the analysis (figure 1c), and the cluster is consistent with ILC2s we initially defined (figure 1a). Both WT and *Regnase-1*$^{-/-}$ cells were found in the cluster, though the numbers of *Regnase-1*$^{-/-}$ ILC2s were much more than the WT (figure 1d). The WT and *Regnase-1*$^{-/-}$ ILC2 clusters also express other markers for ILC2s such as c-kit and CD127 (IL-7R), whereas the expression levels of CD25 and Thy1.2 levels were decreased in *Regnase-1*$^{-/-}$ ILC2s (figure 1e). In addition to ILC2s, populations of effector/memory CD4^{+} T cells and Th2 cells (CD3^{+}CD4^{+}GATA3^{+} cells) were elevated with Regnase-1 deficiency, suggesting that Regnase-1 regulates the number of type 2 immune cells in a cell-intrinsic manner (figure 1b and supplementary figure S1b). When type 2 effector cells were examined, Regnase-1 deficiency increased the basophils, but not eosinophils or mast cells (figure 1b). Conversely, an increase of CD45.2 *Regnase-1*$^{-/-}$ ILC2 population in fat-associated lymphoid cells was not found compared with CD45.1 WT cells, indicating the tissue specific feature of ILC2s increase in the absence of Regnase-1 (supplementary figure S2).

The number of ILC2 was also increased in the lung of *Regnase-1*$^{-/-}$ mice (figure 1f). When we generated mice lacking both Regnase-1 and Rag2, a significant increase in ILC2s was observed in the lung or BAL fluid in the absence of Regnase-1 indicating that the increase of *Regnase-1*-deficient ILC2s does not depend on the secondary effect of T cell activation (figure 1g). Next, the eosinophils in the lungs were examined, which are known to be elicited by ILC2s and Th2 cells. We found that eosinophils were highly increased in the lungs of *Regnase-1*$^{-/-}$ mice (figure 1h). The increased numbers of eosinophils in the lungs and the BAL of *Regnase-1*-deficient mice were observed even when the mice lacked T cells due to *Rag2* deficiency (figure 1i and supplementary figure S3), suggesting the critical role of *Regnase-1* in ILC2s for eosinophilic inflammation.

Regnase-1 regulates ILC2 proliferation and activation

Next, we examined whether Regnase-1 controls ILC2 proliferation or cell death using the competitive transfer model. The expression of a proliferation marker Ki67 was significantly higher in *Regnase-1*-deficient ILC2s compared with WT ILC2s (figure 2a). In contrast, there was no difference in the levels of a cell viability marker (figure 2b), indicating that Regnase-1 negatively regulates the cell proliferation, but not death, of ILC2s.

We then investigated whether this enhanced proliferation is recapitulated in ILC2s cultured *in vitro*. We sorted ILC2s from the lung of WT and *Regnase-1*$^{-/-}$ mice, then cultured them with IL-2 and IL-7, a culture condition inducing homeostatic expansion of ILC2s [26], Unexpectedly, both WT and *Regnase-1*$^{-/-}$ ILC2s proliferated to produce similar numbers of cells after 5 days of culture (figure 2c), suggesting that IL-2 and IL-7 are not sufficient to evoke enhanced proliferation induced under Regnase-1 deficiency *in vivo*.

To further characterise the effect of Regnase-1 deficiency in ILC2 activation, we evaluated ILC2-signature surface molecules on the ILC2s. Interestingly, the expression levels of ICOS and KLRG1 were significantly upregulated in *Regnase-1*-deficient ILC2s in the competitive BM transfer model or in *Rag2*$^{-/-}$*Regnase-1*$^{-/-}$ mice (figure 2d and supplementary figure S4). ICOS stimulation promotes the proliferation of ILC2s by stabilising STAT5 phosphorylation [29], and *Icos* mRNA is directly degraded by Regnase-1 [5]. In contrast, it was shown that KLRG1 stimulation did not induce the proliferation of ILC2s [30]. Therefore, we hypothesised that Regnase-1 suppresses the proliferation of ILC2s through the downregulation of ICOS. To exclude the contamination of T cells, which also express ICOS, we used *Rag2*$^{-/-}$ mice and *Rag2*$^{-/-}$*Regnase-1*$^{-/-}$ mice for the purification and culture of ILC2. Treatment with an ICOS-stimulating antibody significantly increased the number of cultured *Regnase-1*-deficient ILC2s compared with WT (figure 2e), accompanied by the augmentation of STAT5 phosphorylation (figure 2f). These data indicate that the Regnase-1-mediated ICOS downregulation contributes to the maintenance of ILC2 numbers in the lung. In addition, *Regnase-1*-deficient ILC2s were found to show decreased expression of Thy1.2 (figure 2d), which suggested the activation of ILC2s [30, 31]. The secretion of ILC2-signature cytokines such as IL-5 and IL-13 as well as IL-6, which is the established target of Regnase-1, was elevated in cultured *Regnase-1*-deficient ILC2s (figure 2g). These data demonstrate that Regnase-1 is important not only for controlling the numbers of ILC2s but also for maintaining quiescent status of ILC2s in the steady state

