Basophils trigger emphysema development in a murine model of COPD through IL-4–mediated generation of MMP-12–producing macrophages

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Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide. It has generally been considered a non-Th2-type lung disorder, characterized by progressive airflow limitation with inflammation and emphysema, but its cellular and molecular mechanism remains ill defined, compared with that of asthma characterized by reversible airway obstruction. Here we show a previously unappreciated role for basophils at the initiation phase of emphysema formation in an elastase-induced murine model of COPD in that basophils represent less than 1% of lung-infiltrating cells. Intranasal elastase instillation elicited the recruitment of monocytes to the lung, followed by differentiation into interstitial macrophages (IMs) but rarely alveolar macrophages (AMs). Matrix metalloproteinase-12 (MMP-12) contributing to emphysema formation was highly expressed by IMs rather than AMs, in contrast to the prevailing assumption. Experiments using a series of genetically engineered mice suggested that basophil-derived IL-4, a Th2 cytokine, acted on lung-infiltrating monocytes to promote their differentiation into MMP-12–producing IMs that resulted in the destruction of alveolar walls and led to emphysema development. Indeed, mice deficient for IL-4 only in basophils failed to generate pathogenic MMP-12–producing IMs and hence develop emphysema. Thus, the basophil-derived IL-4/monocyte–derived IM/MMP-12 axis plays a crucial role in emphysema formation and therefore may be a potential target to slow down emphysema progression at the initiation phase of COPD.

COPD | emphysema | basophil | IL-4 | interstitial macrophage

Chronic obstructive pulmonary disease (COPD) is estimated to affect 174.5 million people in the global population (1) and is the fourth leading cause of death in the world (2). COPD is characterized by the progressive airflow limitation commonly associated with exaggerated inflammatory responses to inhaled irritants, which leads to the chronic obstructive bronchitis and the destruction of lung parenchyma, termed emphysema (3, 4). Asthma also causes airway obstruction in association with chronic airway inflammation. Nevertheless, these two diseases show different pathologies, clinical manifestations, and responses to therapy (5). In contrast to recent advances in the understanding and management of asthma, the cellular and molecular mechanism underlying COPD remains less well defined.

Several animal models of COPD have been established to elucidate possible mechanisms underlying the initiation and progression of COPD (6). Among them, smoke- and protease-induced models are commonly used. Chronic exposure to cigarette smoke in rodents can mimic some features of COPD, including airway inflammation and emphysema (7). Even though smoke exposure has relevance to humans, long exposure periods are necessary, and even after 6-mo-long cigarette exposure, mice develop mild emphysema with only marginal airspace enlargement and lung dysfunction (8). Moreover, smoking cessation in mice halts disease progression, in contrast to the progressive feature of human COPD even after smoking has stopped (9). As an alternative approach, a protease-induced model was established (10) soon after the discovery of the association between α1-antitrypsin deficiency and emphysema in humans (11), implying that an imbalance between proteases and antiproteases in the lung may result in emphysema formation. Intratracheal or intranasal instillation of elastolytic enzymes, such as papain, human neutrophil elastase, and porcine pancreatic elastase, is utilized to trigger emphysema formation (10, 12, 13). Even though elastase instillation is not a physiologically relevant initiation event in human emphysema, the persistent emphysematous change in this model is reminiscent of human COPD in that emphysema continues to progress even after cessation of smoking (9). Despite the fact that the activity of administered protease is destroyed by endogenous protease inhibitors within 24 h (14), the degree of emphysema progressively worsens after a single protease instillation, suggesting that host endogenous responses triggered by protease-induced acute injury contribute to progressive emphysema formation.

