The microRNA miR-22 inhibits the histone deacetylase HDAC4 to promote T_{H}17 cell–dependent emphysema

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Smoking-related emphysema is a chronic inflammatory disease driven by the T_{H}17 subset of helper T cells through molecular mechanisms that remain obscure. Here we explored the role of the microRNA miR-22 in emphysema. We found that miR-22 was upregulated in lung myeloid dendritic cells (mDCs) of smokers with emphysema and antigen-presenting cells (APCs) of mice exposed to smoke or nanoparticulate carbon black (nCB) through a mechanism that involved the transcription factor NF-κB. Mice deficient in miR-22, but not wild-type mice, showed attenuated T_{H}17 responses and failed to develop emphysema after exposure to smoke or nCB. We further found that miR-22 controlled the activation of APCs and T_{H}17 responses through the activation of AP-1 transcription factor complexes and the histone deacetylase HDAC4. Thus, miR-22 is a critical regulator of both emphysema and T_{H}17 responses.

Pulmonary emphysema is a chronic inflammatory lung condition that, together with other smoking-related chronic obstructive pulmonary diseases, represents the third leading cause of death in the USA. Emphysema is characterized by disruption of the lung matrix support that leads to the enlargement and destruction of the alveoli, which are the main gas-exchange units of the lungs, as well as enhanced collapsibility of conducting airways. These pathological changes, which are often accompanied by loss of the pulmonary microvasculature, combine to severely limit gas exchange and exercise tolerance. Emphysema is, furthermore, the most predictive clinical marker of lung cancer, which is the most deadly malignancy of industrialized societies. Although tobacco smoking is its most important risk factor, emphysema is also seen in people who do not use tobacco but are chronically exposed to smoke derived from other sources.

Susceptible smokers have activated cells of the T_{H}11 and T_{H}17 subsets of helper T cells in their lungs, and circulating elastin-specific autoimmune T cells have been cloned from the peripheral blood of smokers. Elastin is the main structural protein of the lungs that tethers the airways open during exhalation, and accelerated loss of this matrix protein through the enhanced activity of elastases largely accounts for the dynamic airway collapse and lung hyperinflation that are characteristic of emphysema. Interferon-γ (IFN-γ) and interleukin 17A (IL-17A), the canonical T_{H}11 cytokine and T_{H}17 cytokine, respectively, promote the secretion of elastase from constitutive lung cells either directly or indirectly by promoting secretion of chemokines such as KC and macrophage inflammatory proteins that drive the recruitment of elastase-secreting neutrophils and macrophages to the lungs. However, the mechanism underlying the loss of peripheral tolerance to self antigens such as elastin and the induction of autoreactive T_{H}11 and T_{H}17 cells by cigarette smoke remains unclear.

Central to the development of such pathogenic T cells are lung CD11c+ myeloid dendritic cells (mDCs) that assume a highly activated phenotype. CD11c+ mDCs from human smokers with emphysema and CD11c+ antigen-presenting cells (APCs) from mice exposed chronically to cigarette smoke induce the T_{H}17 differentiation of syngeneic and allogeneic T cells through a mechanism that involves production of the cytokines IL-6 and osteopontin and inhibition of the transcription factor PPAR-γ. Indeed, smoke-exposed APCs are alone sufficient to promote the development of emphysema upon adoptive transfer to mice. However, little is currently understood about how cigarette smoke initiates the activation of lung DCs and the initial smoke-activated molecular pathways that drive chronic inflammation and diseases such as emphysema.

MicroRNAs (miRNAs) represent a well-recognized layer of gene regulation that might critically influence immunological diseases such as emphysema. These are endogenous small noncoding RNAs, approximately 23 nucleotides in length, that post-transcriptionally regulate gene expression by binding to target sequences located within the 3′ untranslated regions of select mRNAs. They bind to their 3′ untranslated region targets in association with argonaute proteins within the cytoplasmic RNA-induced silencing complex, which leads to degradation of the mRNA or inhibition of its translation. Over 100 miRNAs are selectively expressed in cells of the mammalian immune system, many of which have been linked to either normal immune function or disease. MicroRNA regulation of T cell responses has been well documented in vitro and in vivo and can modulate the development of both autoreactive and anti-tumour responses. However, the extent to which microRNAs contribute to the development of chronic inflammatory diseases such as emphysema remains poorly understood.

miR-22 is a microRNA that is selectively expressed in cells of the mammalian immune system, including lung APCs. It targets HDAC4, a histone deacetylase that is important for TH1 and TH17 cell development and function. In studies of mice deficient in miR-22, we found that these mice showed attenuated TH17 responses and failed to develop emphysema after exposure to smoke or nCB. We further found that miR-22 controlled the activation of APCs and TH17 responses through the activation of AP-1 transcription factor complexes and the histone deacetylase HDAC4. Thus, miR-22 is a critical regulator of both emphysema and TH17 responses.

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responses or autoimmune disease. Members of the extended let-7 family of miRNAs are pro-inflammatory and promote the development of allergic lung inflammation and disease that resembles allergic asthma.

During a screen of selected miRNAs for immunoregulatory activity, we discovered a potential pro-inflammatory role for miR-22. We found that miR-22 was essential for the activation of DCs and the development of pulmonary emphysema resulting from the inhalation of cigarette smoke or nanoparticulate carbon black (nCB). Of the many targets of miR-22, the diverse immunological effects of miR-22 seemed to be driven through a single major target, the histone deacetylase HDAC4; decreased expression of HDAC4 led to a highly activated DC phenotype and robust Th17 responses that drove the development of pulmonary emphysema. Selective inhibition of miR-22 in the airway thus represents a novel and potentially effective therapy for diverse pulmonary diseases and other inflammatory diseases.

RESULTS
Regulation of cigarette smoke–induced emphysema by miR-22
To establish a potential relationship between miR-22 and human emphysema, we determined how miR-22 expression is regulated in lung mDCs from smokers with and without lung disease. As assessed by reverse transcription followed by quantitative PCR (RT-qPCR), miR-22 expression was higher in CD1a+ lung mDCs from subjects with emphysema than in those from control subjects without emphysema, and this was positively correlated with the severity of obstructive airway disease, as assessed by the forced expiratory volume in 1 s (Fig. 1a,b). We further investigated the relationship between experimental emphysema in mice and miR-22 expression. Mice exposed to 4 months of cigarette smoke had significantly higher expression of miR-22 in their CD11c+ lung APCs than did mice exposed to air alone (Fig. 1c).

