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A protective role for IL-13 receptor α 1 in bleomycin-induced pulmonary injury and repair

D Karo-Atar¹, A Bordowitz¹, O Wand¹, M Pasmanik-Chor², IE Fernandez³, M Itan¹, R Frenkel⁴, DR Herbert⁵, FD Finkelman^{6,7,8}, O Eickelberg³ and A Munitz¹

Molecular mechanisms that regulate lung repair vs. progressive scarring in pulmonary fibrosis remain elusive. Interleukin (IL)-4 and IL-13 are pro-fibrotic cytokines that share common receptor chains including IL-13 receptor (R) α 1 and are key pharmacological targets in fibrotic diseases. However, the roles of IL-13R α 1 in mediating lung injury/repair are unclear. We report dysregulated levels of IL-13 receptors in the lungs of bleomycin-treated mice and to some extent in idiopathic pulmonary fibrosis patients. Transcriptional profiling demonstrated an epithelial cell-associated gene signature that was homeostatically dependent on IL-13R α 1 expression. IL-13R α 1 regulated a striking array of genes in the lung following bleomycin administration and *Il13ra1* deficiency resulted in exacerbated bleomycin-induced disease. Increased pathology in bleomycin-treated *Il13ra1*^{-/-} mice was due to IL-13R α 1 expression in structural and hematopoietic cells but not due to increased responsiveness to IL-17, IL-4, IL-13, increased IL-13R α 2 or type 1 IL-4R signaling. These data highlight underappreciated protective roles for IL-13R α 1 in lung injury and homeostasis.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a devastating disease that is currently on the rise.¹ Despite intensive research, molecular mechanisms that regulate successful lung repair vs. excessive scarring in IPF patients remain elusive. Hence, there is an urgent need to define pathways that regulate IPF pathogenesis.¹

The type-2 cytokines interleukin (IL)-13 and IL-4 exert powerful pro-fibrogenic effects within the liver, intestines and lungs and have been implicated in numerous chronic fibrotic diseases.² Importantly, IL-4 and IL-13 share common receptor chains and signaling intermediates. Therefore, many of their biological functions overlap.³ IL-4 activates a cell surface receptor comprised of IL-4R α and the common γ chain termed the “type 1 IL-4R.” Alternatively, IL-4 can signal through the type 2 IL-4R, comprised of IL-4R α and the IL-13 receptor α 1 chain (IL-13R α 1).^{2,4} IL-13 mediates its effects via IL-13R α 1 and the type-2 IL-4R but can also bind IL-13R α 2 with high affinity.^{4–7} We have recently demonstrated that the development of allergen-induced lung pathology (including

allergen-induced transforming growth factor (TGF)- β induction) is critically dependent on IL-13R α 1.^{8,9} However, IL-13 has been reported to promote pulmonary and intestinal fibrosis via IL-13R α 2 signaling.¹⁰ Thus, the precise roles of IL-13R α 1 in mediating fibrosis are still unclear and require further attention.

Owing to its high expression in structural cells, IL-13R α 1 predominantly shapes the function of non-hematopoietic cells such as epithelial cells, fibroblasts, and smooth muscle cells.¹¹ Notably, IL-13 has been implicated as a key regulator of epithelial cell biology, which normally serves a protective role, but if dysregulated could participate in promoting interstitial lung disease.^{12,13} Directly related, recent data demonstrate that IL-4/IL-13-activated macrophages (i.e., alternatively activated macrophages) or their hallmark mediators and/or metabolic pathways suppress rather than promote tissue remodeling and repair/fibrotic processes.^{14–17} Interestingly, some of the distinguishing features between IPF and other interstitial lung diseases are epithelial cell abnormalities, such as bronchiolar

¹Department of Clinical Microbiology and Immunology, The Sackler School of Medicine, The Tel-Aviv University, Ramat Aviv, Israel. ²Bioinformatics Unit, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel. ³Comprehensive Pneumology Center, Ludwig Maximilians University, University Hospital Grosshadern, and Helmholtz Zentrum München, Member of the German Center for Lung Research, Munich, Germany. ⁴Department of Math, Physics and Computer Science, University of Cincinnati, Cincinnati, Ohio, USA. ⁵Division of Experimental Medicine, University of California, San Francisco, California, USA. ⁶Division of Allergy, Immunology and Rheumatology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. ⁷Department of Medicine, Cincinnati Veterans Affairs Medical Center, Cincinnati, Ohio, USA and ⁸Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA. Correspondence: A Munitz (arielm@post.tau.ac.il)

Received 3 April 2014; accepted 14 May 2015; published online 8 July 2015. doi:10.1038/mi.2015.56

and basal cell hyperplasia, and the ability of epithelial cells to undergo epithelial mesenchymal transition.¹⁸ It is currently perceived that chronic micro-injury to lung epithelial cells triggers rapid repair processes, which are subsequently characterized by the migration, proliferation, and activation of mesenchymal cells to secrete extracellular matrix components.¹⁹ Supporting this notion, targeted damage to type II epithelial cells leads to excessive collagen deposition and subsequent pulmonary fibrosis, establishing a link between the epithelial defects seen in IPF and scar tissue formation.²⁰ Surprisingly, and despite the emerging importance of epithelial cell-associated pathways in pulmonary fibrosis, molecular pathways governing their function are largely unknown.

In this study, we focused on the downstream functions of IL-13R α 1 signaling. We demonstrate altered expression of human (to some extent) and mouse IL-13 receptors in IPF. Transcriptional profiling data revealed a marked homeostatic defect in epithelial cell-associated genes in *Il13ra1*^{-/-} mice. Furthermore, *Il13ra1*^{-/-} mice displayed increased susceptibility to bleomycin-induced lung pathology, which was not due to increased responses to IL-17, increased IL-13R α 2 signaling, or to the requirement of IL-13R α 1 in IL-13/IL-4-induced effects in the lungs. Collectively, these data demonstrate a key protective role for IL-13R α 1 in lung injury and support an important homeostatic function for IL-13R α 1 signaling in this process.

RESULTS

Altered expression of human and mouse IL-13 receptor chains in IPF

To begin defining the role of IL-13R α 1, we examined the expression of IL-13 receptor chains in human IPF biopsies. Under baseline conditions, IL-13R α 1 expression was primarily localized in bronchial epithelium, interstitial fibroblasts, and vascular smooth muscle cells (Figure 1a). IL-13R α 2 was predominantly localized in vascular smooth muscle cells and to a lesser extent in bronchial epithelium (Figure 1a). Pulmonary expression of IL-13R α 1 and IL-4R α were slightly decreased in IPF patients in comparison with healthy controls (Figure 1a–c), whereas IL-13R α 2 expression was increased and localized to fibrotic foci, which were identified by α -smooth muscle actin staining (Figure 1a and d).

Consistently, pulmonary expression of murine IL-13R α 1 (but not IL-4R α) was decreased following bleomycin treatment (Figure 1e and f) and expression of soluble (and to lesser extent, membrane) IL-13R α 2 was significantly increased (Figure 1g and h). The dynamic regulation of IL-13R α 1 expression in IPF and bleomycin-induced lung injury suggested a role for this receptor in IPF.

A homeostatic defect in epithelial cell-associated genes in *Il13ra1*^{-/-} mice

To define the role of IL-13R α 1 in the lungs, we first assessed the contribution of IL-13R α 1 signaling to lung homeostasis. To this end, genome-wide transcriptional profiling was conducted using whole lung tissue of naïve wild-type and *Il13ra1*^{-/-} mice. Under homeostatic conditions, *Il13ra1*^{-/-} mice

displayed marked alteration of 14 transcripts in the lung (Figure 2a, Table 1 and Transcript list #1 in Supplementary Information online). Un-biased STRING analysis, which identifies known and predicted protein interactions, revealed that 5 of 14 altered genes were “hallmark” epithelial cell-associated genes including chloride channel calcium activated, family member 3 (*Clca3/Gob5*), Relm- α (*Retnla*), Anterior gradient 2 (*Agr2*), chitinase 3-like 4 (*Chi3l4*), and trefoil factor 2 (*Tff2*) (17.28-, 2.37-, 2.14-, 2.11-, and 2.58-fold decrease, respectively, $P < 0.001$, Figure 2b, Table 1). Indeed, sorted primary epithelial cells obtained from the lungs of wild-type and *Il13ra1*^{-/-} mice demonstrated an association between *Il13ra1* deficiency and decreased baseline levels of *Clca3/Gob5*, *Retnla*, *Agr2*, and *Chi3l4* but not *Tff2* (Figure 2c–g, Supplementary Figure S1). Notably, these genes have been previously linked with the response to injury and repair,^{21–23} suggesting a role for IL-13R α 1 in lung epithelial cell homeostasis.