Basophils are the least common granulocytes, accounting for less than 1% of peripheral blood leukocytes. While they had long been neglected in immunological research, recent studies revealed that basophils play nonredundant roles distinct from those played by mast cells in various immune responses, including allergies,

Significance

Chronic obstructive pulmonary disease has generally been considered a non-Th2-type lung disease, in contrast to allergic asthma. We identified unexpected roles for basophils, Th2 cytokine IL-4, and interstitial macrophages in triggering emphysema formation in spite of the fact that basophils account for less than 1% of cellular infiltrates in the lung. Our observations in a series of genetically engineered mice illustrated that basophil-derived IL-4 acts on lung-infiltrating monocytes to promote their differentiation into interstitial macrophages that in turn produce a pathogenic enzyme, matrix metalloproteinase-12 (MMP-12), resulting in the destruction of alveolar walls and leading to emphysema. Thus, the present study has identified a previously unappreciated role for the basophil-derived IL-4/monocyte–derived interstitial macrophage/MMP-12 axis at the initiation phase of emphysema development in mice.

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protective immunity against parasitic infections, and regulation of other immune cells (15–17). The contribution of basophils to emphysema development has not yet been reported, to our knowledge.

In the present study, we sought to elucidate the cellular and molecular mechanisms underlying emphysema formation at the initiation phase of COPD. It seems practically difficult to trace back the process of emphysema formation once the emphysema pathology has been established, like in COPD patients. To address this issue, we took advantage of a murine model of COPD in that cells and their products involved in the initial phase of emphysema development elicited by elastase instillation can be analyzed in detail. The present study has identified previously unappreciated roles for basophils and interstitial macrophages (IMs) in the development of emphysema in that basophil-derived IL-4 acts on lung-infiltrating monocytes to promote their differentiation into pathogenic matrix metalloproteinase-12 (MMP-12)–producing macrophages in the lung parenchyma but not alveoli, leading to the destruction of alveolar walls.

Results

MMP-12 Contributes to Emphysema Formation in an Elastase-Induced Murine Model of COPD. BALB/c mice treated with a single intranasal instillation of porcine pancreatic elastase [0.6 unit (U)] progressively showed COPD-like lung pathology by day 21 posttreatment, characterized by cellular infiltration and emphysema (Fig. 1*A*). The mean linear intercept (MLI), an indicator of lung airspace enlargement, increased more than twofold by day 21 in elastase-treated mice (Fig. 1*B*). The lungs of elastase-treated mice showed increased compliance (*SI Appendix*, Fig. S1), as observed in COPD patients. Limjunyawong et al. (18) reported that C57BL/6 mice are less sensitive than BALB/c mice in the development of elastase-induced emphysema. In accordance with this, C57BL/6 mice required a higher dose of elastase (0.9 U) to elicit the same extent of emphysema observed in BALB/c mice (*SI Appendix*, Fig. S2 *A–C*).

MMPs, including MMP-12, have been implicated in emphysema formation by means of the enzymatic destruction of alveolar walls in smoke-induced animal models of COPD and COPD patients (19–21). In accordance with this, treatment of BALB/c mice with a selective MMP-12 inhibitor, MMP408 (22), for seven consecutive days starting 2 d after the elastase instillation almost completely abolished emphysema formation (Fig. 1 *C* and *D*). Moreover, MMP-12–deficient mice showed resistance to elastase-elicited emphysema formation (*SI Appendix*, Fig. S3), clearly indicating a crucial role of MMP-12 in the development of emphysema in this murine model of COPD. Indeed, the expression of MMP-12 in the lung significantly increased at the protein level after elastase instillation (*SI Appendix*, Fig. S4*A*).