To determine if miR-22 is functionally linked to the pathogenesis of emphysema, we initially compared the ability of wild-type and Mir22−/− mice to develop emphysema after 4 months of exposure to cigarette smoke. Wild-type mice exposed to smoke developed canonical features of emphysema, including enlargement of the alveolar spaces and increased total lung volume, accompanied by the accumulation of airway macrophages and neutrophils (Fig. 1d–g). In contrast, all emphysema-related parameters were either attenuated or abolished in Mir22−/− mice (Fig. 1d–g). Quantification of lung helper T cell subsets after exposure to smoke showed significantly fewer Th17 cells in Mir22−/− mouse lungs than in wild-type mouse lungs (Fig. 1h). These data suggested that miR-22 was required for the development of cigarette smoke–induced experimental emphysema.

Requirement for APC miR-22 in nCB-induced emphysema
Cigarette smoking leads to deposition in the lungs of nCB, an insoluble substance that accumulates specifically in and activates APCs that promote pathological Th1 and Th17 responses and emphysema. To determine a potential role for miR-22 in nCB-dependent inflammation, we further compared the ability of wild-type and Mir22−/− mice to develop emphysema after 1 month of exposure to nCB. We treated wild-type and Mir22−/− mice with nCB intranasally twice weekly for 6 weeks in amounts that approximated smokers’ lung nCB burdens (0.1 mg per gram of wet lung). At 4 weeks after the final instillation of nCB, Mir22−/− mice exposed to nCB developed attenuated features of emphysema, including less enlargement of the alveolar spaces and reduced increases in total lung volume, relative to that of wild-type mice (Fig. 2a,b). The recruitment of macrophages, neutrophils and Th17 cells to the airways (Fig. 2c–e) and expression of the elastin-degrading matrix metalloproteinases MMP9 and MMP2 in the airways (Supplementary Fig. 1a) were also lower in Mir22−/− mice than in wild-type mice, while Mir22−/− mice and wild-type mice had similar numbers of lung IL-17A+ γδ T cells (Supplementary Fig. 1b). We then immunized mice with ovalbumin and confirmed that miR-22 was selectively required for the antigen-specific development of Th17 cells, not that of Th1 cells or Th17 cells, in vivo (Supplementary Fig. 2).

Quantification of cytokine production after nCB challenge further showed less secretion of IL-6, KC, MCP-1 and MIP-1β from lung homogenates of Mir22−/− mice than from those of wild-type mice (Supplementary Fig. 3a–d). Activated APCs are a principal cellular source of IL-6 (ref. 12). To determine if APC activation and cytokine secretion were impaired in Mir22−/− mice, we isolated CD11c+ APCs (WT) and Mir22−/− mice (above images) exposed for 4 months to air or cigarette smoke (left margin). Original magnification, ×50 (main images) or ×400 (insets); scale bars, 200 μm (main images) or 25 μm (insets). (e) Lung volume of mice as in d, assessed by micro-CT. Each symbol represents an individual mouse (n = 4–5 per group); small horizontal lines indicate the mean (±s.e.m.). (f–h) Total macrophages (f) and neutrophils (g) in BALF, and total lung IL-17A+ Tβ17 cells (h) in mice as in e, assessed by flow cytometry. *P < 0.05 and **P < 0.01 (Mann Whitney test (a), unpaired t-test (c) or Kruskal-Wallis test (e–h)). Data are from one experiment (a,b) or are representative of two experiments (c) or four experiments (d–h) with similar results (error bars (c,f–h), s.e.m.).
Figure 2 miR-22 deficiency protects mice from nCB-induced emphysema. (a) Hematoxylin- and eosin-stained sections (representative of four total sections) of lungs from wild-type and Mir22−/− mice exposed for 1 month to vehicle (PBS) or nCB (left margin). Original magnification, ×50 (main images) or ×400 (insets); scale bars, 200 μm (main images) or 25 μm (insets). (b) Quantification of lung volume in wild-type and Mir22−/− mice exposed for 1 month to vehicle (−) or nCB (+), assessed by micro-CT. Each symbol represents an individual mouse (n = 4 per group); small horizontal lines indicate the mean (s.s.e.m.). (c,d) Quantification of macrophages (c) and neutrophils (d) in BALF, and total lung IL-17A+ T follicular helper (TH17) cell (e) in mice as in a, assessed by flow cytometry, ND, not detected. (f,g) Enzyme-linked immunosorbent assay (ELISA) of IL-1β (f) and IL-6 (g) in supernatants of lung CD11c+ cells obtained from mice as in b and cultured overnight ex vivo. (h) ELISA of IL-17A in supernatants of co-cultures of lung CD11c+ APCs isolated from in vivo as in a and naive syngeneic CD4+ T cells, cultured for 3 d ex vivo in various combinations (below graph). (i-k) Quantification of macrophages (i) and neutrophils (j) in BALF, and total IL-17A+ TH17 cells (k) in whole lungs of mice lacking miR-22 in CD11c+ APCs (Mir22CD11c−/-) and control mice (Mir22WT−/-) after exposure for 1 month to PBS or nCB. *P < 0.05, **P < 0.01 and ***P < 0.001 (Kruskal Wallis test). Data are representative of four experiments (a-h) or two experiments (i-k) with similar results (error bars (c–k), s.e.m. of n = 4 mice per group).

from nCB-challenged mice (Fig. 2a) and cultured the cells overnight ex vivo. Mir22−/− APCs isolated after exposure to nCB secreted significantly less IL-1β, IL-6, KC, MCP-1 and MIP-1β than did their wild-type counterparts (Fig. 2f,g and Supplementary Fig. 3e–g). Mir22−/− APCs exposed to nCB were also less efficient than their wild-type counterparts at driving the secretion of IL-17A from wild-type T cells in vitro (Fig. 2h). To confirm that APC-specific expression of miR-22 was selectively required for lung TH17 responses and the development of emphysema, we generated mice with deletion of Mir22 in CD11c-expressing cells (mice with loxP-flanked Mir22 alleles (Mir22fl/fl) deleted by Cre recombinease expressed from the gene encoding CD11c (CD11c-Cre); called ‘Mir22CD11c−/-’ here). Similar to Mir22−/− mice, Mir22CD11c−/- mice developed less infiltration of macrophages and neutrophils into bronchoalveolar lavage fluid (BALF) and fewer lung TH17 cells after nCB challenge than did Mir22fl/fl mice (Fig. 2i–k). Furthermore, adoptive transfer of CD11c+ lung APCs isolated from cigarette smoke–exposed wild-type mice induced emphysema, macrophage infiltration and lung TH17 cell development in both naive wild-type recipient mice and naive Mir22−/− recipient mice (Supplementary Fig. 4). Together these findings confirmed that APC-specific miR-22 was necessary and sufficient for the induction of lung TH17 responses and emphysema.