Identification of bleomycin- and IL-13R α 1-regulated genes in the lung

The homeostatic defect in epithelial cell-associated genes in the lungs of *Il13ra1*^{-/-} mice suggested a role for IL-13R α 1 in diseases associated with epithelial cell damage. To examine this possibility, wild-type and *Il13ra1*^{-/-} mice were subjected to lung injury induced by bleomycin. Seven days after bleomycin challenge, total lung RNA was obtained and subjected to global microarray analysis. Lungs from bleomycin-treated wild-type mice displayed differential expression in 273 genes that were changed (increased or decreased more than twofold) in comparison with the genes of saline-treated controls. In contrast, bleomycin-treated *Il13ra1*^{-/-} mice displayed significant alterations in 712 transcripts (involvement of 2.6-fold more transcripts than in bleomycin-treated wild-type mice) compared with saline-treated *Il13ra1*^{-/-} mice (Figure 3a and b, P [false discovery rate] < 0.05 , Transcript list #2–4 in Supplementary Information). Strikingly, 89.7% of the genetic signature that characterized the lungs of bleomycin-treated wild-type mice (245 out of the total 273 transcripts) was at least partially regulated by IL-13R α 1 and only 28 genes were IL-13R α 1-independent (Figure 3b, Transcript list 2–3 in Supplementary Information). Assessment of the 245 genes that displayed altered expression in bleomycin-treated wild-type mice and that were co-regulated by IL-13R α 1 revealed that IL-13R α 1 mainly regulated the extent of expression. Thus, the expression of genes, which were increased by bleomycin in wild-type mice, was further enhanced in bleomycin-treated *Il13ra1*^{-/-} mice (Figure 3c, Transcript list #3 in Supplementary Information). For example, *Timp1* and *Mmp12*, which were upregulated 7.49- and 7.21-fold in bleomycin-treated wild-type mice, were upregulated 13- and 11.53-fold in bleomycin-treated *Il13ra1*^{-/-} mice, respectively (see Table 2, and complete list in Transcript list 3 in Supplementary Information).

Moreover, bleomycin-treated *Il13ra1*^{-/-} mice displayed specific alterations in the expression of 467 genes that were not observed in bleomycin-treated wild-type mice (Figure 3b,

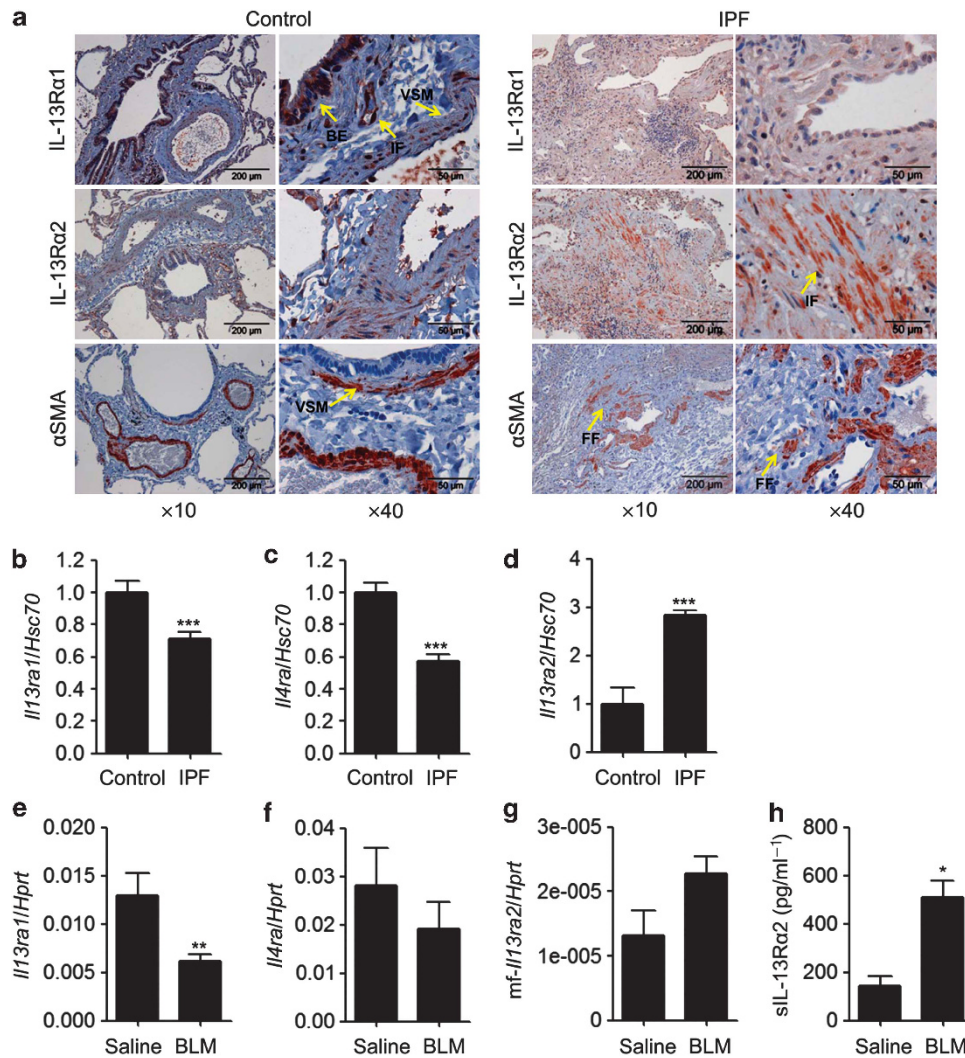


Figure 1 Altered expression of human and mouse IL-13 receptor chains in IPF. The expression of IL-13R α 1, IL-13R α 2, and alpha-smooth muscle actin was assessed by immunohistochemistry in lung biopsies taken from control and IPF patients (**a**). mRNA expression of *IL13ra1* (**b**), *IL4ra* (**c**), and *IL13ra2* (**d**) was determined in human lung cDNA and normalized to the house keeping gene heat shock protein 70 (*Hsc70*). The expression of mouse *IL13ra1* (**e**), *IL4ra* (**f**), and the membrane bound form of *IL13ra2* (**g**, mf-*IL13ra2*) was assessed in lung cDNA obtained from saline- and bleomycin-treated wild-type mice by qPCR analysis and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). The expression of soluble mouse *IL13ra2* (**h**) was assessed by ELISA. Yellow arrows indicate: BE, Bronchial epithelium; IF, Interstitial fibroblasts; VSM, Vascular smooth muscle; FF, fibrocytic foci; in **a–d**, $n = 10$ IPF and 12 control patients, in **e–h** $n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IL, interleukin; IPF, idiopathic pulmonary fibrosis.

Transcript list #4 in **Supplementary Information**) and were entirely induced by bleomycin and dependent on IL-13R α 1. These data reveal a considerable contribution for IL-13R α 1 in bleomycin-induced lung gene expression and suggested an unexpected protective role for IL-13R α 1 in bleomycin-induced lung injury. Bioinformatics STRING-based analysis that was conducted on these 467 genes that were specifically increased in the lungs of bleomycin-treated *IL13ra1*^{-/-} mice revealed three major hubs consisting of multiple genes (see complete gene list in Transcript list 5 in **Supplementary Information**) that were associated with tissue injury and subsequent repair. In fact, GO TERM analysis of the genes that were specifically enriched in each hub revealed that lungs obtained from bleomycin-treated *IL13ra1*^{-/-} mice displayed a markedly altered genetic signature in pathways that are associated with

immune response to wounding (Hub 1, **Figure 3d**, **Table 3**), tissue remodeling and extracellular matrix (Hub 2, **Figure 3d**, **Table 3**) and cell cycle (Hub 3, **Figure 3d**, **Table 3**). Collectively, these data suggest that IL-13R α 1 has a key role in protecting the lung in response to injury and subsequent repair processes by regulating major genetic pathways upstream of wound healing.