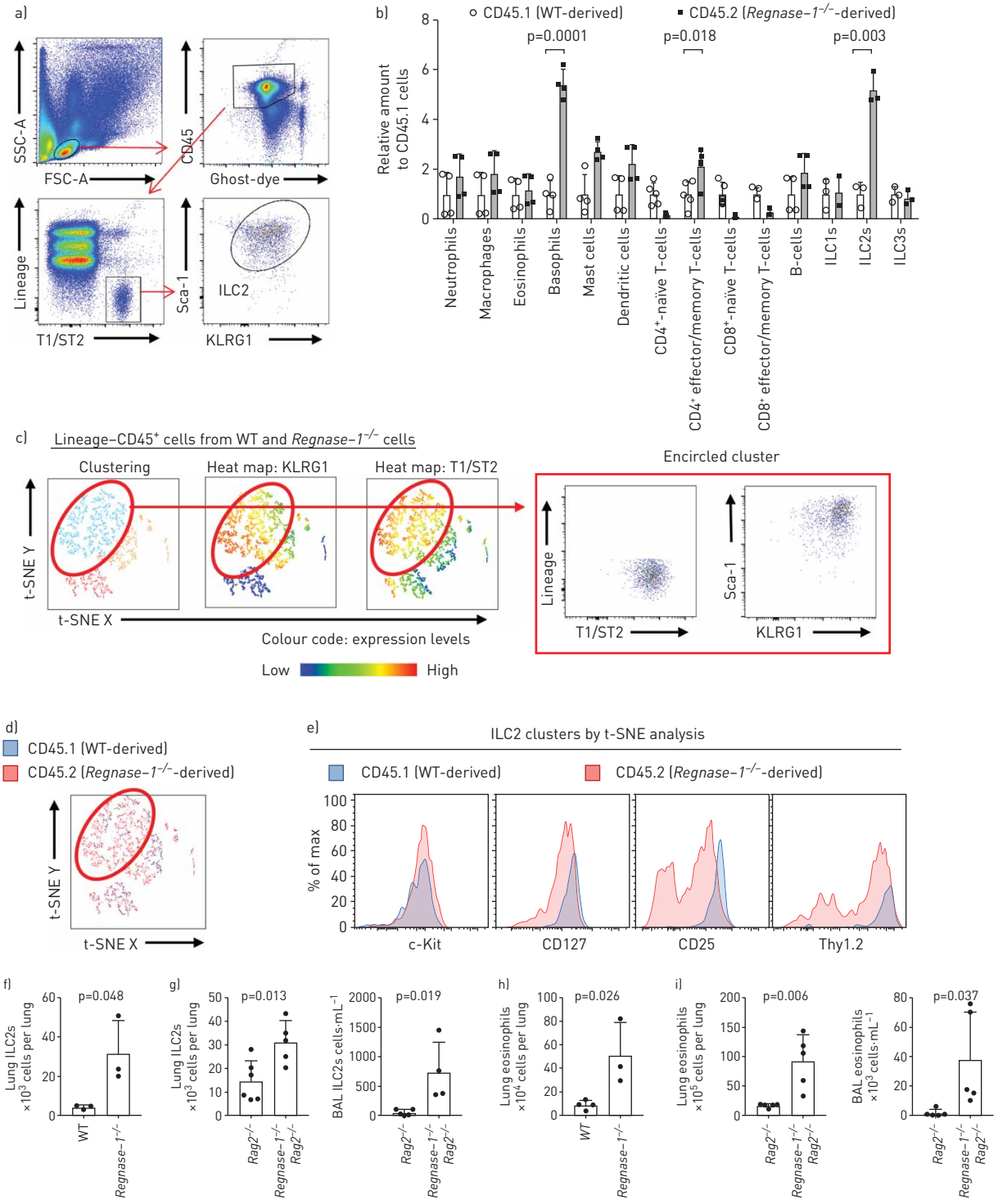

FIGURE 1 Regnase-1 negatively regulates the proliferation of group 2 innate lymphoid cells (ILC2s) in a cell-intrinsic manner. a) Representative figure for gating of ILC2s by flow cytometry. The identification of ILC2s in the lung of mice was done with CD45$^+$Lineage$^-$T1/ST2$^+$KLRG$^+$Sca-1$^+$ cells. Doublet depletion was done by the analysis of forward-scattered (FSC)-A/FSC-H and side-scattered (SSC)-A/SSC-H. b) Ratio of a CD45.1$^+$ (derived from wild-type (WT) mice) and CD45.2$^+$ (derived from *Regnase-1*$^{-/-}$ mice) population among each of cell type (n=3–5). Data are the integrated numbers of two independent experiments. c) A t-distributed stochastic neighbour embedding (t-SNE) analysis and subsequent clustering of CD45$^+$Linage$^-$Ghost-dye$^-$ cells in the lung following competitive bone marrow transfer. The encircled population corresponds with ILC2. d) Comparison of CD45.1$^+$ (WT mice-derived, blue) and CD45.2$^+$ (*Regnase-1*$^{-/-}$ mice-derived, red) CD45$^+$Linage$^-$Ghost-dye$^-$ cells by t-SNE analysis. The encircled population corresponds with ILC2. e) Histograms of expression of c-Kit, CD127, CD25 and Thy1.2 on WT and *Regnase-1*$^{-/-}$ ILC2s. f and h) Total number of f) ILC2s or h) eosinophils in the lung of WT mice (n=4) and *Regnase-1*$^{-/-}$ mice (n=3). Data are the representative of two independent experiments. g and i) Comparison for the total number of g) ILC2s or i) eosinophils in the lung (left panel, n=5) and bronchoalveolar lavage (BAL) (right panel, n=4) in the lung of *Rag2*$^{-/-}$ mice and *Regnase-1*$^{-/-}$*Rag2*$^{-/-}$mice. Data are representative of two independent experiments. Data are shown as mean±SD. The t-test was used for analyses.