Transcription factor NF-κB regulates miR-22 expression

The expression of miR-22 in human CD1a+ mDCs and monocyte-derived DCs from subjects without emphysema was enhanced after exposure to nCB ex vivo (Fig. 3a,b). We observed similarly enhanced miR-22 expression in mouse bone marrow–derived DCs (BMDCs) treated with a variety of activating stimuli, including various Toll-like receptor ligands and agonistic ligation of the costimulatory receptor CD40, in addition to nCB (Fig. 3c).

The expression of miRNAs typically correlates with that of the host genes from which they derive3. The miR-22 primary RNA transcript (pri-miRNA) host gene transcription unit (Mir22hg) was also induced by nCB and several, but not all, Toll-like receptor ligands (including lipopolysaccharide (LPS), the synthetic lipoprotein FSL-1 (Pam3CGDPPKHPKSF), the synthetic RNA duplex poly(I:C), the lipopeptide Pam2,CSK4 and lipoteichoic acid) in mouse BMDCs (Fig. 3d). Ligation of CD40 with antibody to CD40 (anti-CD40) conspicuously failed to induce Mir22hg expression (Fig. 3d), which suggested that the increase in mature miR-22 due to this form of stimulation might have been the result of enhanced processing of extant Mir22hg transcripts or diminished consumption of miR-22. To understand how the Mir22hg is regulated in APCs, we cloned the 2.3-kilobase promoter region upstream of the Mir22hg transcriptional start site into a reporter plasmid encoding enhanced green fluorescent protein (eGFP) and generated multiple deletion mutants, which we transfected into RAW264.7 mouse macrophages. Both basal promoter activity and promoter activity stimulated with LPS (1 ng/ml) progressively decreased as the promoter was shortened (Fig. 3e), which suggested that all regions of this promoter domain contained elements that regulated Mir22hg expression.

Through the use of TFSearch, a server for the prediction of transcription factor–binding sites, we found six predicted potential consensus sequences for the transcription factor AP-1 and one predicted consensus sequence for the transcription factor NF-κB within the 2.3-kilobase promoter region of Mir22hg (Fig. 3e). To determine if Mir22hg promoter activity is regulated by AP-1 and NF-κB, we treated RAW264.7 cells carrying a 2.3-kilobase Mir22hg promoter
plasmid either with an inhibitor of the c-Jun (AP-1 subunit) kinase Jnk (SP600125) to block AP-1 activity or with the proteasome inhibitor MG132 to block NF-κB activity. MG132 significantly inhibited eGFP expression with and without LPS stimulation (Fig. 3f). Moreover, chromatin-immunoprecipitation assays revealed more binding of the NF-κB subunit RelA (p65) but less binding of c-Jun within the Mir22hg promoter region in LPS-treated BMDCs than in unstimulated BMDCs (Fig. 3g). MG132 further impaired the expression of both Mir22hg and miR-22 in nCB-treated BMDCs (Fig. 3h.i). Together these observations indicated that NF-κB increased the activity of the Mir22hg promoter in APCs, which suggested that miR-22 was an NF-κB-responsive miRNA in these cells.

To further understand the role of NF-κB and related transcription factors in regulating miR-22 expression, we assessed the abundance of miR-22 in PPAR-γ-deficient lung APCs. PPAR-γ is an inhibitor of NF-κB activity and the development of emphysema14,15. Consistent with those observations, miR-22 expression was upregulated in PPAR-γ-deficient lung APCs compared with that of PPAR-γ-sufficient control APCs (Supplementary Fig. 5a). Moreover, intranasal treatment of smoke-exposed mice with the PPAR-γ agonist ciglitazone reduced the expression of miR-22 in lung APCs (Supplementary Fig. 5b). Together these observations indicated that PPAR-γ inhibited miR-22 expression in APCs, probably through the inhibition of NF-κB activity. These findings provide insight into the transcriptional mechanism underlying the enhanced miR-22 expression seen in APCs derived from subjects with emphysema.

Requirement for miR-22 in APC activation

To further investigate the immunological importance of miR-22 in APCs, we assessed the expression profiles of miRNAs encoding pro-inflammatory molecules in CD11c+ APCs isolated from the lungs of wild-type and Mir22−/− mice that were exposed to nCB. Mir22−/− lung APCs had lower expression of mRNA encoding c-Jun (a subunit of AP-1), the co-stimulatory molecules CD86 and CD40, the chemokine CCL8, and the metalloproteases MMP13 and MMP14 than did their wild-type counterparts (Fig. 4a and Supplementary Fig. 6). After treatment overnight with LPS or agonistic antibody to CD40, Mir22−/− BMDCs further showed significantly lower surface