Interestingly, *IL13ra1* deficiency did not protect from initial bleomycin-induced lung epithelial cell injury as administration of bleomycin caused rapid epithelial cell apoptosis, which was equivalent in wild-type and *IL13ra1*^{-/-} mice (**Figure 3e**). However, 72 h following bleomycin treatment, apoptosis was still apparent in wild-type epithelial cells, whereas nearly no apoptotic cells were observed in epithelial cells of *IL13ra1*^{-/-} mice. The marked elevation in pathways that were associated with the lung wounding response in bleomycin-treated

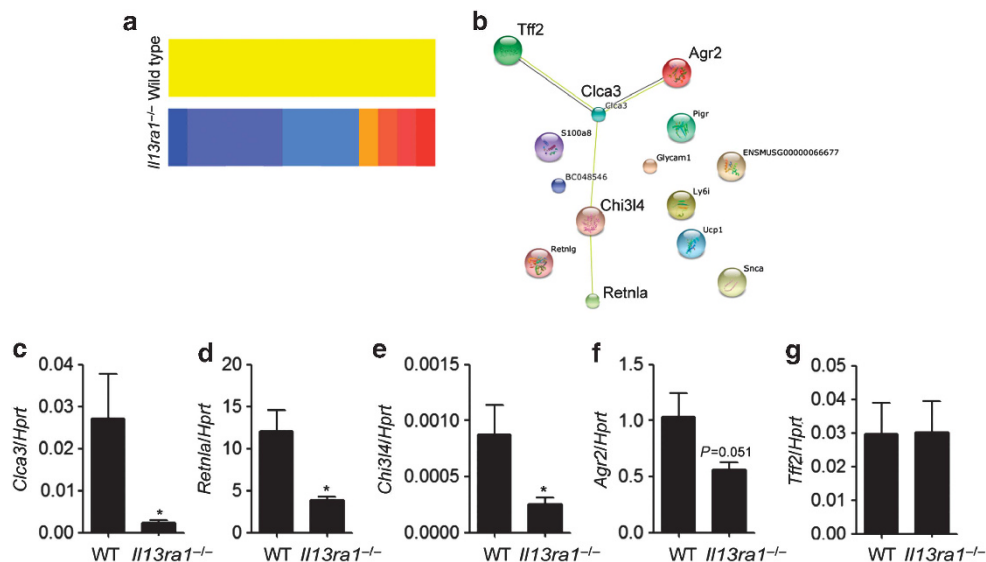


Figure 2 A homeostatic defect in epithelial cell-associated genes in *Il13ra1*^{-/-} mice. Heat plot (a) and STRING (b) analysis of 14 altered transcripts ($n = 2$ lungs per microarray false discovery rate P value < 0.05 , twofold change) as identified by global transcriptome lung profiling of naïve wild-type and *Il13ra1*^{-/-} mice is shown. DAPI⁻/CD45⁻/Podoplanin⁺ alveolar epithelial cells were sorted from the lungs of naïve wild-type and *Il13ra1*^{-/-} mice and the expression of *Clca3* (c), *Retnla* (d), *Chi3l4* (e), *Agr2* (f), and *Tff2* (g) was assessed by qPCR analysis and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*); $n = 5$ mice, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 1 Genes that are homeostatically dysregulated in the lungs of *Il13ra1*^{-/-} mice

Gene symbol	Baseline	
	Fold change	Regulation direction
<i>Clca3</i>	17.28	Down
<i>Snca</i>	6.51	Up
<i>Ucp1</i>	3.29	Up
<i>Glycam1</i>	2.68	Up
<i>Ly6i</i>	2.58	Down
<i>Tff2</i>	2.58	Down
<i>E430029J22Rik</i>	2.41	Down
<i>Retnla</i>	2.37	Down
<i>Retnlg</i>	2.22	Down
<i>Agr2</i>	2.14	Down
<i>LOC672291</i>	2.12	Up
<i>Chi3l4</i>	2.11	Down
<i>S100a8</i>	2.04	Down
<i>Pigr</i>	2.04	Down

Il13ra1^{-/-} mice suggested exaggerated lung repair in response to damage in the absence of IL-13R α 1.

Increased bleomycin-induced pathology in *Il13ra1*^{-/-} mice

To further explore the role of IL-13R α 1 in response to lung injury, kinetic analysis of bleomycin-induced lung pathology

was conducted in wild-type and *Il13ra1*^{-/-} mice. Bleomycin-treated *Il13ra1*^{-/-} mice displayed increased lung histopathology and fibrosis in comparison with bleomycin-treated wild-type animals (Figure 4a). In fact, bleomycin-treated *Il13ra1*^{-/-} mice produced greater amounts of soluble and lung collagens (Figure 4b and c). To substantiate our findings, the levels of lung hydroxyproline, a major component and stabilizer of collagen were determined. Indeed, similar to our findings using soluble collagen measurements (Figure 4c), hydroxyproline levels were significantly increased in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice (Figure 4d). Moreover, bleomycin-treated *Il13ra1*^{-/-} mice exhibited increased numbers of lung fibroblasts (as determined by flow cytometric analysis of CD45⁻/FSP-1⁺ cells) (Figure 4e). Consistent with our microarray data, bleomycin-treated *Il13ra1*^{-/-} mice expressed higher levels of numerous pro-fibrogenic mediators including *Timp1*, *Mmp12*, Relm- α , and the key pro-fibrogenic molecule *Tgfb1* compared with wild-type controls (Figure 4f-i). Notably, the aggravated response to lung injury in *Il13ra1*^{-/-} mice was not accompanied by any alterations in inflammatory cell infiltration or expression of IL-13, IL-4, and IL-17 (data not shown).

Although IL-13R α 1 is predominantly expressed in structural cells (e.g., epithelial cells and fibroblasts), it is also expressed in macrophages and additional cells of the hematopoietic system. Thus, we were interested to define whether increased pathology in bleomycin-treated *Il13ra1*^{-/-} mice is due to its expression in structural or hematopoietic cells. To this end, bone marrow chimeric mice were generated and subjected to bleomycin-induced lung injury. Consistent with our previous findings, the adoptive transfer of bone marrow from *Il13ra1*^{-/-} mice to sub-lethally irradiated *Il13ra1*^{-/-} mice that were further

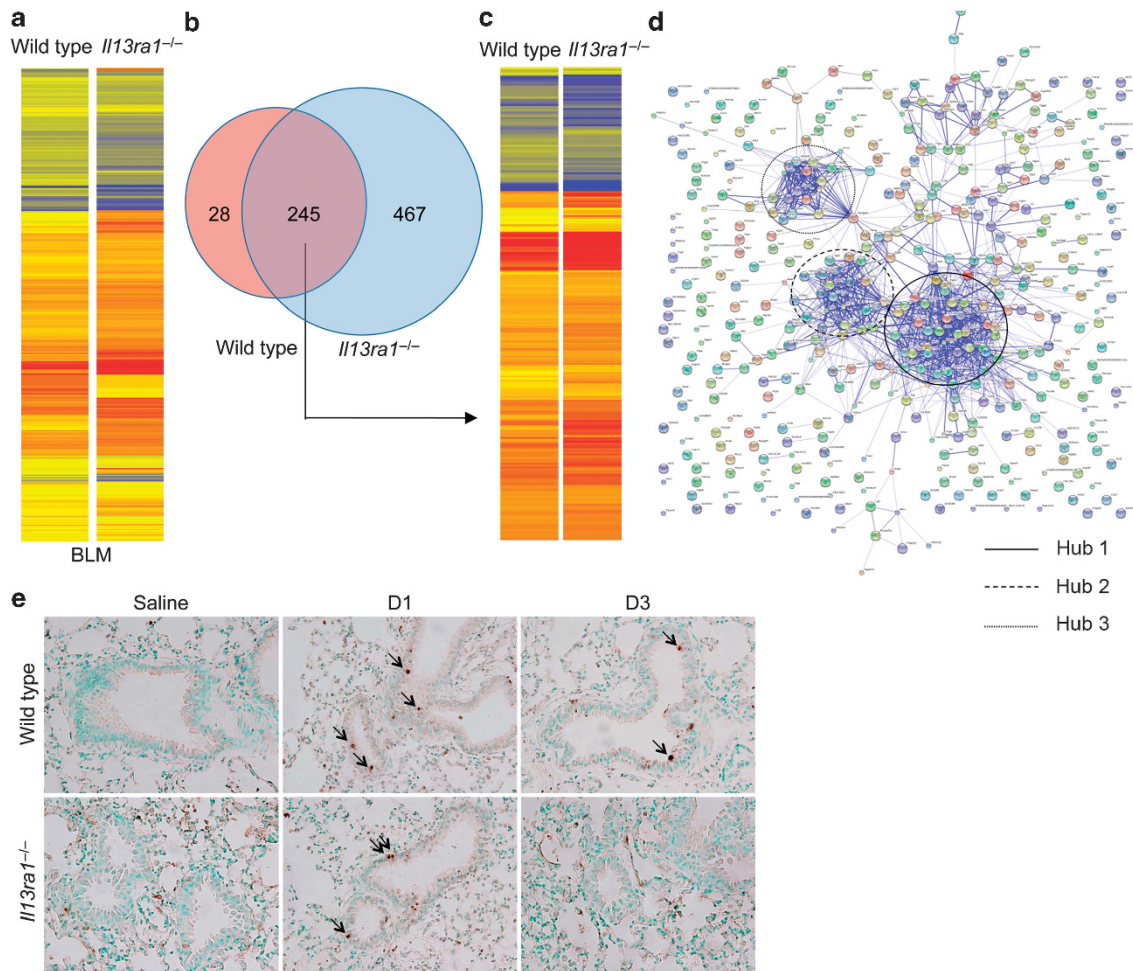


Figure 3 Global transcriptome analysis of bleomycin-induced genes in wild-type and *Il13ra1*^{-/-} mice. Heat (a) and Venn plot (b) analyses of 740 differentially expressed transcripts ($n = 2$ mice per treatment, fold change > 2 , false discovery rate P value < 0.05) is shown. In c, Heat plot of 245 transcripts that are commonly expressed in the lungs of bleomycin-treated wild-type and *Il13ra1*^{-/-} lungs. STRING analysis of the 467 genes that were exclusively dysregulated in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice (d). Representative photomicrographs of TUNEL-stained lung slides of saline- and bleomycin-treated lungs taken from wild-type and *Il13ra1*^{-/-} mice (e). Arrows indicate TUNEL-positive epithelial cells. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

challenged with bleomycin was associated with enhanced lung collagen content, as compared with mice that had wild-type bone marrow-derived and/or non-bone marrow-derived cells (Figure 4j). Collectively, these data suggest that IL-13R α 1 expression in both structural and hematopoietic cells regulates lung injury and subsequent repair in response to bleomycin.