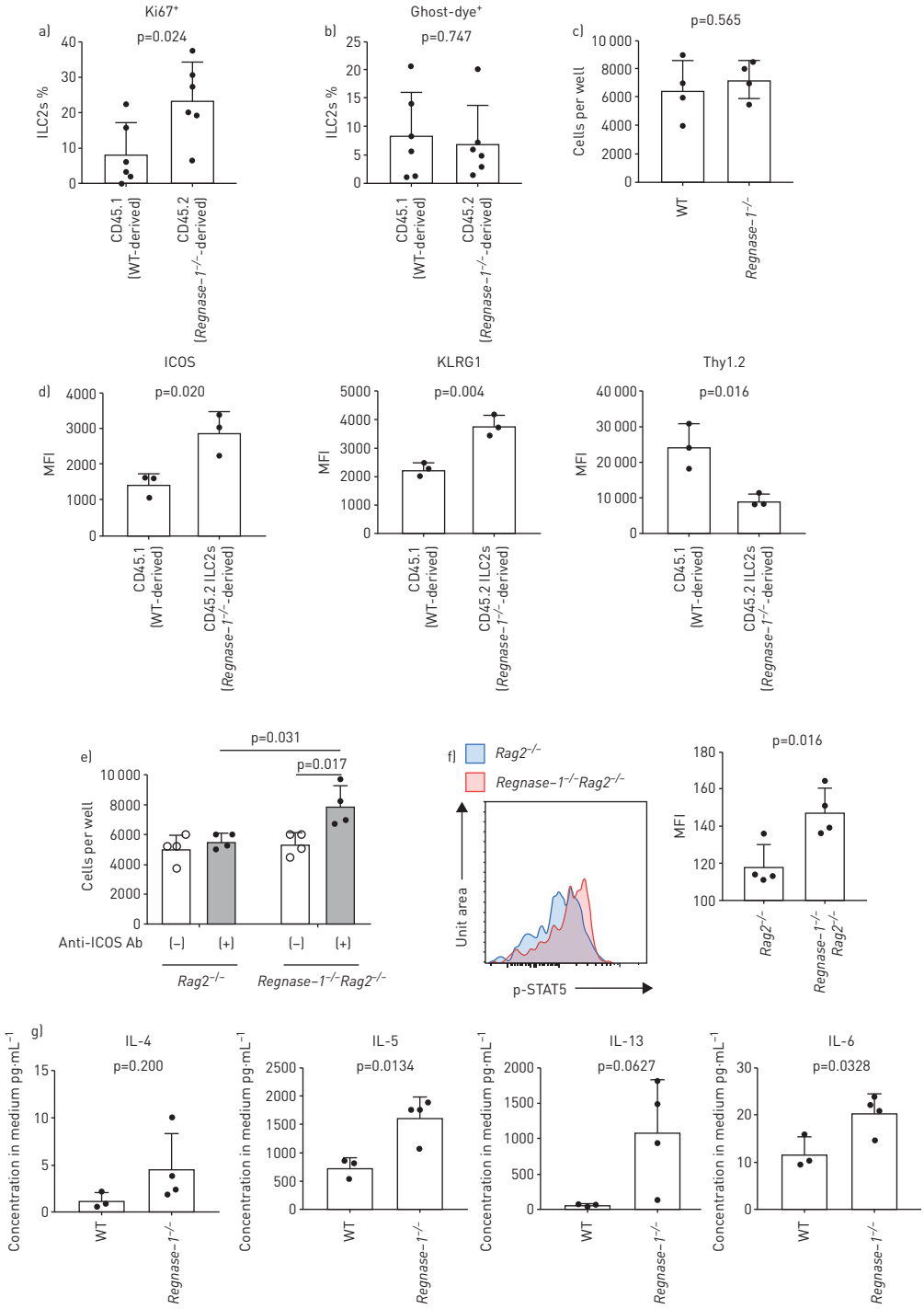

FIGURE 2 *Regnase-1*-deficient innate lymphoid cells (ILC2s) exhibit spontaneous activation. a and b) Ratio of the a) Ki67$^+$ population and b) viability marker (Ghost-dye) among wild-type (WT) (CD45.1$^+$) and *Regnase-1*-deficient ILC2s (CD45.2$^+$) from the lung of competitively transferred mice (n=6, the integrated numbers of two independent experiments). Data are shown as mean±SD. The t-test was used for analyses. c) *Ex vivo* cell proliferation of ILC2s isolated from the lung of WT or *Regnase-1*$^{-/-}$ mice cultured with 10 ng·mL^{-1} recombinant mouse IL-2 plus IL-7 (n=4). On day 1, 5000 cells were seeded and stimulated, and on day 5 the numbers of cells were evaluated. Data are representative of two independent experiments. d) Cell-surface expression levels of the indicated proteins among WT (CD45.1$^+$) and *Regnase-1*-deficient (CD45.2$^+$) ILC2s from the lung of competitively transferred mice. Expression levels were evaluated by flow cytometry. Data are representative of three independent experiments. e) *Ex vivo* cell proliferation and f) phospho-STAT5 protein levels evaluated by flow cytometry of ILC2s isolated from the lung of *Rag2*$^{-/-}$ or *Regnase-1*$^{-/-}$*Rag2*$^{-/-}$ mice cultured with 10 ng·mL^{-1} recombinant mouse IL-2 plus IL-7 supplemented with or without 3 µg·mL^{-1} anti-ICOS stimulating antibody (n=4). On day 1, 5000 cells were seeded and stimulated. On day 5, the number of cells and phospho-STAT5 expression levels were evaluated. Data are representative of two independent experiments. g) Concentrations of indicated cytokines in the culture supernatant of ILC2s from the lungs of WT or *Regnase-1*$^{-/-}$ mice. In total, 5000 cells were suspended with 200 µL of medium supplemented with 10 ng·mL^{-1} recombinant mouse interleukin (IL)-2 and IL-7, cultured and samples were collected on day 5. The concentrations were measured by Bioplex. MFI: mean fluorescence intensity.

condition [30, 31]. Collectively, these data demonstrate that Regnase-1 is important not only for controlling the numbers of ILC2s but also for maintaining quiescent status of ILC2s.

Regnase-1-*deficient ILC2s promote pulmonary fibrosis*

We next investigated the relevance of Regnase-1 expressed in ILC2s in pulmonary diseases. For this purpose, we simultaneously isolated WT and *Regnase-1*-deficient ILC2s from the lung of competitively transferred mice, and performed transcriptome analysis. We found 289 differentially expressed genes between WT and *Regnase-1*-deficient ILC2s. Among them, 221 genes showed increased expression in *Regnase-1*-deficient ILC2s compared with WT ILC2s (hereafter "upregulated genes") (figure 3a and supplementary tables S3 and S4). The upregulated genes included cytokines such as *Il4*, *Il5*, *Il6* and *Il13* (figure 3b). We then analysed the associations between the upregulated genes and pulmonary diseases by using the Comparative Toxicogenomics Database (CTD) [32]. Consistent with the activation of ILC2s, genes categorised to "asthma" [33, 34] were significantly enriched (figure 3c and supplementary table S5). In addition, the genes categorised to "pulmonary fibrosis" were also highly enriched (figure 3c and supplementary table S5). Analysis with human orthologues of the upregulated genes reproduced the same set of diseases (supplementary figure S5a). While pulmonary fibrosis- and asthma-related genes overlapped each other (supplementary table S6), pulmonary fibrosis-specific genes and overlapping genes were significantly more frequent compared with asthma-specific genes (figure 3d), implying that Regnase-1 regulates pulmonary fibrosis-related genes more broadly than asthma-related genes. These data suggest the association of Regnase-1 in ILC2s with pulmonary diseases, especially fibrosis.

Therefore, the relationship between Regnase-1 in ILC2s and pulmonary fibrosis in mice was investigated. First, reverse-transcriptase quantitative PCR analysis revealed that bleomycin treatment decreased *Regnase-1* expression in lung ILC2s, suggesting that Regnase-1 expression is decreased following the development of pulmonary fibrosis in mice (figure 3e). Then we administered bleomycin intratracheally in *Rag2*$^{-/-}$*Il2rc*$^{-/-}$ mice, which lack all mature T cells, B cells and ILCs, followed by the intratracheal transfer of cultured ILC2s derived from *Regnase-1*$^{-/-}$*Rag2*$^{-/-}$ or control (*Rag2*$^{-/-}$) mice [22] (supplementary method and supplementary figure S5b). Mice receiving *Regnase-1*-deficient ILC2s (KO-transfer) exhibited more severe fibrotic lesions compared with mice without ILC2 transfer (NO-transfer) or receiving the transfer of control ILC2s (WT-transfer) (figure 3f and g). In addition, collagen deposition in the lung as well as the levels of IL-4 and IL-13, potent inducers of fibrosis [35], were elevated in the BAL fluid of mice receiving *Regnase-1*-deficient ILC2s (figure 3g and i and supplementary table S2). Collectively, these data indicate that Regnase-1 expressed in ILC2s is critical for the regulation of pulmonary fibrosis in mice.

Regnase-1 negatively regulates the expression of genes associated with pulmonary fibrosis

Since fibrosis-associated genes *Il4* and *Il13* are not directly degraded by Regnase-1, we hypothesised that Regnase-1 primarily regulates the transcriptional programme(s) of ILC2s which subsequently control cytokines and fibrosis-associated genes. To identify the Regnase-1-regulated transcriptional programmes, we employed two approaches. First was the analysis on the association between the upregulated genes and CGAP BioCarta Pathways [36, 37], which revealed a significant enrichment of the GATA3 pathway, which is critical for the regulation of IL-4, IL-5 and IL-13 (figure 4a) [38]. Intriguingly, *Gata3* mRNA levels were significantly increased in *Regnase-1*-deficient ILC2s (figure 4b), which is consistent with the previous report that *Gata3* mRNA is a direct target of Regnase-1 [7]. As the second approach, we screened transcription factor binding sites of upregulated genes associated with pulmonary fibrosis in the CTD (supplementary methods), and isolated a set of transcription factors potentially regulating pulmonary fibrosis-associated genes (figure 4c and supplementary table S7). Among identified transcription factors, the expression of *Egr1*, *Nfkb1* and *Fos* was significantly increased in *Regnase-1*-deficient ILC2s (figure 4d and supplementary table S8). Especially, the expression level of *Egr1* was more than 60 times higher in *Regnase-1*-deficient ILC2s (figure 4d). A luciferase reporter harbouring *Egr1* 3′UTR was suppressed by the overexpression of WT, but not nuclease activity-inactivated mutant (D141N), Regnase-1 (figure 4e), indicating that the *Egr1* mRNA is directly degraded by Regnase-1. Taken together, profibrotic function of ILC2s in the lung is critically suppressed by Regnase-1 *via* the post-transcriptional regulation of transcriptional networks including GATA3 and EGR-1.