Figure 3  miR-22 expression is transcriptionally regulated through NF-κB and AP-1 complexes. (a) miR-22 expression in human lung CD1a+ mDCs before (Naive) and after (nCB) 2 d of in vitro challenge with 10^4 ng nCB (key); results (relative to those of U6) are presented relative to those of naive cells. (b) miR-22 expression in naive human monocyte-derived DCs (MDCC) from healthy subjects after 2 d of in vitro challenge with 0–10^4 ng (horizontal axis) of nCB; results (relative to those of U6) are presented relative to those of untreated cells (0 ng nCB). (c,d) Expression of miR-22 (e) and Mir22hg (d) in naive mouse BMDCs (N) or mouse BMDCs stimulated overnight with 10^4 ng carbon black (C), 1 ng/ml of LPS (L), 100 ng/ml of polyI:C (C), 100 ng/ml FSL-1 (F), heat killed Listeria monocytogenes at a density of 1 × 10^6 cells per ml (H), 100 ng/ml of Pam3CSK4 (P), 1 µg/ml of lipoteichoic acid (T), or agonistic antibody to CD40 (40); results are presented relative to those of U6 (c) or 18S RNA (d). (e) Proximal 2.3 kilobases of the Mir22hg promoter (top left), including binding sites for AP-1 and NF-κB (key), and eGFP reporter constructs containing the full-length or truncated promoter (below left); numbers adjacent to diagrams indicate position relative to the transcription start site. Right, eGFP expression in RAW264.7 cells transfected with reporter plasmids encoding the constructs at left, left unstimulated (Naive) or stimulated with LPS (key), assessed by flow cytometry. (f) Mean fluorescence intensity (MFI) of eGFP in cells transfected with reporter plasmid containing the proximal 2.3-kilobase promoter in e, left unstimulated (Naive) or stimulated with LPS (key), and treated with the vehicle DMSO or with the Jnk inhibitor SP600125 (JNKi) or the NK-κB inhibitor MG132 (horizontal axis). (g) Binding of RelA (p65) and c-Jun to the Mir22hg promoter, as well as histone H3 acetylated at Lys9 (Acetyl-H3K9), in naive (−) and LPS-stimulated (+) mouse BMDCs, assessed by chromatin immunoprecipitation (IP); bottom, PCR of 10% of the input DNA without immunoprecipitation. (h,j) Expression of miR-22 (h) and Mir22hg (j) in naive (nCB −) or nCB-treated (nCB +) mouse BMDCs with (MG132 +) or without (MG132 −) treatment with MG132; results are presented relative to those of U6 (h) or 18S RNA (j). *P < 0.05, **P < 0.01 and ***P < 0.001 (Mann-Whitney test (a), Kruskal Wallis test (b–d,h,i) or two-way analysis of variance (f)). Data are from one experiment representative of at least two experiments with similar results (error bars, s.e.m. of n = 4 subjects per group (a,b), n = 4 mice per group (c,d), n = 5 replicates per group (e), n = 3 replicates per group (f) or n = 4 replicates per group (h,j)).
expression of CD86 and CD40 and less secretion of IL-6 than did their wild-type counterparts (Fig. 4b-d). In vitro, nCB also induced less secretion of IL-6 from Mir22−/− BMDCs than from wild-type BMDCs (Fig. 4e). nCB-treated miR-22-deficient BMDCs induced less secretion of IL-17A from co-cultured naive T cells than did nCB-treated miR-22-sufficient APCs (Supplementary Fig. 7). However, this defect in IL-17A secretion was abrogated by the addition of IL-6 to the culture conditions (Supplementary Fig. 7). Conversely, transfection of miR-22 mimics into unstimulated mouse BMDCs was alone sufficient to induce the expression of Il1b, Il6, Il23a and Cdb6 mRNA (Fig. 4f) and also enhanced the secretion of IL-6 dependent on LPS or agonistic antibody to CD40 (Fig. 4g). In a dose-dependent manner, miR-22 was similarly required for robust LPS-dependent secretion of IL-6 from human monocyte-derived DCs (Fig. 4h). Thus, miR-22 drove the production of cytokines such as IL-6 and co-stimulatory molecules that are required for robust T\(_{HI}\)17 responses.

Impaired AP-1 activity in Mir22−/− APCs

Ligation of CD40 triggers APC maturation mainly through NF-κB and AP-1, two transcription factors that promote the expression of inflammatory cytokines and co-stimulatory molecules.\(^{16-19}\) To determine if miR-22 regulates the activation of NF-κB and AP-1 in APCs, we treated wild-type and Mir22−/− mouse BMDCs with agonistic antibody to CD40. As assessed by immunoblot analysis, there was no substantial difference between wild-type cells and Mir22−/− cells in their production of the NF-κB p50 or p52 fragments from their precursors p105 (NF-κB1) or p100 (NF-κB2) (Fig. 5a). We further assessed the phosphorylation status of major signaling proteins in BMDCs from wild-type and Mir22−/− mice by reverse-phase protein array. Phosphorylation of c-Jun increased in wild-type BMDCs, but not in Mir22−/− BMDCs, after treatment with anti-CD40, while phosphorylation of the upstream mitogen-activated protein kinases MEK1, p38, Erk1/2 and Jnk showed similar patterns in wild-type and Mir22−/− BMDCs (Fig. 5b), which suggested that the lack of c-Jun phosphorylation in Mir22−/− BMDCs was not due to disruption of upstream kinase pathways. We next assessed the expression of c-Jun in wild-type and Mir22−/− BMDCs by immunoblot analysis. Expression of c-Jun increased in wild-type BMDCs but decreased in Mir22−/− BMDCs after treatment with anti-CD40 (Fig. 5c). After 24 h of stimulation, there was less c-Jun in Mir22−/− BMDCs than in wild-type BMDCs (Fig. 5c), which suggested that miR-22 might be an important regulator of c-Jun expression in APCs. To address this further, we transfected an AP-1 reporter plasmid (containing sequence encoding GFP controlled by a promoter element specific to the gene encoding AP-1) into both wild-type BMDCs and Mir22−/− BMDCs. Wild-type BMDCs generated a significantly stronger GFP signal than did Mir22−/− BMDCs upon stimulation...
miR-22 promotes the transcriptional activity of AP-1 in APCs. (a,b) Immunoblot analysis of NF-κB1 components p105 and p50 (top) and NF-κB2 components p100 and p52 (bottom) and of c-Jun and β-actin (loading control) (b) in wild-type and Mir22−− BMDCs (below blots) stimulated for 0, 1, 2 or 24 h (above lanes) in vitro with agonistic antibody to CD40. (c) Reverse-phase protein array of the phosphorylation (p-) of signaling proteins (left margin; phosphorylated residues in parentheses) in BMDCs as in a. (d) Mean fluorescence intensity of AP-1–GFP in wild-type and Mir22−− BMDCs transfected with a reporter plasmid encoding AP-1–GFP and left unstimulated (Naive) or activated for 24 h with antibody to CD40 (key), quantitated by flow cytometry. *P < 0.01 (unpaired t-test). Data are from one experiment representative of two experiments (a,b), one experiment (c) or two experiments (d), error bars, s.e.m. of n = 3–4 mice per group.

with anti-CD40 (Fig. 5d). Collectively, these data confirmed that miR-22 promoted the transcriptional activity of AP-1 in APCs.