Increasing concentrations of IL-13 does not suppress bleomycin-induced pathology

Recent data suggest an inhibitory role for IL-4R signaling in IL-17-induced responses,²⁴ a pathway that has been implicated in bleomycin-induced lung pathology.²⁵ Thus, we assessed whether *Il13ra1*^{-/-} mice display increased IL-17-induced pathology. To this end, IL-17 was administered to wild-type and *Il13ra1*^{-/-} mice alone or in combination with IL-13 and lung pathology was assessed. IL-17-induced lung collagen was similar in wild-type and *Il13ra1*^{-/-} mice (Figure 5a). Furthermore, we examined the expression of *Timp1* and

Mmp12, as surrogate markers for lung fibrosis because they comprised two of the most highly induced genes in lungs of bleomycin-treated mice (see Table 2, and complete list in Transcript list 3 in Supplementary Information). *Timp1* and *Mmp12* expression was similar in IL-17-treated wild-type and *Il13ra1*^{-/-} mice (Figure 5b and c). As expected, IL-13 administration augmented IL-17-induced lung responses in an IL-13R α 1-dependent manner. To this end, IL-13 + IL-17-treated wild-type mice displayed elevated levels of *Timp1* and *Mmp12* and showed increased lung collagen deposition in comparison with IL-13 or IL-17 alone (Figure 5a-c). Amplification of IL-17-induced responses by IL-13 was completely abolished in *Il13ra1*^{-/-} mice (Figure 5a-c). Thus, increased pathology in bleomycin-treated *Il13ra1*^{-/-} mice is likely not due to increased IL-17 signaling in the absence of IL-13R α 1 signaling. Alternatively, increased pathology in bleomycin-treated *Il13ra1*^{-/-} mice is not due to suppressive activity of IL-13 towards IL-17-induced lung responses.

Consistent with the inability of IL-13 to suppress IL-17-induced lung pathology, co-administration of IL-13 with bleomycin resulted in substantially augmented lung fibrosis as determined by soluble and lung collagen levels (Figure 5d and e) and increased expression of the pro-fibrogenic marker *Mmp12* (Figure 5f). Thus, these data indicate that homeostatic IL-4/IL-13 signaling through IL-13R α 1 is required to limit bleomycin-induced pathology, but increasing such signaling by administering IL-13 is not protective.

Increased fibrosis in bleomycin-challenged *Il13ra1*^{-/-} mice is independent of IL-13R α 2 signaling

In the absence of IL-13R α 1, IL-13 may mediate IL-13R α 1-independent effects in the lungs, which might augment bleomycin-induced lung injury. To examine this possibility, we first assessed the expression of IL-13R chains in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice. IL-13R α expression

was significantly decreased in bleomycin-treated *Il13ra1*^{-/-} mice (Figure 6a). In addition, under baseline conditions, *Il13ra1*^{-/-} mice displayed markedly reduced levels of membrane IL-13R α 2 mRNA expression (Figure 6b and c). Yet, following bleomycin treatment, the expression of membrane and soluble IL-13R α 2 was significantly increased (Figure 6b and c). Thus, the upregulation of IL-13R α 2 following bleomycin treatment is predominantly IL-13R α 1-independent. The finding that IL-13R α 2 expression is still increased in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice raised the possibility that increased fibrosis in bleomycin-challenged *Il13ra1*^{-/-} mice might be due to IL-13:IL-13R α 2 interactions. Thus, IL-13 was neutralized in the lungs of bleomycin-challenged *Il13ra1*^{-/-} mice using anti-IL-13-neutralizing antibodies, subsequently blocking any IL-13:IL-13R α 2-induced responses during the course of bleomycin-induced lung injury. Neutralization of IL-13 in bleomycin-treated *Il13ra1*^{-/-} mice did not revert the increased fibrotic response that was observed in these mice as they still displayed elevated levels of collagens in the lungs and bronchoalveolar lavage fluid (Figure 6d and e) and had increased *Timp1* mRNA expression (Figure 6f). Therefore, IL-13R α 2 does not mediate IL-13 signaling in the lungs even in the absence of IL-13R α 1.

Table 2 Examples of genes with increased expression in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice

Gene symbol	Wild type		<i>Il13ra1</i> ^{-/-}	
	Fold change	Regulation direction	Fold change	Regulation direction
<i>Timp1</i>	7.49	Up	13.00	Up
<i>Mmp12</i>	7.21	Up	11.53	Up
<i>Cxcl10</i>	3.85	Up	11.35	Up
<i>Arg1</i>	2.28	Up	7.10	Up
<i>Mmp8</i>	3.46	Up	6.79	Up
<i>Cxcl9</i>	2.87	Up	4.88	Up
<i>Mmp19</i>	2.34	Up	3.51	Up
<i>Col1a1</i>	2.06	Up	3.32	Up
<i>Col5a2</i>	2.30	Up	3.29	Up
<i>Tgfb1</i>	2.19	Up	3.20	Up
<i>Mmp14</i>	2.20	Up	2.94	Up
<i>Areg</i>	2.23	Up	2.37	Up

Identification of IL-13-induced IL-13R α 1-dependent genes

Next, IL-13 was administered to wild-type and *Il13ra1*^{-/-} mice, and after 7 days, whole lungs were obtained and subjected to global transcriptome analysis. IL-13-treated wild-type mice displayed a distinct genetic signature involving alterations in 143 transcripts (Transcript list #6 in **Supplementary Information**, P [false discovery rate] < 0.05) including various pro-fibrogenic genes (e.g., *Timp1* and *Mmp12*), which were also increased in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice. In striking contrast, IL-13-treated *Il13ra1*^{-/-} mice displayed no significant alterations in any lung transcripts and the genetic signature following IL-13 administration was identical to the signature observed in saline-treated *Il13ra1*^{-/-} mice (Figure 7a and b). Thus, IL-13-induced effects in the lungs are exclusively mediated via IL-13R α 1. Therefore, increased

Table 3 Top 3 GO TERM pathways that were enriched in each identified hub

		GO TERM	P-value
Hub 1	Immune response to wounding	Leukocyte activation	2.50E-10
		Defense response	1.80E-10
		Immune effector process	5.70E-06
Hub 2	Tissue remodeling and ECM	RNA binding	3.40E-04
		ECM and collagen	5.50E-07
		Innate immune response	8.70E-03
Hub 3	Cell cycle	Cell cycle	1.90E-14
		Nucleotide binding	8.20E-04
		Spindle	4.70E-03

Abbreviation: ECM, extracellular matrix.