Regnase-1 expression levels negatively correlate with the ILC2 population in human BAL

These results prompted us to investigate the clinical significance of Regnase-1-mediated regulation of ILC2s in IPF patients. Human ILC2s were defined as CD45^{+}lineage^{-}CD127^{+}CRTH2^{+}CD161^{+} cells (supplementary figure S6). We analysed Regnase-1 expression levels in BAL ILC2s from IPF patients and control subjects by newly generating a flow cytometry-based method of intracellular Regnase-1 staining (supplementary method, supplementary figure S7a–c and supplementary table S9). Interestingly, a significant negative association was found between Regnase-1 expression levels and the population as well

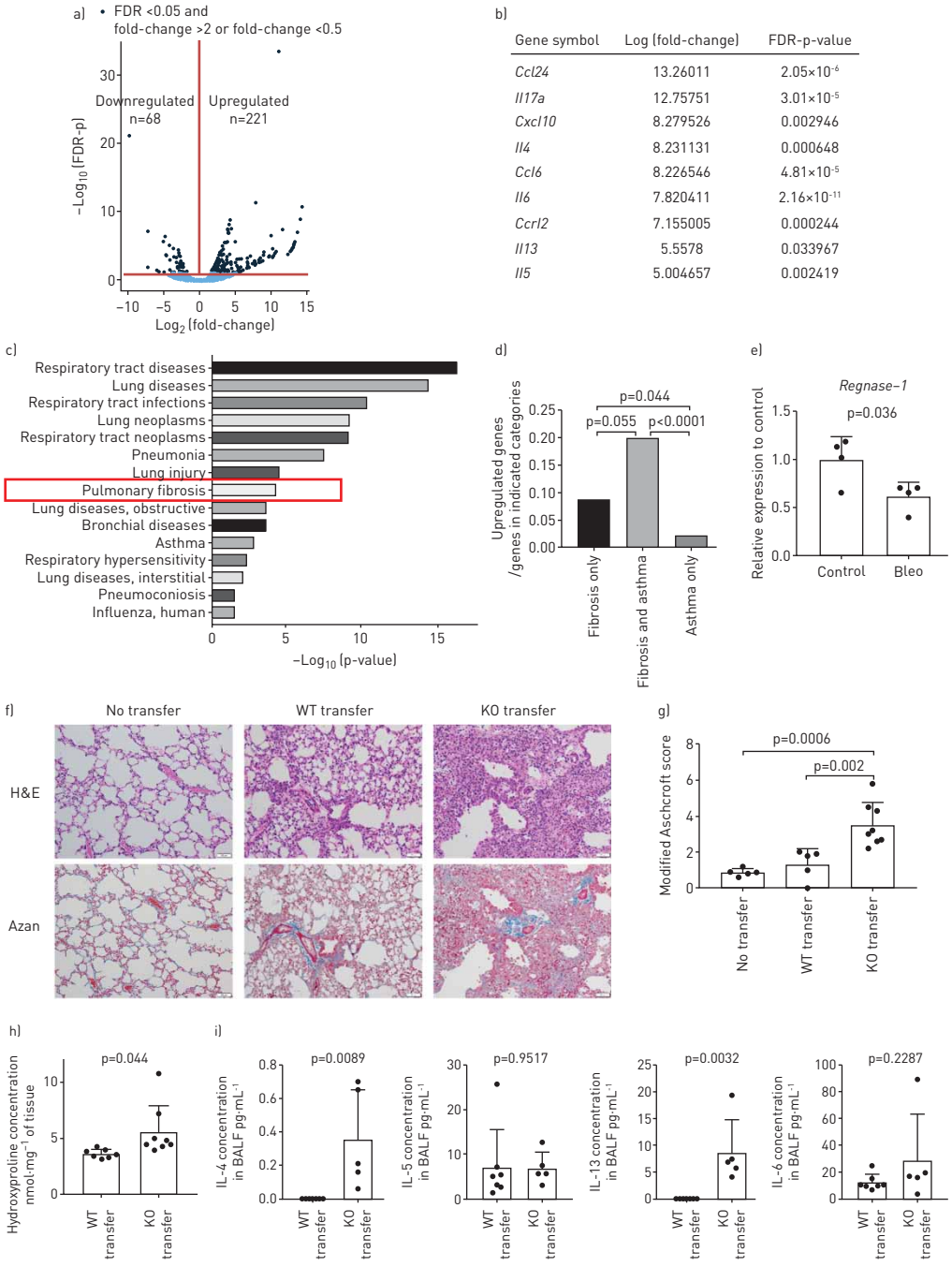

FIGURE 3 *Regnase-1*-deficient group 2 innate lymphoid cells (ILC2s) promote pulmonary fibrosis. a) Volcano plot comparing the mRNA levels in wild-type (WT) (CD45.1$^+$) and *Regnase-1*-deficient (CD45.2$^+$) cells among ILC2s in the lung of competitive bone marrow (BM) transfer model mice (n=2). Expression levels in CD45.1$^+$ cells were regarded as the control, and differentially expressed genes (DEGs) were defined as genes with false discovery rate (FDR) p-values of <0.05 and fold changes of <0.5 or >2.0, and highlighted. b) Upregulated genes coding cytokines. FDR p-values and fold-changes (expression levels in *Regnase-1*-deficient ILC2s compared with WT ILC2s) were calculated by edgeR. c) Top 15 human diseases that are associated with the upregulated genes. The Comparative Toxicogenomics Database (CTD) analyser was used for the analysis. d) Ratio of upregulated genes within "pulmonary fibrosis" but not in "asthma" (categorised as fibrosis only), within asthma but not in pulmonary fibrosis (asthma only) or both of pulmonary fibrosis and asthma (fibrosis and asthma). Fisher's exact test was used for the analysis, and Bonferroni's adjustment was performed for multiple comparisons. e) Quantitative PCR analysis for the expression levels of *Regnase-1* for ILC2s in the lungs of intratracheal bleomycin-treated *Rag2*$^{-/-}$ mice. *Rag2*$^{-/-}$ mice without bleomycin treatment were used as controls (n=4). Data are shown as mean±SD. f) Histological images and g) pathological lung fibrosis scores of the bleomycin-induced pulmonary fibrosis model mice without cell transfer (no transfer, n=5) or transferred with ILC2s derived from *Rag2*$^{-/-}$ or *Regnase-1*$^{-/-}$*Rag2*$^{-/-}$ mice (WT-transfer, n=5 and knockout (KO)-transfer, n=8, respectively). Representative images with haematoxylin and eosin staining (H&E, upper) and Azan staining (lower) are shown in f). Scale bars=50 µm. Data are the cumulative of three independent experiments. h) Amount of collagen deposition measured with hydroxyproline assay (n=7 for WT-transfer, n=8 for KO-transfer). Data are the cumulative of three independent experiments. i) Concentrations of indicated cytokines in the bronchoalveolar lavage fluid (BALF) of WT-transfer and KO-transfer mice. The concentrations were measured by Bioplex. Fisher's exact test and t-test were used for the statistical analyses. For the adjustment of multiple comparisons, Holm's method was used.