miR-22 targets HDAC4 in APCs

miR-22 putatively regulates several hundred genes (as suggested by the TargetScanHuman website for the prediction of miRNA targets). To begin to determine the critical miR-22-dependent genes that underlie Th17 responses and emphysema, we again compared the gene-expression profiles of CD11c+ APCs isolated from the lungs of wild-type and Mir22−− mice exposed to nCB, focusing on putative miR-22 target genes that were downregulated in wild-type APCs but downregulated less in Mir22−− APCs (Fig. 6a). To screen for functional relevance to APC activation, we then used small interfering RNA (siRNA) to knock down Mir22−− BMDCs the genes (Ogn, Hdac4 and Acrv2b) that were most differentially expressed in lung APCs from nCB-exposed Mir22−− mice relative to their expression in those from nCB-exposed wild-type mice. Of these putative targets, the impairment in IL-6 secretion shown above for Mir22−− BMDCs upon stimulation with anti-CD40 (Fig. 4c) was partially restored only by knockdown of Hdac4 (Fig. 6b), which has been shown to be a true miR-22 target20–22. To confirm that miR-22 regulated HDAC4 expression in APCs, we first showed that neutralization of miR-22 in naive mouse BMDCs with an antisense locked nucleic acid (LNA) enhanced the expression of both Hdac4 mRNA and HDAC4 protein (Fig. 6c,d). Moreover, after stimulation of cells with anti-CD40, HDAC4 expression decreased in wild-type BMDCs but increased in Mir22−− BMDCs (Fig. 6e). Finally, knockdown of Hdac4 by siRNA in Mir22−− BMDCs or BMDCs transfected with LNA targeting miR-22 restored their ability to induce IL-17A secretion from co-cultured naive wild-type T cells after stimulation with nCB (Fig. 6f,g). Collectively, these studies confirmed that Hdac4 was a major target of miR-22 in APCs and suggested the existence of an obligatory regulatory pathway in which activated APCs enhance the expression of miR-22 that then inhibits HDAC4 expression to promote Th17 responses.

HDAC4 inhibits APC activation and emphysema development

HDAC4 is a class II histone deacetylase and is therefore a potential epigenetic regulator23,24. The deacetylation of histones in gene

**Figure 5** miR-22 promotes the transcriptional activity of AP-1 in APCs. (a,b) Immunoblot analysis of NF-κB1 components p105 and p50 (top) and NF-κB2 components p100 and p52 (bottom) (a) and of c-Jun and β-actin (loading control) (b) in wild-type and Mir22−− BMDCs (below blots) stimulated for 0, 1, 2 or 24 h (above lanes) in vitro with agonistic antibody to CD40. (c) Reverse-phase protein array of the phosphorylation (p-) of signaling proteins (left margin; phosphorylated residues in parentheses) in BMDCs as in a. (d) Mean fluorescence intensity of AP-1–GFP in wild-type and Mir22−− BMDCs transfected with a reporter plasmid encoding AP-1–GFP and left unstimulated (Naive) or activated for 24 h with antibody to CD40 (key), quantitated by flow cytometry. *P < 0.01 (unpaired t-test). Data are from one experiment representative of two experiments (a,b), one experiment (c) or two experiments (d), error bars, s.e.m. of n = 3–4 mice per group.

**Figure 6** miR-22 targets HDAC4 in APCs to promote IL-6 production. (a) Expression of genes predicted to be targets of miR-22 (right margin) in wild type and Mir22−− CD11c+ APCs stimulated with PBS or nCB (above plot). (b) ELISA of IL-6 in wild-type or Mir22−− BMDCs transfected with control siRNA with a scrambled sequence (siScr) or siRNA specific for the gene encoding OGN (siOGN), HDAC4 (siHdac4) or Acrv2b (siAcrv2b), left unstimulated (−) or stimulated overnight with anti-CD40 (+); dashed horizontal line indicates IL-6 secretion by Mir22−− BMDCs transfected with control siRNA and activated with anti-CD40. (c,d) RT-qPCR analysis of Hdac4 mRNA (c) and immunoblot analysis of HDAC4 (d) in wild-type BMDCs transfected with LNA with a scrambled sequence (Scr) or LNA targeting miR-22; RNA results (c) are presented relative to those of 18S RNA. Numbers above lanes (d) indicate the density of HDAC4 relative to that of β-actin. (e) Immunoblot analysis of HDAC4 in wild-type or Mir22−− BMDCs stimulated for 0–24 h (above lanes) with anti-CD40. (f,g) ELISA of IL-17A secreted by wild-type naive CD4+ T cells cultured for 3 days together with wild-type or Mir22−− BMDCs (f) or wild-type BMDCs transfected with LNA with a scrambled sequence or LNA targeting miR-22 (g), left unprimed (nCB −) or primed with 1,000 ng nCB (nCB +) and transfected with siRNA with a scrambled sequence (siHDAC4 −) or HDAC4-specific siRNA (siHDAC4 +). *P < 0.05, **P < 0.01 and ***P < 0.001 (Kruskal-Wallis test (b,f,g) or unpaired t-test (c)). Data are from one experiment (a) or are from one experiment representative of two or three experiments with similar results (b–g; error bars, s.e.m. of n = 5 mice per group (b), n = 3 mice per group (c) or n = 4 mice per group (f,g)).
promoter regions results in chromosomal condensation, which diminishes the potential for genes in these regions to be transcriptionally activated. Published studies have shown that loss of HDAC4 in myeloid cells results in exacerbated inflammation in mice fed a high-fat diet, which would suggest an anti-inflammatory role for HDAC4. Indeed, we found higher c-Jun expression 26,27. Upon stimulation with nCB, HDAC4-deficient BMDCs treated for 0–12 h (above lanes) with anti-CD40 (below), and cumulative density measurements of each band (above). (*P < 0.05, **P < 0.01, ***P < 0.001 (Mann-Whitney test (a-f) or Kruskal-Wallis test (g,h,i)). Data are from one experiment representative of three experiments with similar results (error bars, s.e.m. of n = 4 subjects (a) or mice (b) or n = 4–5 mice per group (c-f)).

Reversal of mouse emphysema by anti-miR-22

The studies reported above suggested that either neutralization of miR-22 or enhanced expression of HDAC4 in vivo would attenuate the development of emphysema. As it is not technically possible to enhance the activity of constitutive HDAC4 in vivo, we instead neutralized miR-22 in vivo by delivering an LNA targeting miR-22 intranasally into emphysematous mice that were previously exposed to nCB. We exposed 2-month-old wild-type mice to nCB or PBS for 1 month and allowed them to ‘rest’ for another month. At the age of 4 months, mice previously exposed to nCB developed severe emphysema, as determined by quantification of lung volume through the use of micro-computerized tomography (micro-CT) (Fig. 8a). nCB-exposed mice then continued to develop severe emphysema after 1 month of treatment with a control LNA with a scrambled sequence, whereas nCB-exposed mice treated with the LNA targeting miR-22 showed evidence of resolution of emphysema, as determined by lung volume and lung histology (mouse age, 5 months; Fig. 8a,b).
We continued to treat the two mouse groups similarly for 2 additional months and found that after a total of 3 months of treatment with LNA, mice treated with the control LNA showed continued disease progression, whereas mice treated with the LNA targeting miR-22 had significantly lower lung volumes (mouse age, 7 months; Fig. 8a). At 3 months after the cessation of LNA treatment, emphysema returned fully in the mice treated with the LNA targeting miR-22 (mouse age, 10 months; Fig. 8a,b).