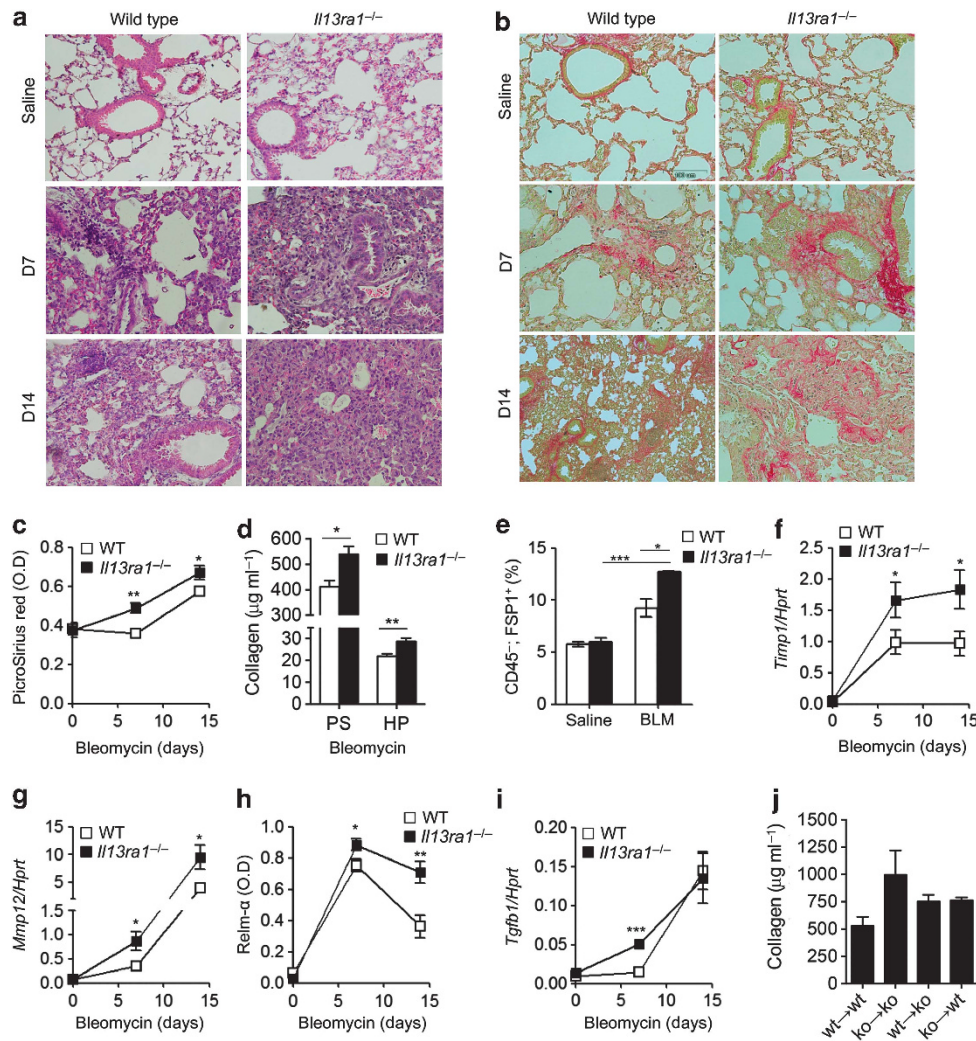


Figure 4 Increased bleomycin-induced pathology in *Il13ra1*^{-/-} mice. Representative photomicrographs of H&E (a) and Picro-Sirius red staining (b), as well as quantitative assessment of soluble collagen (c) and hydroxyproline content (d) in saline- and bleomycin-treated lungs at day 7 (D7) and 14 (D14) are shown. (e) Flow cytometric analysis of CD45⁺/FSP1⁺ fibroblasts in saline- and bleomycin (BLM)-treated wild-type and *Il13ra1*^{-/-} mice. In j, soluble collagen levels in bone marrow chimeric mice at day 14 after bleomycin-treatment are shown. The expression of *Timp1* (f), *Mmp12* (g), and *Tgfb1* (i) following saline or BLM treatment was assessed by qPCR analysis and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). (h) The secretion of Relm- α was assessed by ELISA; n = 3, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. H&E, hematoxylin and eosin; HP, Hydroxyproline; PS, Picro Sirius red.

pathology and profibrogenic transcripts in the lungs of bleomycin-treated *Il13ra1*^{-/-} mice were not due to injury-induced IL-13 expression.

Identification of IL-4-induced IL-13R α 1-dependent genes

The fact that IL-4 can also utilize the type 2 IL-4R to induce its effects raised the remote possibility that IL-4-induced responses may be augmented in *Il13ra1*^{-/-} mice, possibly by increased availability and binding of IL-4 to the type 1 IL-4R. Subsequently, increased IL-4 signaling may account for the augmented pathology, which was observed in bleomycin-treated *Il13ra1*^{-/-} mice. Utilizing our global microarray screening approach, we compared the genetic signature of IL-4-treated wild-type mice with IL-4-treated *Il13ra1*^{-/-} mice (Figure 7c, Transcript list #7–9 in Supplementary

Information). This analysis revealed that IL-4-treated *Il13ra1*^{-/-} mice displayed decreased responsiveness to IL-4. IL-4-treated *Il13ra1*^{-/-} mice showed an approximately ~37% reduction in the number of IL-4-induced transcripts (Figure 7d). Importantly, although IL-4 was capable of inducing the expression of 55 genes both in wild-type and in *Il13ra1*^{-/-} mice (Figure 7d, Transcript list #7 in Supplementary Information), the overall magnitude of induction of these genes was markedly reduced in *Il13ra1*^{-/-} mice (i.e., the expression of these genes was impaired in *Il13ra1*^{-/-} mice). Of note, these IL-4-induced transcripts included the fibrosis-associated genes *Timp1*, *Mmp12*, and *Arg1*, all of which were augmented in bleomycin-treated *Il13ra1*^{-/-} mice (Figure 7d, Transcript list #3, #7–9 in Supplementary Information).

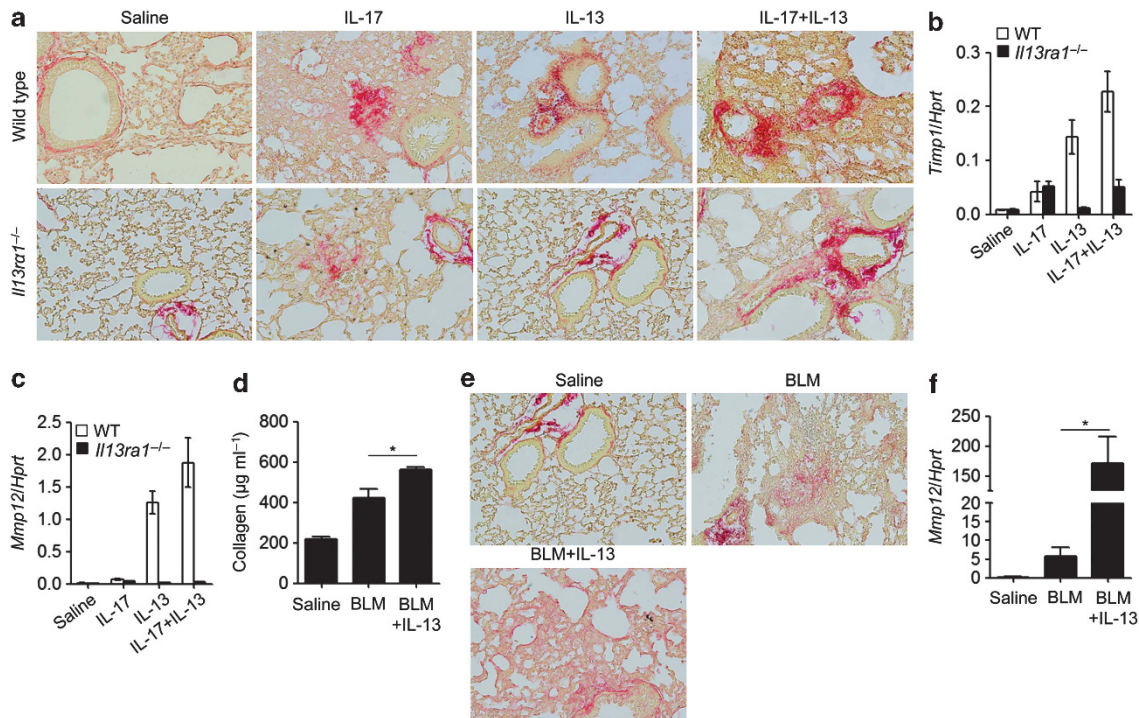


Figure 5 IL-13 does not suppress IL-17 or bleomycin-induced pathology. Representative photomicrograph of Picro-Sirius red staining of lungs from IL-17 +/- IL-13 and bleomycin (BLM) +/- IL-13-treated mice is shown (a, e). The expression of *Timp1* (b), *Mmp12* (c, f) was assessed in IL-17 +/- IL-13 - or BLM +/- IL-13-treated mice by qPCR analysis and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). (d) Soluble collagen lung content is shown; $n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IL, interleukin.

Although IL-4-induced responses are significantly reduced in the absence of IL-13R α 1, it is still possible that increased pathology in bleomycin-treated *Il13ra1*^{-/-} mice may be mediated by a shift from activation of the type 2 IL-4R to activation of the type 1 IL-4R in the absence of IL-13R α 1. To test this hypothesis, we neutralized IL-4R α in bleomycin-treated *Il13ra1*^{-/-} mice. If increased pathology was mediated by increased IL-4 signaling via the type 1 IL-4 receptor, this experimental regimen would result in attenuated pathology in comparison with isotype control-treated bleomycin-challenged *Il13ra1*^{-/-} mice. Assessment of lung collagen (Figures 7e and h), *Timp1* (Figure 7f), and *Mmp12* (Figure 7g) revealed no difference between anti-IL-4R α - and isotype-treated bleomycin-challenged mice. Collectively, these data demonstrate that increased bleomycin-induced gene expression and subsequent pathology in *Il13ra1*^{-/-} mice was not due to increased responsiveness to IL-4 in *Il13ra1*^{-/-} mice or increased signaling by the type 1 IL-4R.