MMP-12 Is Produced Mainly by Interstitial Rather than Alveolar Macrophages in the Affected Lung. Expression of *Mmp12* but not *Mmp2* or *Mmp9* mRNAs in the lung was progressively and significantly up-regulated until day 5 after elastase instillation (Fig. 1*E* and *SI Appendix*, Fig. S5). Among various types of cells present in the lung on day 5, monocyte-macrophage lineage cells showed the highest levels of *Mmp12* mRNA expression (Fig. 1*F*), and their number increased approximately sevenfold in the lung by day 5 after elastase instillation (Fig. 1*G*). Flow cytometric analysis identified three major subpopulations among monocyte-macrophage lineage cells in the affected lung based on differential expression of F4/80, CD11b, and CD11c (Fig. 1*H* and *SI Appendix*, Fig. S6 *A–C*). Monocytes, IMs, and alveolar macrophages (AMs) were defined as cells expressing F4/80^{low}CD11b^{+}CD11c^{int}, F4/80^{+}CD11b^{+}CD11c^{+}, and F4/80^{+}CD11b^{int}CD11c^{+}, respectively, in this study. AMs but not IMs expressed Siglec-F (*SI Appendix*, Fig. S6*D*), further distinguishing them as separate subpopulations (23, 24). The number of monocytes increased in the lung after elastase instillation, as detected as early as on day 1 postelastase treatment (Fig. 1 *H* and *I*). Of note, although few or no IMs were detected in the lung before elastase instillation, they appeared on day 3 and further accumulated on day 5 (Fig. 1 *H* and *I*). In contrast, the number of AMs remained relatively unchanged (Fig. 1 *H* and *I*).