a)

	p-value	Fold-enrichment
Cytokine pathway	0.00243	8.07644
Gata3 pathway	0.00781	9.04561
Cdmac pathway	0.00943	8.48026
IL1R pathway	0.01296	5.13955
4-1-BB pathway	0.01321	7.53801
Tnfr2 pathway	0.01773	6.78421
IL-5 pathway	0.02014	12.72039
HIV Nef pathway	0.02233	3.50907
NFκB activation by nontypeable *Haemophilus influenzae* pathway	0.03242	5.42737
MAPK pathway	0.03384	2.72928
Cytokines and inflammatory response pathway	0.03593	5.21862
Erythropoietin-mediated neuroprotection through NF-κB pathway	0.03747	9.25120
IL-10 pathway	0.04416	8.48026

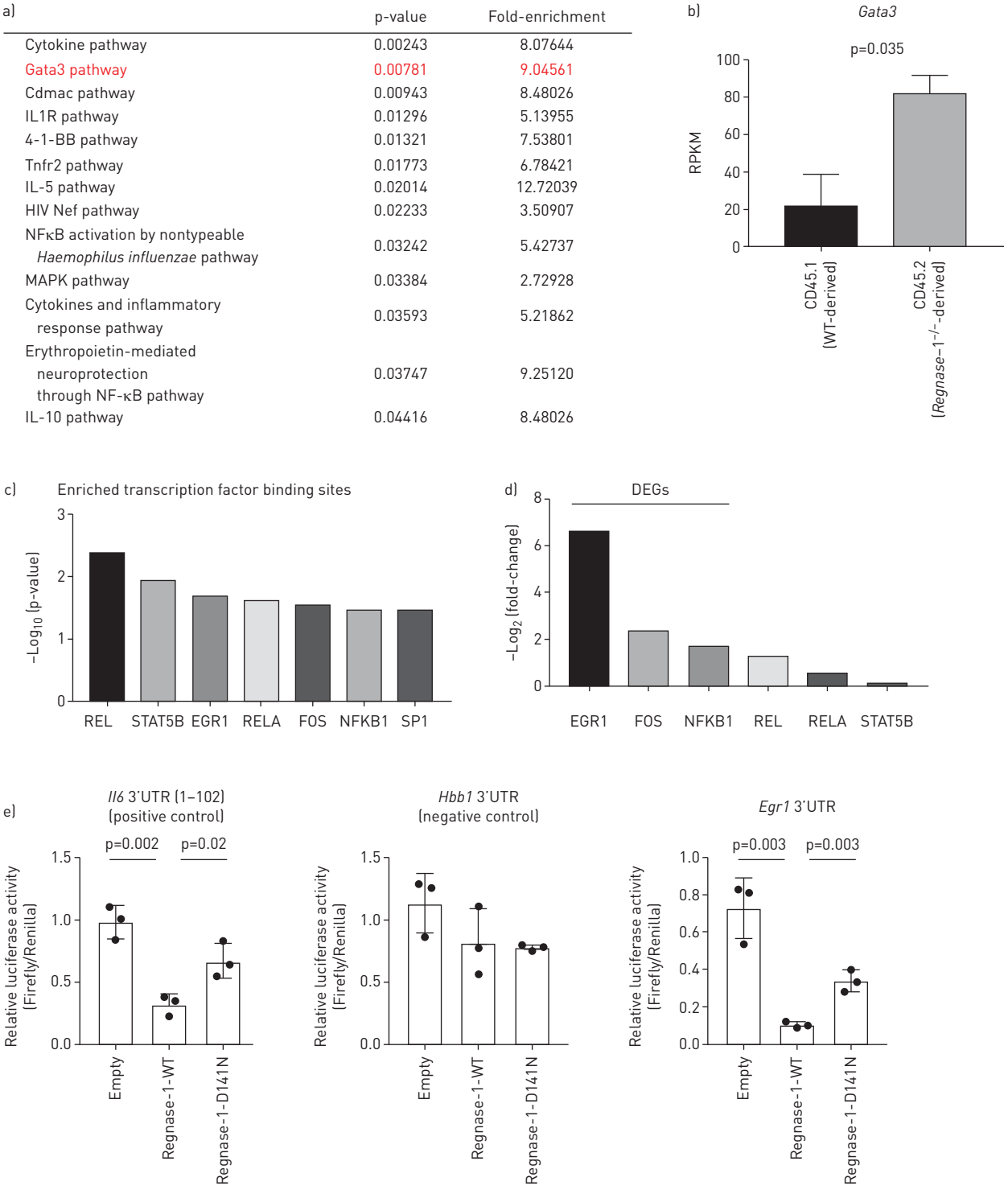

FIGURE 4 Loss of Regnase-1 induces the expression of genes associated with pulmonary fibrosis. a) Significantly enriched pathways for differentially expressed genes (DEGs) in the BioCarta Pathway. A p-value <0.05 was regarded as significant. The analysis was done using DAVID 6.8 Bioinformatics Resources (https://david.ncifcrf.gov/). b) Comparisons for RPKM (reads per kilo base per million mapped reads) values of *Gata3* between wild-type (WT) (CD45.1$^+$) and *Regnase-1*-deficient (CD45.2$^+$) cells. p-values (false discovery rate (FDR)-p-values) were calculated by edgeR. c) Enriched transcription factor binding motifs associated with the upregulated genes categorised in "pulmonary fibrosis" in the Comparative Toxicogenomics Database. Screening was performed based on the JASPAR (http://jaspar.genereg.net/) and TRANSFAC (https://genexplain.com/transfac/) databases. The analysis was done by using Enrichr. Transcription factors with p-values <0.05 are shown. d) Fold changes of the expression levels for the genes shown in figure 4c in Regnase-1-deficient cells compared with WT cells. *Sp1* is not shown because it had been excluded from the analysis due to low RPKM values. e) Luciferase reporter assay for analysing Regnase-1 mediated suppression of gene expression through the 3′ untranslated region (UTR) sequence of the indicated genes. Either an empty plasmid, murine Regnase-1 WT or a nuclease-dead mutant (D141N) were transfected into HEK293 cells, and luciferase activity was measured on day 2 (n=3). Data representative of two independent studies are shown. The t-test was used for the analysis.

as the number of ILC2s in BAL (figure 5a and supplementary figure S8), suggesting that Regnase-1 regulates ILC2 numbers in the lungs both in mouse and human. However, Regnase-1 expression levels or ILC2 populations were not significantly different between IPF patients and controls (figures 5b and c). Whether BAL ILC2 populations were associated with the clinical course among IPF patients was then investigated. We found that ILC2 populations in BAL tend to be increased in IPF patients who exhibited complications with disease progression within 1 year, although the difference did not reach statistical significance (figure 5d).