Assessing the contribution of IL-4/IL-13 signaling to bleomycin-induced transcriptional changes

Comparing the genetic signatures of IL-13-, IL-4-, and bleomycin-treated lungs revealed that IL-13 and IL-4 induce a genetic signature that is comprised of 48 and 59 genes, respectively, which are shared with those of bleomycin-treated wild type lungs (Supplementary Figure S2A-B and transcript list #10–11 in Supplementary Information). However, analyzing the expression of these transcripts in the lungs of

bleomycin-treated *Il13ra1*^{-/-} mice revealed that while IL-13 and IL-4 have the potential to regulate (i.e., increase or decrease) the expression of the aforementioned genes, their expression following bleomycin-treatment is predominantly IL-13R α 1-independent (Transcript list #10–11 in Supplementary Information). In fact, this analysis revealed that only three genes (e.g., *Cla3*, *LOC672291*, and *Mgl2*) were IL-13-, IL-4-, and bleomycin-induced, IL-13R α 1-dependent (Table 4). Taken together, these data establish a minor contribution of “acquired” signaling of IL-13R α 1 (mediated by bleomycin-induced IL-4 and/or IL-13 expression) to the bleomycin-induced transcriptional signature and suggest that the absence of IL-13R α 1 leads to homeostatic defects which give rise to increased susceptibility to bleomycin-induced lung injury.

DISCUSSION

Over the last decade, the IL-13/IL-4 signaling axis has drawn considerable attention in various lung diseases including asthma and IPF.^{26,27} Yet, thorough examination of the IL-4/IL-13:IL-13R α 1 signaling axis requires further attention because agents that target this pathway can be and are actively developed for the treatment of fibrotic diseases such as IPF. We report altered expression of IL-13 receptor chains in the lungs of bleomycin-treated mice, a phenomenon that was also observed in IPF patients (albeit in a small patient sample size). Integrating the data obtained from multiple *in vivo* models with global microarray analyses suggested that IL-13R α 1 has a central role in lung epithelial cell homeostasis. Furthermore, in

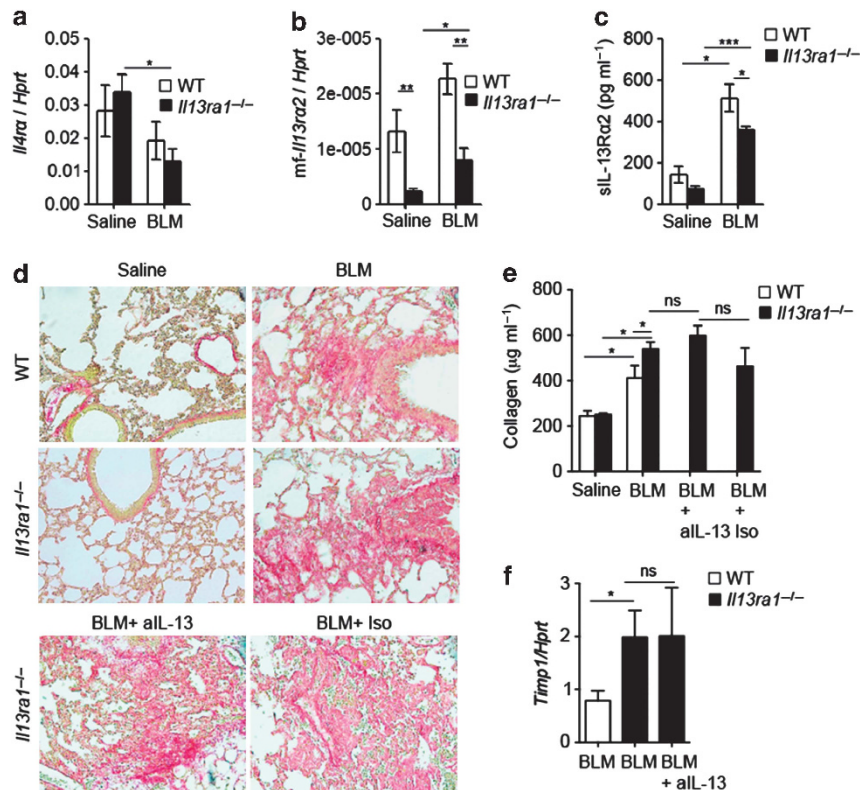


Figure 6 Increased fibrosis in bleomycin-treated *Il13ra1*^{-/-} mice is independent of IL-13R α 2 signaling. The expression of mouse *Il4ra* (a) and membrane form (mf)-*Il13ra2* (b) was assessed in lung cDNA obtained from saline- and bleomycin (BLM)-treated wild-type and *Il13ra1*^{-/-} mice by qPCR analysis and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). Expression of soluble mouse *Il13ra2* (c) was assessed using ELISA. Representative photomicrograph of Picro-Sirius red staining of lungs obtained from bleomycin-treated *Il13ra1*^{-/-} mice that were treated with anti-IL-13 neutralizing antibodies (aIL-13) or isotype control antibodies (Iso) are shown (d). The expression of lung soluble collagen levels is shown (e). *Timp1* expression (f) is shown and normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). $n=3$, * $P<0.05$, ** $P<0.01$; IL, interleukin; ns, non-significant.

response to bleomycin-induced injury, *Il13ra1*^{-/-} mice displayed augmented pathology that was dependent on the lack of IL-13R α 1 expression in both structural and hematopoietic cells. Importantly, excessive fibrosis in *Il13ra1*^{-/-} mice was not due to increased responsiveness to IL-17, to suppressive activity of IL-13, to increased IL-13R α 2 signaling in the absence of IL-13R α 1, or to increased responsiveness of the type 1 IL-4 receptor. We further demonstrated that IL-13R α 1 was required for absolutely all the transcriptional changes induced by IL-13 in the lungs and that additional IL-13R chains or IL-4 signaling do not mediate the increased pathology that was observed in bleomycin-treated *Il13ra1*^{-/-} mice. Collectively, these data suggest that the lack of homeostatic signaling by IL-13R α 1 may predispose towards fibrotic lung disease.

One of the most interesting findings of this study was the finding that in response to bleomycin-induced lung injury, *Il13ra1*^{-/-} mice displayed an increased fibrotic disease. This result is of specific interest because it is counter intuitive. Multiple data including our own have clearly demonstrated a potent pro-fibrogenic role for IL-13:IL-13R α 1 interactions, especially in settings of Th2 immunity and parasite infections.²⁸ For example, we have recently shown that IL-13R α 1 was required for allergen-induced lung TGF- β 1.^{8,9} The marked

dichotomy between the function of IL-13R α 1 in asthma and lung injury is likely due to the “acquired” levels of IL-13 and/or IL-4 during disease pathogenesis. It is clearly established that IL-4 and IL-13 are markedly increased in asthma and can mediate many of the pathological features that are associated with asthma (e.g., mucus production, airway hyper responsiveness, and fibrosis).^{29,30} Thus, in settings where IL-13 and IL-4 are highly increased (e.g., asthma), IL-13R α 1 is required to deliver the effector functions of these cytokines.^{8,9} In contrast to the aforementioned settings, the roles of IL-13 and its respective receptors in lung injury and repair (which is independent of Th2 immunity) received much less attention and are largely unclear. Importantly, bleomycin-induced lung injury is largely independent of IL-13/IL-4 induction.²⁵ Interestingly and consistent with our findings, *Il13*^{-/-} mice displayed increased mortality and lung injury in response to hyperoxia and *Il13ra1*^{-/-} mice displayed residual liver fibrosis and increased expression of pro-fibrotic markers following parasite infection.^{31,32} In addition, chronic proliferative dermatitis was substantially increased in the absence of IL-4/IL-13 signaling.³³

Mechanistically, our unbiased microarray analyses highlight a possible function for IL-13R α 1 in the regulation of the immune response to damage (likely by myeloid cells such as