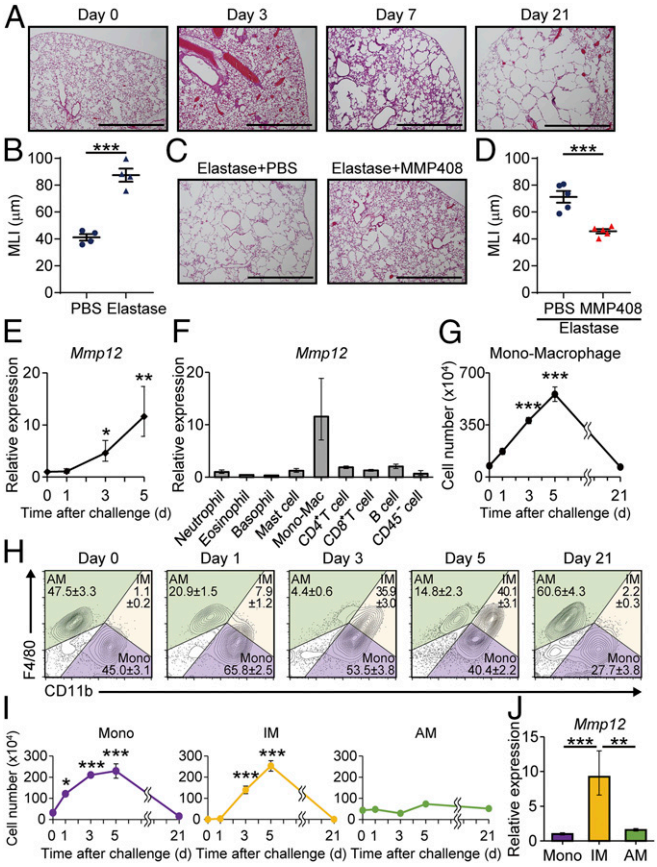

Fig. 1. MMP-12 plays a crucial role in the development of elastase-induced emphysema and is produced mainly by IMs in the lung. BALB/c mice were intranasally treated once with porcine pancreatic elastase to elicit emphysema. (*A*) Hematoxylin/eosin–stained sections of lung samples isolated at the indicated time points after the elastase treatment. (Magnification: 40×.) (Scale bars: 500 μm.) (*B*) The extent of emphysema development on day 21 was evaluated with MLI (mean ± SEM, $n = 4$ each) in lung sections. (*C* and *D*) MMP408, a selective MMP-12 inhibitor, or PBS alone was intragastrically administrated to mice twice a day for seven consecutive days starting 2 d after the elastase instillation. On day 21, lung pathology (*C*) and MLI (*D*; mean ± SEM, $n = 5$ each) were examined as in *A* and *B*. (*E*) Relative expression of *Mmp12* mRNA in the lung at indicated time points after elastase instillation is shown (mean ± SEM, $n = 5$ each), where the value on day 0 is set to 1. (*F*) Relative expression of *Mmp12* mRNA in indicated cell lineages isolated from the lung on day 5 is shown (mean ± SEM, $n = 3$ each), where the value in neutrophils is set as 1. (*G*) Cell numbers of monocyte-macrophage lineages (Ly6G^{-}CD11b$^{int\text{-}high}$CD11c$^{int\text{-}high}$) isolated from the lung at indicated time points are shown (mean ± SEM, $n = 3$–5 each). (*H–J*) Differential expression of F4/80 and CD11b identified three major subsets, namely, monocyte (Mono), interstitial macrophage (IM), and alveolar macrophage (AM), among monocyte-macrophage lineage cells. (*H*) The frequency (percentage) of each subset at indicated time points is shown (mean ± SEM, $n = 3$ each). (*I*) The time course of cell number in each subset is shown (mean ± SEM, $n = 5$ each). (*J*) The relative expression of *Mmp12* mRNA in each subset isolated on day 5 is shown (mean ± SEM, $n = 4$ each), where the value in Mono is set to 1. Data shown are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, measured by an unpaired Student's *t* test or one-way ANOVA with a post hoc Tukey HSD test.

It has been generally thought that AMs are the major producer of MMP-12 in the affected lung in COPD patients and animal models (25–27). Nevertheless, we found that IMs displayed the

highest levels of *Mmp12* mRNA expression on day 5 postelastase treatment among the three subpopulations of the monocyte–macrophage lineage (Fig. 1*J*). Expression of *Mmp12* mRNAs in the lung was up-regulated in parallel with the accumulation of IMs in the lung (Fig. 1 *E* and *I*). In accordance with this, the immunohistochemical examination of the lung sections revealed an increased number of MMP-12–expressing macrophages in the interstitium of the affected lung (*SI Appendix*, Fig. S4*B*). Thus, IMs rather than AMs appeared to be the major producer of MMP-12 in the affected lung and therefore to contribute to emphysema formation, in contrast to the prevailing assumption.

Monocytes Migrate to the Lung and Differentiate into IMs, Leading to Emphysema Formation. The emergence and accumulation of monocytes and IMs in a sequential order (Fig. 1 *H* and *I*) prompted us to examine the possibility that monocytes recruited from the peripheral blood to the lung may differentiate into MMP-12^{+} IMs. To this end, we analyzed mice deficient for the chemokine receptor CCR2, which exhibit defective monocyte recruitment to peripheral tissues during immune responses (28). As expected, monocyte accumulation in the lung after elastase instillation was barely detected in CCR2-deficient mice unlike in WT mice (Fig. 2*A* and *SI Appendix*, Fig. S7). CCR2-deficient mice also showed little or no appearance of IMs in the lung, in contrast to WT mice, whereas no significant difference was observed in the number of AMs between these two mouse strains (Fig. 2*A*). Of note, CCR2-deficient mice were resistant to elastase-elicited emphysema formation (Fig. 2 *B* and *C*). Expression of *Mmp12* mRNAs in the lung was significantly lower in CCR2-deficient mice than in WT mice (Fig. 2*D*). These results strongly suggested that monocytes recruited to the lung differentiated to IMs but not AMs, leading to MMP-12–mediated emphysema formation. Consistent with this assumption, when monocytes were isolated from WT mice and i.v. transferred to CCR2-deficient mice before elastase instillation, they migrated to the lung and then differentiated into IMs (Fig. 2*E*). In contrast, monocytes derived from CCR2-deficient mice failed to migrate to the lung (*SI Appendix*, Fig. S8*A*), whereas the number of transferred cells detected in the spleen was comparable, irrespective of the donor mice (*SI Appendix*, Fig. S8*B*). Importantly, adoptive transfer of WT but not CCR2-deficient monocytes reconstituted emphysema in CCR2-deficient mice (Fig. 2 *F* and *G*). These results clearly demonstrated that monocytes recruited to the lung can differentiate into IMs but rarely AMs and contribute to the development of emphysema.