After 1 month of treatment with the LNA targeting miR-22 (mouse age, 5 months), miR-22 was essentially undetectable in mouse lungs (Supplementary Fig. 8a). These mice further showed less infiltration of macrophages and neutrophils into the lungs, fewer T\(_{h}17\) cells from whole lung, and diminished secretion of IL-6 and IL-17A into lung homogenates than did mice treated with the control LNA (Fig. 8c–e and Supplementary Fig. 8b,c). We also found higher expression of Hdacho1 mRNA and, conversely, lower expression of Ifi6 and Cdad10 mRNA in CD11c\(^{+}\) lung APCs from mice treated with LNA targeting miR-22 than in those from mice treated with the control LNA (Supplementary Fig. 8d–f). Mice previously treated with LNA targeting miR-22 still showed less infiltration of macrophages and neutrophils into the lungs than did mice previously treated with the control LNA, but they had numbers of T\(_{h}17\) cells from whole lung similar to those of mice previously treated with the control LNA (Fig. 8c–e). Thus, prolonged treatment with an LNA that inhibited miR-22 reversed fully established emphysema and the activation of lung APCs.

**DISCUSSION**

We have identified miR-22 as a critical regulator of the activation of DCs in response to cigarette smoke and smoke-derived nCB that underwent phagocytosis by diverse lung APCs. Lung DCs activated with nCB secreted IL-6 and expressed discrete co-stimulatory molecules that are required for the differentiation and effector function of T\(_{h}17\) cells and that we found were controlled through miR-22. Although miR-22 probably influences many genes encoding products related to immunity, much of the pro-inflammatory activity of miR-22 was mediated through the post-translational suppression of HDAC4. Together our findings expand the understanding of miRNAs related to immunity, much of the pro-inflammatory activity of miR-22 has been linked to control of the cardiac response to acute stress\(^{28,29}\) and regulation of the differentiation of mesenchymal stem cells\(^{30}\), but this is the first demonstration to our knowledge of a critical immunological function for this miRNA. Like many other miRNAs, miR-22 has also been linked to cancer phenotypes. In some contexts, miR-22 is viewed as a tumor suppressor\(^{31}\), with various human tumors showing repression of miR-22 expression relative to its expression in surrounding normal tissue\(^{32}\). In contrast, overexpression of miR-22 induces myelodysplastic syndrome and bone marrow malignancies in mice\(^{33}\), which would suggest that this miRNA might, under other circumstances, act as a tumor promoter. Over the approximately 5 years that we have monitored our miR-22-deficient mouse colony, we have not noticed early mortality or the development of tumors of any kind. Moreover, we have not noticed unusual mortality or tumors in wild-type mice treated with LNA targeting miR-22. Thus, whereas manipulation of any miRNA or other genetic element carries a theoretical risk of cellular transformation, our experience does not indicate that loss of miR-22 in vivo is alone sufficient to promote cancer.

In contrast to miR-22, HDACs have been linked to the pathogenesis of emphysema. HDAC activity in lung biopsies and lung macrophages of subjects with emphysema who smoke was found to be suppressed, relative to its activity in such samples from non-smokers, in a manner positively correlated with disease severity\(^{2}\). Those observations were linked to HDAC2, HDAC5 and HDAC8, and subsequent studies have suggested that less deacetylation of the glucocorticoid receptor...
by HDAC2 in emphysema antagonizes the ability of corticosteroids to inhibit the pro-inflammatory potential of NF-κB. Corticosteroids are notoriously ineffective in chronic obstructive pulmonary disease and/or emphysema; thus, this mechanism suggests that interventions that increase activity of HDAC2 might enhance the effectiveness of corticosteroids. Subsequent study of HDAC inhibitors in rodents and in vitro has further suggested an anti-inflammatory role for HDACs in emphysema. However, the broad-spectrum nature of these inhibitors precludes delineation of the most relevant anti-inflammatory HDACs.

Independent lines of evidence have, however, established clear links to immunological regulation for HDAC4, which functions as a co-repressor of multiple immunologically related transcription factors, including c-Jun, NF-κB and Bcl-6, and regulates IL-5 expression. HDAC4 also promotes activation of STAT6 and expression of arginine-1 that is required for the differentiation of myeloid DCs. Our studies have now shown that HDAC4 is essential for suppressing Th17 responses and nCB-dependent emphysema in mice. Together with our analyses of miR-22, our findings suggest the existence of a pro-inflammatory regulatory loop in which noxious stimuli such as carbon black enhance the expression miR-22 that then suppresses HDAC4 sufficiently to promote the differentiation of Th17 cell–promoting DCs. We have emphasized the role of HDAC4 in controlling DC-derived IL-6, but the expression of CD40, CD86 and c-Jun was also impaired in the context of less miR-22 and enhanced HDAC4 activity. As all of these factors are required for its development, our findings explain the requirement for a pleiotropic suppressive factor such as HDAC4 in the regulation of the highly complex Th17 response.

Our findings do not rule out the possibly of a role for HDAC2 and other HDACs in the regulation of inflammation in emphysema. Nonetheless, our mechanistic delineation of the pro-inflammatory role of miR-22 in emphysema has revealed that HDAC4 specifically regulated smoking-related lung inflammation and Th17 responses and might account for much of the HDAC-related anti-inflammatory activity that has been reported on the basis of studies with inhibitors. IL-17A and Th17 cells have also been linked to many chronic diseases other than emphysema that are related to dysregulation of the immune system, including asthma, inflammatory bowel disease, rheumatoid arthritis and others. Interventions that inhibit miR-22 or enhance HDAC4 activity might thus be broadly useful in the therapy of many human diseases caused by chronic inflammation.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE72734.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.L. and D.B.C. conceptualized the project and all studies and wrote the manuscript with assistance from all co-authors; W.L. designed and performed all experiments, with R.Y., X.Y. and T.Y. contributing to selected mouse experiments and micro-CT analysis; E.L.G.S., D.C.M., W.K.A.S. and J.M.T. prepared nCB. A.R. provided miR-22 deficient mice; and F.K. and D.B.C. provided grant support.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Mice.** C57BL/6 and B6-Albino (B6(Cg)-Tyr<sup>−/−</sup>) were purchased from Jackson Laboratory. The MiR<sup>22<sup>−/−</sup> (miR-22-null) mice used here were generated as described<sup>22</sup>; they were backcrossed at least five generations onto the C57BL/6 background and then were maintained as a homozygous stock. Hdac4<sup>−/−</sup> mice were provided by E. Olsson<sup>43</sup>. Mice with transgenic expression of CD11c-Cre (backcrossed for eight generations onto the C57BL/6 background) were provided by J. Levitt. For all mouse experiments, wild-type syngeneic littermates were used as controls. All mice were bred and maintained in the specific pathogen-free Transgenic Mouse Facility at Baylor College of Medicine, were provided water and standard rodent chow for ad libitum consumption and were used in accordance with all federal and institutional guidelines for the humane treatment of animals.