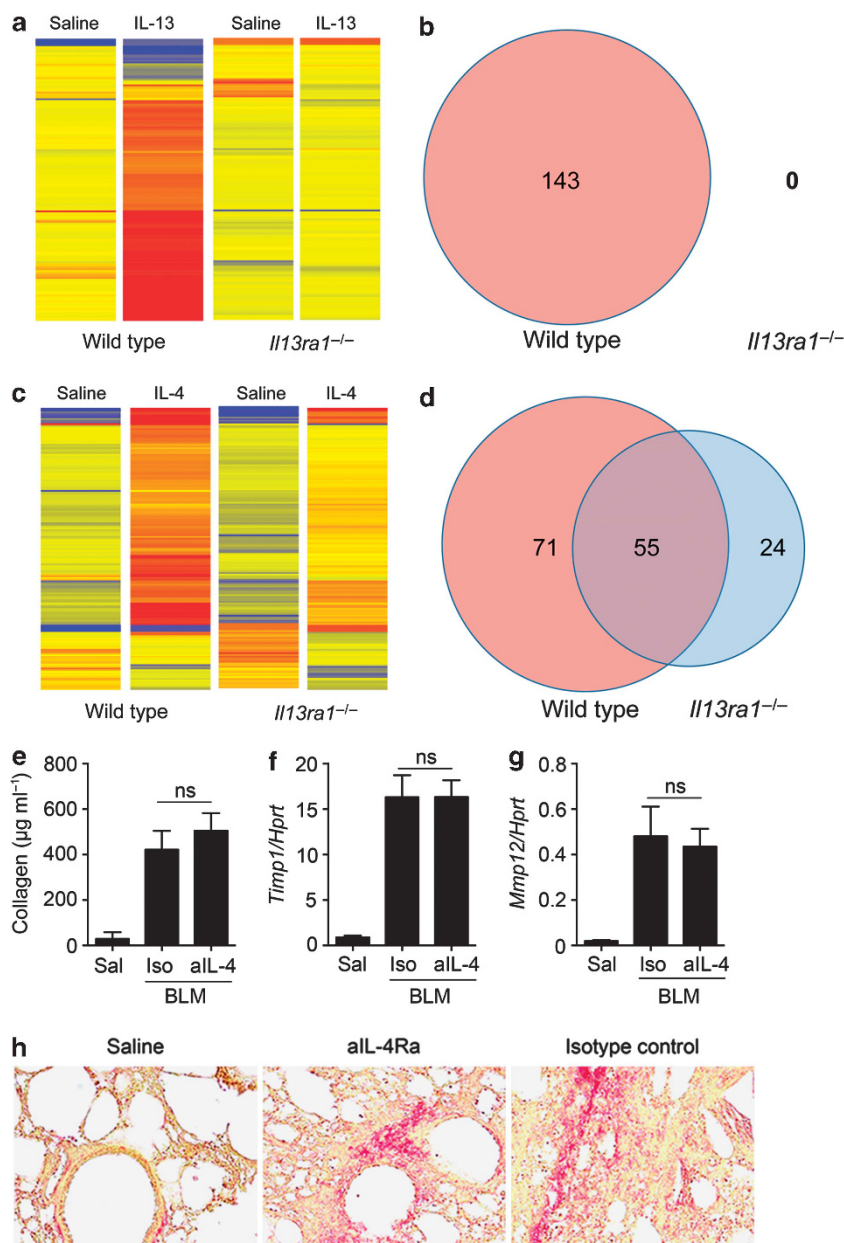


Figure 7 Global Transcriptome analysis of IL-13 and IL-4-induced genes. Heat (a, c) and Venn plot (b, d) analyses of IL-13- and IL-4-treated wild-type and *Il13ra1*^{-/-} lungs ($n = 2$ mice, fold change > 2 , false discovery rate $P < 0.05$). The expression of soluble collagen content (e), *Timp1* (f), and *Mmp12* (g) in bleomycin-challenged *Il13ra1*^{-/-} mice that were treated with neutralizing anti-IL-4R α antibody (aIL-4) or isotype control (Iso). In f, g, data were normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). Representative photomicrograph of Picro-Sirius red staining of lungs from bleomycin-challenged *Il13ra1*^{-/-} mice that were treated with neutralizing anti-IL-4R α antibody (aIL-4) or isotype control (Iso) (h). IL, interleukin; ns, non-significant.

macrophages) and by governing collagen and extracellular matrix synthesis. For example, bleomycin-treated *Il13ra1*^{-/-} mice displayed marked alterations in the expression of inhibitory and activating Ig-like receptors (e.g., *Clec*-family receptors, *Lair1*, *Lilrb3*, *Clec4a3*, *Cd300lf*, *Cd300ld*, and *Pilra*) that have been associated with myeloid cell activities and provide counter regulatory signals for their cellular activities including fibrogenic properties.^{34–36} In addition, IL-13R α 1 regulates the expression of numerous genes that are involved in the subsequent repair response. These genes include collagens

(e.g., *Col3a1*, *Col6a3*, *Col1a2*, *Col8a1*, *Col12a1*) and enzymes (e.g., *Adam8*, *Mmp12*, *Mmp13*, *Mmp3*, *Timp1*). Collectively, these data implicate IL-13R α 1 as a novel regulator of the lung damage-repair response by regulating immune and structural cell activities.

The pleotropic effects of IL-13 and IL-4 are mediated via an overlapping set of receptors that display differential distribution among structural and hematopoietic cells. Thus, the available gene-targeted mice that target the IL-13/IL-4 signaling pathway (e.g., *Il4ra*^{-/-}, *Il13*^{-/-}, *Il4*^{-/-}, or

Table 4 A list of genes that are induced by bleomycin, IL-4 and IL-13, and are regulated by IL-13R α 1

Gene symbol	BLM		IL-4		IL-13	
	Fold change	Regulation direction	Fold change	Regulation direction	Fold change	Regulation direction
<i>Wild type</i>						
Clca3	4.41	Down	3.15	Up	4.12	Up
LOC672291	2.18	Up	2.60	Up	3.00	Up
Mgl2	2.44	Up	4.06	Up	4.03	Up
<i>Il13ra1^{-/-}</i>						
Clca3	UC	UC	UC	UC	UC	UC
LOC672291	UC	UC	2.73137	Down	UC	UC
Mgl2	UC	UC	5.19279	Up	UC	UC

Abbreviations: IL, interleukin; UC, unchanged.

Il13^{-/-}/Il4^{-/-}) may display unique and non-overlapping phenotypes. For example, *Il13ra1^{-/-}* mice may have a distinct phenotype in response to bleomycin in comparison with *Il4ra^{-/-}*, *Il13^{-/-}*, *Il4^{-/-}*, or *Il13^{-/-}/Il4^{-/-}* mice. IL-13R α 1 is predominantly and highly expressed by structural cells and mediates both IL-4 and IL-13 signaling in such cells.¹¹ Therefore, in the absence of IL-13R α 1, IL-4 signaling in hematopoietic cells will remain largely intact owing to their high expression of IL-4R α .¹¹ In contrast, *Il13^{-/-}/Il4^{-/-}* mice will display aberrant responses also in their hematopoietic compartment, which may affect disease outcome owing to impaired myeloid cell responses. In addition, structural cells in *Il13^{-/-}* mice, which express the IL-13R α 1 chain will still mediate signals via IL-4 and may not display any homeostatic alteration such as the ones we observed in *Il13ra1^{-/-}* epithelial cells. Finally, *Il4ra^{-/-}* mice will lose the signals driven by both the type 1 and type 2 IL-4R's via IL-13 and IL-4, and *Il4^{-/-}* mice will still have integral IL-13:IL-13R α 1 signaling. To the best of our knowledge, the findings of this study are unique and non-redundant with any experimental systems to date.

Recent data aimed at assessing the roles of IL-13 in IPF patients reported elevated levels of IL-13 (and IL-4) in IPF patients.^{37,38} Furthermore, and in contrast to our findings, increased expression of IL-13R α 1 was reported.³⁷ The discrepancy between those reports and ours may be due to differences in mediators that are capable of increasing IL-13R α 1 expression. For example, Murray and colleagues³⁸ have shown that TGF- β 1, IL-13, and CCL2 can increase the expression of IL-13 receptors in lung fibroblasts, and lung fibroblasts, which were obtained from IPF patients, were hyperresponsive to IL-13 treatment. In addition, adoptive transfer of fibroblasts from IPF patients into SCID mice generated a fibrotic disease that was attenuated by anti-IL-13 treatment.³⁹ The aforementioned human studies suggest a pathogenic role for IL-13 in IPF. It is important to note that our study does not contradict previous findings regarding the pathogenic and pro-fibrogenic functions of IL-13. In fact, our data show that when present in the lungs, IL-13 induces

substantial transcriptional alterations and production of fibrotic mediators. This is also consistent with established data from IL-13 transgenic mice,⁴⁰ which demonstrate the ability of IL-13 to promote fibrosis. The undisputed findings regarding the pro-fibrotic function of IL-13 in combination with our data using bleomycin-treated *Il13ra1^{-/-}* mice strengthen the notion that increased fibrotic pathology in *Il13ra1^{-/-}* mice is likely due to a “homeostatic” defect and that injury-induced IL-13 has a minor role in the bleomycin mouse model as suggested previously by Wilson *et al.*²⁵

Several studies suggest IL-13R α 2-dependent TGF- β induction and subsequent fibrosis.^{10,41} We demonstrate that *Il13ra1^{-/-}* mice displayed markedly reduced baseline levels of IL-13R α 2. However, following bleomycin treatment, both membrane and soluble forms of IL-13R α 2 were still increased in the absence of IL-13R α 1. Importantly, blockade of IL-13:IL-13R α 2 interactions in bleomycin-treated *Il13ra1^{-/-}* mice revealed that increased fibrosis in *Il13ra1^{-/-}* mice was not due to increased signaling of IL-13R α 2. These results are consistent with recent data showing that IL-13R α 2 does not mediate IL-13-induced responses in human fibroblasts and with reports demonstrating that IL-13R α 2 serves predominantly as a decoy receptor.^{42,43} Furthermore, we demonstrate that soluble IL-13R α 2 was induced to a greater extent in comparison with that of membrane IL-13R α 2. This ratio will likely favor its function as a decoy receptor and not a signaling molecule. Finally, and consistent with previous reports,²⁵ we established a minor IL-13/IL-4-induced gene signature in association with bleomycin treatment.