High number of peripheral blood ILC2s predicts poor prognosis in IPF patients

Although ILC2s are considered tissue-resident, the number of ILC2s in the peripheral blood is suggested to correlate with the local inflammatory conditions contributing the increase in ILC2s [34, 39]. Therefore, to increase the number of patients, we quantified the number of ILC2s in the peripheral blood of IPF patients and analysed the association between the clinical indices. We found that ICOS expression levels were positively associated with the number of blood ILC2s in IPF patients (figure 6a). However, no statistically significant correlations were found between peripheral blood ILC2 numbers and pulmonary function indices (figure 6b). Also, no correlations were observed between the ILC2 numbers and plasma levels of IL-4, IL-5 and IL-13 (supplementary figure S9a). These results suggest that the peripheral blood ILC2s do not reflect the intensity of systemic type 2 inflammation among IPF patients.

We next analysed the associations between peripheral blood ILC2s and the survival of IPF. Based on a receiver operating characteristic curve to identify respiratory death (supplementary figure S9b), we divided patients into two groups according to a peripheral blood ILC2 number of 1500 cells·mL^{-1}. Patients with ILC2s >1500 cells·mL^{-1} showed lower forced vital capacity (FVC) percentage, but otherwise the two groups showed no significant differences (table 1). Notably, patients with peripheral blood ILC2s >1500 cells·mL^{-1} showed strikingly worse respiratory mortality and all-cause mortality (figure 6c). The 3-year survival rates of the patients with the higher and lower ILC2 numbers were 0.239 and 0.734, respectively. By setting these values, the power of analysis was calculated as 0.891. Death due to chronic respiratory failure was significantly common among the patients with peripheral blood ILC2s

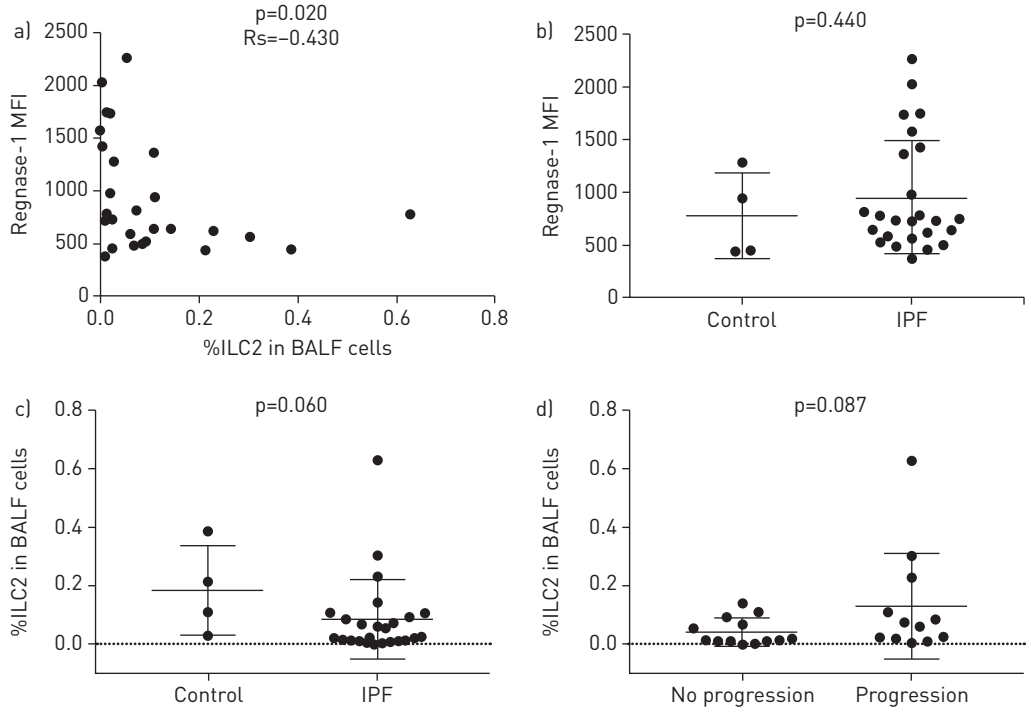

FIGURE 5 Regnase-1 expression negatively correlates with the group 2 innate lymphoid cells (ILC2s) population in human bronchoalveolar lavage fluid (BALF). a) Correlation between the ratio of ILC2s among the BALF cells and Regnase-1 protein expression levels of ILC2s in BALF measured by flow cytometry. b and c) Comparisons of b) the Regnase-1 protein expression levels of ILC2s in BALF measured by flow cytometry or c) ratio of ILC2s among the BALF cells between controls and idiopathic pulmonary fibrosis (IPF) patients. d) Comparisons of the ratio of ILC2s among the BALF cells between IPF patients without and with progression in 1 year after BAL. Mann–Whitney U-test and Spearman's rank correlation test were used. MFI: mean fluorescence intensity.