**Human subjects.** Non-atopic current or former smokers (<i>n</i> = 28) with a substantial history of smoking (>20 pack-years, where one pack-year is the equivalent of smoking one pack of cigarettes per day each year) were recruited for studies from the chest or surgical clinics at Methodist and Michael E. DeBakey Houston Veterans Affairs Medical Center hospitals. Studies were approved by the Institutional Review Board at Baylor College of Medicine, and informed consent was obtained from all patients. Control patients with and without emphysema were diagnosed according to the criteria recommended by the National Institutes of Health–World Health Organization workshop summary<sup>49</sup>.

**Preparation of DCs from bone marrow.** Bone marrow isolated from 8-week old mice underwent lysis of red blood cells (RBCs) through the use of ammonium chloride–potassium bicarbonate (lysis buffer), after which the bone marrow cells were washed with complete medium (RPMI-1640 medium supplemented with 10% Hyclone heat-inactivated FBS (GE Healthcare Life Sciences)) and then were plated in six-well plates at a density of 5 × 10<sup>5</sup> to 10 × 10<sup>5</sup> cells per ml. The culture medium was supplemented with the cytokines mouse GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) (R&D Systems). On day 5 or 6, both adherent cells and non-adherent cells were harvested by gentle scraping. CD11c<sup>+</sup> BMDCs were isolated with bead-conjugated anti-CD11c (Miltenyi) and were isolated from lung single-cell suspensions with an AutoMACS Separator (Miltenyi).

**Preparation of human samples.** Single-cell suspensions of human lungs were prepared as described<sup>6</sup>. Fresh lung tissue was cut into pieces 0.1 cm in diameter in Petri dishes and was treated for 30–40 min at 37 °C with 2 mg/ml of dNTPs (Takara) and DNase I (Roche) in a solution of 10 × 10<sup>5</sup> cells per ml. The culture medium was supplemented with the cytokines mouse GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) (R&D Systems). On day 5 or 6, both adherent cells and non-adherent cells were harvested by gentle scraping. CD11c<sup>+</sup> RBC-free lung cells were isolated with bead-conjugated anti-CD11c (Miltenyi) through the use of an autoMACS.

**Isolation of cells of the immune system.** Single-cell suspensions of mouse lung and spleen were prepared, and RBCs in the samples were lysed with ammonium chloride–potassium bicarbonate (lysis buffer) as described<sup>35</sup>. RBC-free samples of lung cells were washed with complete medium and then were labeled with bead-conjugated anti-CD11c (Miltenyi) for isolation of lung APCs with an autoMACS. RBC-free samples of splenic cells were labeled with bead-conjugated anti-CD4 (Miltenyi) for isolation of CD4<sup>+</sup> T cells.

**Neutralization of miR-22 in vivo.** An antisense LNA targeting miR-22 (5′-CTT CAACCTGGCACCT-3′) and a control LNA (th scrambled sequence 5′-AGCT CTATACGCCCA-3′) were synthesized by Exiqon. The LNAs were first reconstituted in saline and then were injected into mice per os. The concentration of total RNA was determined with a NanoDrop 2000 (Thermo Fisher Scientific), and the cDNA of total mRNA or miRNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit or a TaqMan MicroRNA Reverse Transcription Kit, respectively, according to the manufacturer's guidelines (Life Technologies). Quantitative PCR was performed with the ABI 7500 Real-Time PCR System (Applied Biosystems) by standard protocols. All Taqman probes were from Applied Biosystems.

**Quantitative flow cytometry.** Flow cytometry was performed with a BD LSRll or Accuri C6 (BD Biosciences) and results were analyzed with FlowJo software. For quantification of the absolute number of specific cell types in samples, Flow-Seq Fluospheres beads (Beckman Coulter) were mixed into all samples before as input controls. Event numbers of all cell populations were normalized to the number of fluorospheres beads for generation of the absolute number.

**Antibodies and other reagents.** Mouse specific antibodies were as follows: Pacific Blue–anti-CD3 (500A2), allopurinol–indocarbocyanine (Cy7)–anti-CD8 (53-67), phycoerythrin (PE)–anti-IL17A (TC11-18H10), allopurinol–anti-IFN-γ (XMGL2) and PE–indocarbocyanine (Cy5)–anti-CD4 (PM4-5) (all from BD Pharmingen); and fluorescein isothiocyanate (FITC)–anti-γδ TCR (eBioGL3), eFluor 450–anti-B220 (RA3-6B2), PE–anti-CD11b (M1/70) and allopurinol–anti-CD11c (N418) (all from eBioscience).

Cigarette smoke–exposure model of pulmonary emphysema. Mice were exposed to cigarette smoke as described<sup>3</sup>. 8-week old mice were exposed to four commercial cigarettes (Marlboro 100's; Philip Morris USA) per day, 5 d a week, for 4 months. 1 week after the final exposure to cigarette smoke, lung volumes were assessed by micro-CT imaging as described<sup>9</sup>. Mice were anesthetized with etomidate (30 mg per kg body weight) and were scanned in an animal CT scanner (Gamma Medica) in the Animal Phenotyping Core at Baylor College of Medicine. Three-dimensional models of mouse lung were reconstructed and the volume was calculated with Amira 3.1.1 software. BALF cell differential counting, lung-tissue collection and lung histopathology were performed as described<sup>33</sup>. Mice were anesthetized with etomidate, BALF was collected by instillation and withdrawal of 1 ml of sterile PBS twice through the tracheal cannula. Mouse lungs were either dissected for the preparation of single cell suspensions or fixed with 4% paraformaldehyde solution for histopathological studies. CD11c<sup>+</sup> lung APCs were labeled with CD11c MicroBeads (Miltenyi) and were isolated from lung single-cell suspensions with an AutoMACS Separator (Miltenyi).