Although the underlying cause of IPF is unclear, it is currently perceived that chronic micro-injury to alveolar epithelial cells plays a central role.^{19,28} In support of this notion, genome-wide association studies identified various epithelial cell-associated genes that may have a role in response to injury. For example, surfactant protein C mutations were linked to specific familial cases of pulmonary fibrosis and large genome-wide association studies linked the mucin gene MUC5B with increased susceptibility to development of

fibrosis.⁴⁴ Thus, it is quite striking that our unbiased microarray experiments and subsequent STRING analysis revealed an IL-13R α 1-regulated, epithelial cell-associated gene signature that is closely linked to mucins and epithelial cell integrity such as *Retnla* (Relm- α), *Agr2* (Anterior grade 2), *Clca3* (Gob5), *Chi3l4* (YM2), and *Tff2* (Trefoil factor 2). Interestingly, many of these genes have been already associated with wound healing and repair processes in mucosal surfaces. For example, deletion of *Agr2*, an epithelial cell gene that is associated with mucins, renders the mice highly susceptible to colonic injury in response to epithelial cell damage.⁴⁵ In addition, *Clca3* expression was significantly reduced in the intestines of mice with cystic fibrosis and correction of *Clca3* deficiency resulted in amelioration of the fibrotic disease.⁴⁶ It is tempting to speculate that the increased susceptibility of *Il13ra1*^{-/-} mice may be a consequence of alterations in one (or more) of these epithelial cell-associated pathways. Interestingly, our bone marrow chimeric experiments suggest that increased pathology in bleomycin-treated *Il13ra1*^{-/-} mice is likely due to its expression in both structural and hematopoietic cells. This finding is not surprising because macrophages, which express IL-13R α 1 and the type 2 IL-4 receptor, have been implicated in fibrotic diseases of the lung and gastrointestinal tract, kidney and liver. In these tissues, numerous studies have shown that IL-4/IL-13-activated macrophages may actually prevent fibrosis and act to resolve tissue damage.^{14,16,17} Thus, the absence of IL-13R α 1 in hematopoietic cells can indeed contribute to increased bleomycin-induced pathology. Further research in our laboratory is currently assessing the contribution of IL-13R α 1 in these distinct cell types.

Although our study shows a clear protective role for IL-13R α 1 in mice, these data need to be considered with caution, as there are marked differences between bleomycin-induced fibrosis and human IPF. While the typical features of human IPF including usual interstitial pneumonia, scattered α -smooth muscle actin-positive fibroblastic foci in collagen-rich areas, and alternating normal lung areas are nicely reproduced in the bleomycin mouse model,⁴⁷ the subsequent development of fibrosis is relatively rapid and at least partially reversible, independent from any intervention.⁴⁸ Thus, the aspect of chronic and irreversible progression of IPF in patients, one of the most important aspects of human IPF, is not reproduced in the bleomycin model.⁴⁹ Notably, none of the currently available animal models of pulmonary fibrosis (bleomycin, radiation, silica, transgenic, viral vectors) fully mimic human IPF and many candidate drugs proved to be effective in the bleomycin model but are ineffective in IPF.⁵⁰ Despite these limitations, these mouse models provide an outstanding opportunity to dissect the cellular and molecular events underlying pulmonary fibrosis.

In summary, our results establish a protective role for IL-13R α 1 in lung injury and subsequent repair in response to bleomycin. Taken together, our data suggest that basal levels of IL-4 and/or IL-13, acting through the type 2 IL-4 receptor, are protective against fibrosis, probably by regulating epithelial cell healing and immune cell responses to damage in the lungs.

METHODS

Mice. Male and female 6–8-week-old *Il13ra1*^{-/-} mice (backcrossed >F9 to C57BL/6) were generated as previously described.⁸ C57BL/6 wild-type mice were obtained from Harlan Laboratories (Rehovot, Israel). In all experiments, age-matched, weight-matched, and gender-matched mice were housed under specific pathogen-free conditions, according to institutionally approved protocols of the Animal Care Committee at Tel Aviv University.

Patients. Lung tissue biopsies were obtained from 10 patients with IPF (usual interstitial pneumonia pattern; mean age 51.3 \pm 11.4 years) and 12 control subjects (organ donors, mean age 47.5 \pm 13.9 years). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

Bleomycin-induced pulmonary fibrosis. Mice were anesthetized with xylazine and ketamine, and intratracheally challenged with either bleomycin sulfate (0.03–0.05 U per mouse) or saline (50 μ l per mouse). Mice were killed 0–14 days after challenge, and bronchoalveolar lavage fluid was obtained as described.³⁴

Bone marrow chimera mice. Syngeneic BM chimeras were generated as described.⁵¹ Briefly, wild-type C57BL/6 mice were exposed to a single sub-lethal total body irradiation of 900 rad. One day after irradiation 5×10^6 bone marrow cells obtained from *Il13ra1*^{-/-} or wild-type mice were injected into the retro-orbital sinus of the irradiated mice. Four weeks after engraftment, the mice were intranasally challenged with lipopolysaccharide (20 μ g kg⁻¹) in order to deplete the resident alveolar macrophage population.⁵¹ Thereafter, the mice were allowed to rest for an additional 4 weeks (for a total of 8 weeks post irradiation). Thereafter, the mice were treated with bleomycin. Engraftment was validated in peripheral blood samples using anti-CD45.1 and anti-CD45.2 staining.

IL-13 and IL-4R α neutralization. Anti-IL-13 neutralizing antibodies (R&D Systems, Minneapolis, MN, MAB413, 150 μ g per mouse in 50 μ l saline), anti-IL-4R α (clone M1 1.5 mg per mouse in 50 μ l saline), and isotype control antibodies (GL117 and MAB006 R&D Systems, MAB006) were administered intratracheally on days 0, 3 and 5 following bleomycin instillation (a total of). Bronchoalveolar lavage fluid was assessed 48 h after the final administration for collagen levels and lungs were obtained for mRNA analysis and histological assessment.

Real-time quantitative PCR. Lung cDNA was subjected to quantitative PCR, as previously described.³⁴ A complete list of primers used in this study is provided in **Supplementary Table S1**.

Histopathology. Mouse lungs were fixed, paraffin-embedded, and stained with hematoxylin and eosin (Pioneer Research Chemicals, Essex, UK) or Picro Sirius red as described.³⁴

Assessment of collagen levels. Soluble collagen levels were assessed using the modified Sircoll assay (based on Picro Sirius red staining) as described.⁵² Alternatively, hydroxyproline levels were measured using a commercially available kit (Sigma, Rehovot, Israel).

IL-4-, IL-13- and IL-17-induced airway inflammation. IL-17 was administered every day for 4 days (8 μ g per mouse). IL-4 and IL-13 were administered as previously described.³⁴ Bronchoalveolar lavage fluid was assessed either 24 or 48 h after the final challenge for differential cell counts, Resistin-like molecule α (Relm- α) expression and collagen levels.¹¹ Lungs were obtained for RNA analysis and histological assessment.

Affymetrix cDNA microarray. Mouse Affymetrix (Santa Clara, CA) microarrays (1.1 ST GeneChip) were performed and analyzed using established protocols of the Tel-Aviv University Bioinformatics Unit

and according to the manufacturer's instructions. Data were analyzed using Genespring (Agilent, Santa Clara, CA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Saline- and bleomycin-treated lungs were fixed, paraffin-embedded, and stained for the detection of apoptotic cells using ApopTag Plus Peroxidase *In Situ* Apoptosis Kit (Millipore, Billerica, MA) according to the manufacturer's instructions.

Statistical analysis. Data were analyzed by analysis of variance followed by Tukey *post hoc* test or Student's *t*-test using GraphPad Prism 4 (San Diego, CA). Data are presented as mean \pm s.e.m., and values of $P < 0.05$ were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

ACKNOWLEDGMENTS

This study was supported by research funding to AM by the FP7 Marie-Curie Reintegration grant (grant no. 256311), the US-Israel Bina-tional Science Foundation (grant nos. 2009222 and 2011244), the Israel Science Foundation (grant no. 955/11), the Israel Cancer Research Foundation (ICRF) Research Career Development Award (RCDA), The Fritz Thyssen Stiftung, and The Tel-Aviv University Stolz Award, and research funding to FDF from the U.S. Department of Veterans Affairs. Danielle Karo-Atar performed this work in partial fulfillment of the requirements for a PhD degree at the Sackler Faculty of Medicine, Tel-Aviv University, Israel. We thank Dr. Katharina Heinzelmann for her technical assistance.

AUTHOR CONTRIBUTIONS

DKA, AB, OW, IEF, KH, MI, OE, and AM performed experiments; DKA, MPC, OE, DRH, and AM designed the experiments and analyzed the data; FDF designed experiments and provided critical reagents; RF analyzed the data; DKA, RF, OE, DRH, FDF and AM wrote the manuscript.

DISCLOSURE

Ariel Munitz is consultant for Compugen and Augmanity Nano LTD. The authors declared no conflict of interest.

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