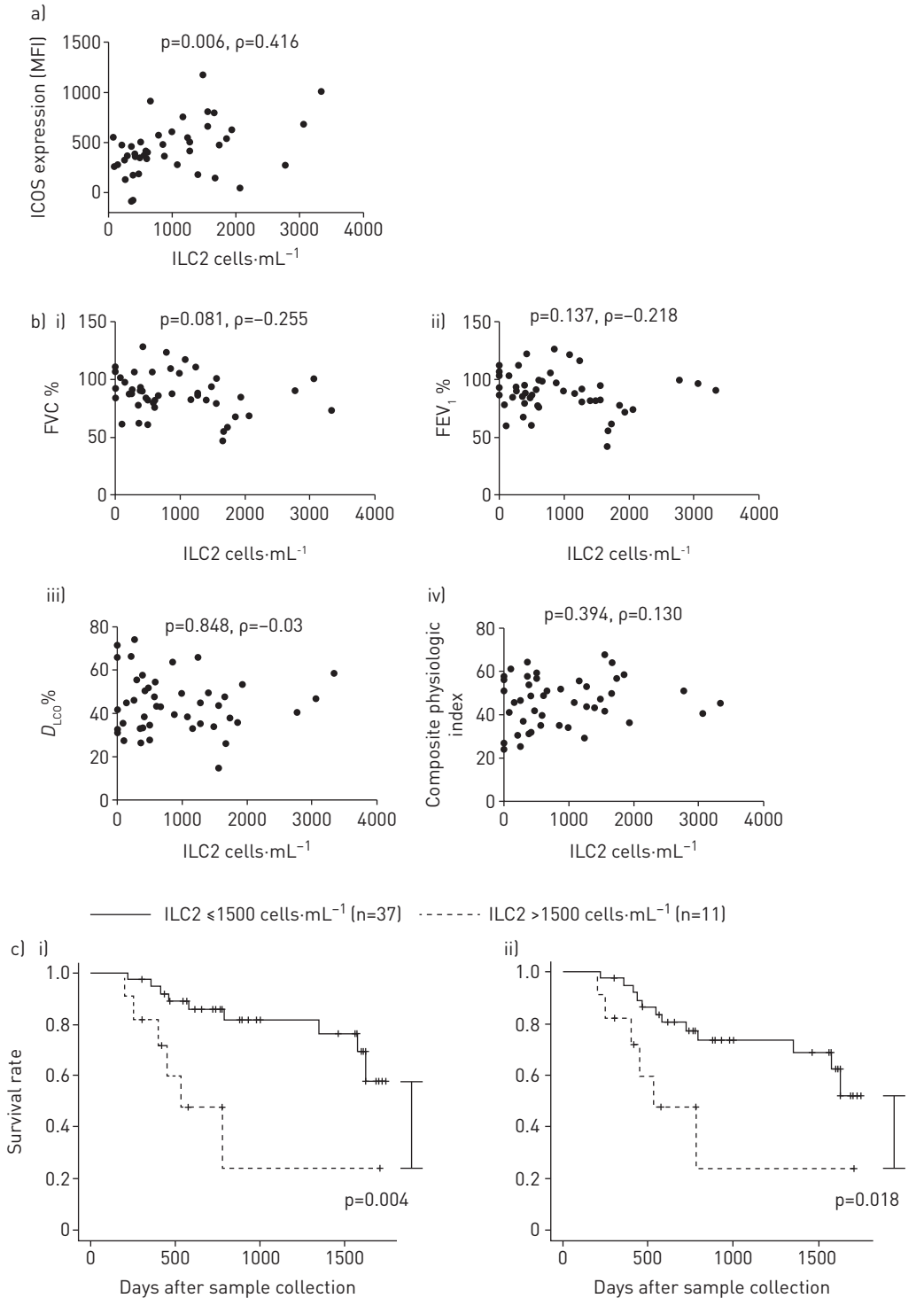

FIGURE 6 Associations between the concentrations of group 2 innate lymphoid cells (ILC2s) in peripheral blood and clinical indices among idiopathic pulmonary fibrosis (IPF) patients. a) Associations between the number of ILC2s in peripheral blood and ICOS protein expression levels evaluated as mean fluorescence intensity (MFI) with flow cytometry. b) Associations between the number of ILC2s in peripheral blood and i) forced vital capacity (FVC) % predicted, ii) forced expiratory volume in 1 s (FEV_1) % predicted, iii) diffusing capacity of the lung for carbon monoxide (D_{LCO}) % predicted, and iv) composite physiologic index. Spearman's rank correlation test was used for the analysis. c) Kaplan–Meier curves for i) respiratory death and ii) all-cause mortality. The log rank test was used for the analysis.

>1500 cells·mL^{-1} (supplementary table S10). These data suggest that higher ILC2 numbers in the blood are associated with disease severity and poor prognosis.

To further investigate whether ILC2s could predict prognosis independent of other confounding factors, we performed univariate and multivariate analyses for respiratory mortality. In univariate analysis, ILC2s >1500 cells·mL^{-1}, FVC percentage, diffusing capacity of the lung for carbon monoxide % predicted and the lowest saturation of peripheral oxygen (S_{pO_2}) value in the 6-min walk test (6MWT) were significantly associated with respiratory mortality. Conversely, multivariate analysis revealed that the peripheral blood ILC2 number >1500 cells·mL^{-1} was the independent factor for respiratory mortality, while other factors except the lowest S_{pO_2} value in the 6MWT failed to remain as significant factors (table 2). These data suggest that an increase in the number of peripheral blood ILC2s can correlate with IPF disease severity, and is an independent and strong predictive factor for survival (supplementary figure S10).

Discussion

In this study, we discovered that Regnase-1 is critical for the regulation of proliferation and activation of ILC2s, and inhibition of pulmonary fibrosis progression. Compared with the roles of ILC2s in allergic diseases such as bronchial asthma, a small number of studies have focused on their significance in pulmonary fibrosis, and the regulatory mechanisms for profibrotic functions is largely unknown [40]. In this regard, mice lacking Regnase-1 is a novel mouse model for the functional analysis of ILC2s in suppressing pulmonary fibrosis.

Although Regnase-1 degrades a set of cytokine genes, such as *Il6*, *Il12b* and *Il2* in macrophages and T cells, it fails to recognise *Il4* and *Il13* genes [5], which are abundantly produced by ILC2s. Instead, Regnase-1 seems to regulate these cytokines *via* suppression of transcription factors such as *Gata3* and *Egr1*. It has been shown that increased GATA3 expression promoted the production of IL-4, IL-5 and IL-13 in ILC2s [38]. These cytokine genes were included in the categories of both pulmonary fibrosis- and asthma-associated genes in the CTD, suggesting that increased GATA3 expression contributes to the development of both diseases [35]. A very recent paper reported that Regnase-1 degradation in ILC2 through IL-25 or IL-33 stimulation enhanced papain-induced respiratory tract inflammation, which

TABLE 1 Background of cases in the analysis of group 2 innate lymphoid cells (ILC2s) in peripheral blood

	ILC2 ⩽1500 cells·mL^{-1}	ILC2 >1500 cells·mL^{-1}	p-value
Subjects	37	11	
Male	33 (89.2)	9 (81.8)	0.609
Age years	73.00 (69.00–79.00)	71.00 (67.50–75.50)	0.350
Never-smoker	3 (8.3)	0 (0.0)	>0.99
Brinkman index	740.00 (460.00–1055.00)	900.00 (655.00–1840.00)	0.175
Diabetes mellitus	11 (29.7)	4 (36.4)	0.720
Bronchial asthma	4 (10.8)	1 (9.1)	>0.99
Pirfenidone	6 (16.2)	5 (45.5)	0.095
Corticosteroid	3 (8.1)	1 (9.1)	>0.99
P_{aO_2} mmHg	82.80 (74.90–93.70)	77.80 (68.85–80.20)	0.128
P_{aCO_2} mmHg	39.10 (36.30–40.40)	41.40 (38.20–42.60)	0.113
FVC %	87.90 (82.40–105.30)	72.90 (63.10–87.20)	0.010
D_{LCO} %	43.30 (34.20–53.20)	42.00 (36.40–47.43)	0.594
FEV_1 %	90.20 (82.20–103.40)	77.90 (66.75–92.80)	0.032
FEV_1/FVC %	81.19 (77.39–85.21)	83.57 (78.26–86.72)	0.454
Composite physiologic index	46.41 (34.93–55.64)	50.98 (43.31–61.05)	0.138
6MWT distance m	479.00 (412.00–532.00)	420.00 (357.50–469.50)	0.116
Lowest S_{pO_2} in 6MWT %	86.50 (82.75–90.00)	88.00 (77.50–91.00)	0.548
SP-D ng·mL^{-1}	213.00 (108.00–353.00)	285.00 (242.00–479.00)	0.031
KL-6 U·mL^{-1}	726.00 (621.00–1190.00)	986.00 (472.00–1535.00)	0.787
Death	12 (32.4)	6 (54.5)	0.288
Respiratory death	9 (24.3)	6 (54.5)	0.095

Data are presented as n, n (%) or median (interquartile range), unless otherwise stated. P_{aO_2}: partial pressure of oxygen; P_{aCO_2}: partial pressure of carbon dioxide; FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; FEV_1: forced expiratory volume in 1 s; 6MWT: 6-min walk test; S_{pO_2}: saturation of peripheral oxygen; SP-D: surfactant protein D; KL: Krebs-von den Lungen. Fisher's exact test and Mann–Whitney U-test were used for analyses.