**nCB-exposure model of pulmonary emphysema.** Commercial grade carbon black (15-μm; lot 1278105; Cabot), composed of 15-μm particles in clusters of three to five particles, was suspended in tert-butyl alcohol with 1% sucrose (sucrose was added to prevent aggregation in aqueous suspension), and then was lyophilized overnight at −80 °C. Dried nCB nanoparticles were resuspended in sterile PBS to a concentration of 10 mg/ml. 50 μl reconstituted nCB (0.5 mg) was delivered intranasally to deeply anesthetized mice on a schedule of three times a week for 6 weeks (total dose delivered, 9 mg). Lung volume and airway inflammation were assessed 4 weeks after the final nCB challenge.

**APC–T cell co-culture.** Isolated human or mouse CD4<sup>+</sup> T cells were cultured together with APCs at a ratio of 1:1 in 96-well U-bottomed tissue culture plates with complete medium supplemented with anti-CD3ε (1 μg/ml; 145-2C11; BD Bioscience). The medium and cells were collected on day 3 for further analysis.

**ELISA and the Bioplex system.** Mouse and human IL-1β, IL-6 and TNF were measured with BD OptEIA ELISA kits according to the manufacturer's guidelines (BD Bioscience). Mouse IL-4, IFN-γ, IL-17A and human IL-17A were measured by sandwich ELISA with antibody pairs from R&D Systems. Other human and mouse cytokines and chemokines were measured by Milliplex Assays (EMD Millipore) performed on the Bioplex 100 system (Bio-Rad Laboratories).

**RNA isolation and quantitative PCR.** Fresh cell pellets were collected and stored in TRIzol (Invitrogen). Total RNA was extracted with chloroform (Sigma-Aldrich) and was precipitated in isopropanol (Sigma-Aldrich). The concentration of total RNA was determined with a NanoDrop 2000 (Thermo Fisher Scientific), and the cDNA of total mRNA or miRNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit or a TaqMan MicroRNA Reverse Transcription Kit, respectively, according to the manufacturer's guidelines (Life Technologies). Quantitative PCR was performed with the ABI 7500 Real-Time PCR System (Applied Biosystems) by standard protocols. All Taqman probes were from Applied Biosystems.
Gene expression in lung APCs. Gene-expression profiling of mouse CD11c+ lung APCs was performed with an Illumina MouseWG-6 v2 Expression BeadChip Array (Expression Analysis). Probes with a signal of less than 20 units were pre-filtered. Data were analyzed with variance-stabilizing transform and quantile normalization. The heat maps were generated with Cluster 3.0 clustering software (http://bonsai.hgc.jp/~mdehoon/software/cluster/) as a hierarchical cluster through the use of Pearson correlation and a centered metric. The resulting heat map was visualized and presented with Treeview program for displaying phylogenies (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Chromatin immunoprecipitation (ChIP) assay. ChIP experiments were performed with a Pierce Agarose ChIP Kit according to the manufacturer’s protocols (Thermo Fisher Scientific). For each sample, 4 × 10⁶ mouse BMDCs were cross-linked by incubation for 10 min at 24 °C with 1% formaldehyde, followed by the addition of 0.2 M glycine and further incubation for 5 min at 24 °C. Chromatin was digested with micrococcal nuclease and was immunoprecipitated by incubation overnight at 4 °C with ChIP-grade anti-p65 (D14E12; 8242; Cell Signaling Technology), anti-Jun (60A8; 9165; Cell Signaling Technology), antibody to acetylated histone H3 (C3B8E1; 9648; Cell Signaling Technology) or rabbit IgG (provided with the Pierce Agarose ChIP Kit), together with agarose beads. DNA was recovered thereafter with a DNA column. The Mirt22hg promoter region was amplified by PCR (forward primer, 5′-GGGCCAGTCAGATTTCG-3′, reverse primer, 5′-CCTCTGCTTACTCCCGCCC-3′).

Reverse-phase protein array. Mouse BMDCs were activated in the presence of agonistic antibody to CD40 (1C10; R&D Systems) and then were collected by gentle scraping followed by ‘snap freezing’ in liquid nitrogen. All samples were analyzed in the reverse-phase protein array Core Facility at MD Anderson Cancer Center and were processed according to standard procedures. Serially diluted cellular lysates were denatured with 1% SDS and β-mercaptoethanol before being loaded onto nitrocellulose-coated array slides (Grace Biolab) with a 2470 Arrayer (Aushon BioSystems). Each slide was probed with primary antibodies (listed below) plus biotin-conjugated secondary antibodies (customized ‘cocktails’ provided by the reverse-phase protein array Core Facility at MD Anderson Cancer Center). The primary antibodies were as follows: antibody to RelA phosphorylated at Ser536 (3033; Cell Signaling Technology), antibody to MEK1 phosphorylated at Ser217/Ser221 (9154; Cell Signaling Technology), antibody to p38 phosphorylated at Thr180 and Tyr182 (9211; Cell Signaling Technology), antibody to ERK1/2 phosphorylated at Thr202 and Tyr204 (4377; Cell Signaling Technology), antibody to Jnk phosphorylated at Thr183 and Tyr185 (4668; Cell Signaling Technology) and antibody to c-Jun phosphorylated at Ser73 (9164; Cell Signaling Technology). Probed array slides were scanned, analyzed and quantified using Microvigene (VigeneTech). Each dilution curve was fitted to a standard fitting curve and was normalized with a loading-correction factor to generate the expression of all analytes for each sample. The heat maps of log₂-transformed antibody-expression data were generated in Cluster 3.0 as a hierarchical cluster using Pearson correlation and a centered metric. The resulting heat map was visualized and presented with Treeview software.

Gelatin gel zymography. The proteinase activity of MMP2 and MMP9 in BALF was quantified by zymography as described. BALF (15 µl) was initially separated by electrophoresis through a 10% SDS-polyacrylamide gels with 2% gelatin. SDS was removed from gels through the use of 2.5% Triton X-100 immediately after electrophoresis. SDS-free gels were incubated overnight at 37 °C in developing buffer (50 mM Tris-HCl, pH 8, 5 mM CaCl₂, and 0.02% NaCl). Gels were then fixed and were stained with Coomassie blue. MMP2 and MMP9 appeared as transparent bands on a blue background around 70-kilodalton range and 100-kilodalton range, respectively.

Ovalbumin sensitization. Mice were intraperitoneally sensitized with ovalbumin precipitated in alum once a week for 3 continuous weeks as described. Helper T cell subsets among splenocytes were quantified by the end of the fourth week.

Statistics. Significance of differences was determined with the Mann-Whitney test for paired groups or with the Kruskal-Wallis test for multiple comparisons.

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