TABLE 2 Univariate and multivariate analysis for respiratory mortality

	Hazard ratio (95% CI)	p-value
Univariate analysis		
ILC2s >1500 cells·mL^{-1}	4.21 (1.45–12.19)	0.008
Age	1.00 (0.92–1.08)	0.938
Male sex	0.44 (0.12–1.57)	0.206
Corticosteroid usage	2.38 (0.67–8.46)	0.182
FVC %	0.95 (0.92–0.99)	0.006
D_{LCO} %	0.91 (0.86–0.97)	0.002
P_{aO_2}	0.97 (0.93–1.00)	0.062
Brinkman index	1.00 (0.99–1.00)	0.484
6MWT S_{pO_2} minimum	0.88 (0.82–0.94)	<0.0001
Multivariate analysis (stepwise)		
ILC2s >1500 cells·mL^{-1}	7.17 (1.66–30.97)	0.008
Brinkman index	1.00 (0.99–1.00)	0.082
P_{aO_2}	1.06 (0.98–1.13)	0.130
6MWT S_{pO_2} minimum	0.74 (0.62–0.88)	0.0008

ILC2s: group 2 innate lymphoid cells; FVC: forced vital capacity; D_{LCO}: diffusing capacity of the lung for carbon monoxide; P_{aO_2}: partial pressure of oxygen; 6MWT: 6-min walk test; S_{pO_2}: saturation of peripheral oxygen. Cox proportional hazards test was used for the analyses.

indicates the relationship between Regnase-1 in ILC2s and asthma [41]. Conversely, Donovan *et al.* [42] reported that exposure of *Rora*$^{fl/fl}$ *Il7r*^{Cre} mice, which lack ILC2s, to cigarette smoking showed increased *Il13* expression and collagen deposition, although they are protected from emphysema. Thus, the functions of ILC2s in the lungs may be different depending on the disease models, although further studies are required to uncover the precise mechanisms how ILC2s contribute to these lung diseases.

In addition to GATA3, we discovered that Regnase-1 profoundly suppressed *Egr1* mRNA expression *via* the 3′UTR. Previous studies showed that EGR-1 enhances the transcription of various fibrosis-associated genes such as *Tgfb1* [43, 44]. Indeed, we found that the mRNA levels of *Tgfb1* in Regnase1-deficient ILC2s were much higher than WT ILC2s, although they failed to show statistical significance owing to the large deviation (supplementary table S3). Thus, it is plausible that Regnase-1 regulates the profibrotic function of ILC2s by modifying transcriptional networks including GATA3 and EGR-1, although further studies are required to uncover the entire transcriptional and post-transcriptional gene expression networks that control pulmonary fibrosis *via* ILC2s.

We found that Regnase-1 regulates the number of lung ILC2s in a cell-intrinsic manner in mice by controlling the ICOS expression. While ICOS ligand (ICOSL) was shown to be expressed on myeloid cells such as dendritic cells and macrophages or ILC2s [29, 45], this study found that ICOSL expression on ILC2s was much lower than the myeloid cells in the lungs (data not shown). Therefore, ILC2 proliferation likely requires additional ICOS stimulation *via* cells expressing ICOSL or the supplementation of ICOS-stimulating antibody even in the absence of Regnase-1. In contrast, ICOS-stimulating antibody did not enhance the proliferation of WT ILC2s. We speculate that Regnase-1 controls the threshold of responses to ICOS stimulation in ILC2s by suppressing the expression of ICOS and its signalling molecules, although further studies are required to elucidate the detailed mechanism.

Furthermore, the ILC2 population in human BAL was negatively correlated with Regnase-1 expression levels, suggesting that mice and humans share Regnase-1-mediated regulation of the ILC2 number in the lung. Considering that ILC2s could promote fibrosis and that a high number of ILC2s was associated with poor prognosis among IPF patients, it is suggested that an inadequate activation and proliferation of ILC2s accelerates the progression of IPF. In addition, we discovered that ICOS expression on the surface of blood ILC2s was positively correlated with the number of ILC2s, which evokes the speculation that enhanced ICOS signalling contributes to an increased number of ILC2s in IPF patients. Because Regnase-1 restricts ILC2 proliferation, potentially through suppressing ICOS expression *via* the 3′UTR [5], the Regnase1–ICOS axis might play a role in the regulation of blood ILC2s in IPF patients. In addition, Regnase-1 can critically restrict the profibrotic function of ILC2s, therefore the stabilisation of Regnase-1 in ILC2s may have a therapeutic property. In future studies, it is expected that an interventional method to induce Regnase-1 expression *in vivo* will be established and its efficacy in protection against pulmonary fibrosis will be evaluated.

The limitation of this study is that the number of clinical samples available was quite small, which reduced the validity of the clinical data in this study. In particular, validation of the cut-off value of peripheral blood ILC2 number for the prediction of prognosis among IPF patients using an independent cohort is required. To overcome this limitation, prospective studies with larger number of cases are required to elucidate the clinical significance of Regnase-1 and ILC2s in IPF.

Despite these limitations, this study showed for the first time that Regnase-1 regulates the proliferation and profibrotic function of ILC2s in the lungs, and an increase in the number of ILC2s in the blood was associated with poor survival, suggesting that Regnase-1 is a critical post-transcriptional regulator of the profibrotic function of ILC2s both in mouse and human. Further studies are required to confirm the clinical significance of ILC2s in IPF with a larger cohort and to evaluate the therapeutic potential of Regnase-1 stabilisation for ILC2s.

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Author contributions: Y. Nakatsuka and O. Takeuchi developed the study concept and design. Y. Nakatsuka performed the majority of experiments, clinical data collection and analysis of data. A. Yaku, Y. Hikichi and Y. Motomura also developed experimental design and performed experiments. M. Yoshinaga, T. Uehata and T. Mino helped with the experiments. A. Vandenbon and Y. Suzuki performed data analysis. A. Sato and T. Tsujimura performed histological analysis. T. Handa supervised clinical study. K. Tanizawa, K. Watanabe, T. Hirai and K. Chin performed clinical data collection and helped with clinical data analysis. K. Moro helped with the development of study concept and experimental design, and provided critical materials. Y. Nakatsuka, T. Handa and O. Takeuchi wrote the manuscript. O. Takeuchi led the whole project.

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