IL-4 Is Involved in the Generation of IMs and the Development of Emphysema. We next compared the profile of gene expression between monocyte-derived IMs and lung-resident AMs isolated from the affected lung (Fig. 3*A* and *SI Appendix*, Fig. S9*A*). Both cell types expressed comparable levels of a macrophage marker *Mertk*, while IMs expressed higher levels of the chemokine receptor *Cx3cr1* compared with AMs (*SI Appendix*, Fig. S9*A*), as reported previously (24). Besides *Mmp12* mRNA expression (Fig. 1*J*), markers of M2 macrophages such as *Arg1* and *Fizz1* were more highly expressed in IMs than in AMs (Fig. 3*A*). This suggested the possible involvement of Th2 cytokines in the generation of IMs. Indeed, the expression of *Il4* but not *Il10*, *Il13*, or *Tgfb* mRNAs was significantly up-regulated in the lung after elastase instillation (Fig. 3*B* and *SI Appendix*, Fig. S9*B*), prompting us to analyze the IM generation and emphysema formation in the absence of IL-4. IL-4–deficient mice showed highly diminished emphysema (Fig. 3 *C* and *D*) in conjunction with poorer generation of IMs and reduced *Mmp12* expression in the lung compared with WT mice (Fig. 3 *E* and *F*). These results suggested that IL-4 plays a key role in the generation of MMP-12–producing IMs and therefore contributes to the development of emphysema.

Basophils Are the Major Producer of IL-4 in the Affected Lung and Crucial for IM Generation and Emphysema Formation. We then examined the cellular source of IL-4 in the affected lung. Expression of *Il4* mRNAs was detected predominantly in basophils among cell types analyzed on day 5 postelastase instillation in both BALB/c and C57BL/6 mice (Fig. 4*A* and *SI Appendix*, Fig. S2*E*). Flow cytometric analysis using IL-4 reporter G4 mice confirmed this observation (*SI Appendix*, Fig. S2*F*), demonstrating that basophils are the major producer of IL-4. After elastase instillation, basophils accumulated in the lung with a peak on day 5 in both BALB/c and C57BL/6 mice (Fig. 4*B* and *SI Appendix*, Fig. S2*D*), even though they accounted for only 0.3% of hematopoietic cells isolated from the affected lung. To examine whether basophils indeed play an important role in IL-4 production, generation of MMP-12^{+} IMs, and hence emphysema formation, we used two distinct methods to deplete basophils in vivo, namely, diphtheria toxin (DT) treatment of *Mcpt8*^{DTR} mice (29) and anti-CD200R3 antibody (Ba103) treatment of WT mice (30, 31). Treatment of *Mcpt8*^{DTR} mice with a single administration of DT but not its inactive mutant (mDT) 1 d before elastase instillation ablated the vast majority of basophils in the lung for 5 d after the instillation (*SI Appendix*, Fig. S10). *Mcpt8*^{DTR} mice treated with DT but not mDT were resistant to emphysema formation, while WT mice treated with DT normally developed emphysema (Fig. 4 *C* and *D*). DT-mediated basophil depletion resulted in diminished *Il4* expression in the affected lung (Fig. 4*E*) as well as impaired generation of IMs (Fig. 4*F*) and reduced *Mmp12* expression (Fig. 4*E*) as observed in IL-4–deficient mice (Fig. 3 *E* and *F*). Impaired emphysema formation was also observed in mice treated with a basophil-depleting anti-CD200R3 antibody (Ba103), as shown in *SI Appendix*, Fig. S11, confirming the important role of basophils in emphysema formation. Taken together, the basophil-derived IL-4/monocyte–derived IM/MMP-12 axis appears to play a crucial role in the development of elastase-elicited emphysema formation in that basophil-derived IL-4 acts on lung-infiltrating monocytes to promote their differentiation into pathogenic MMP-12–producing IMs.

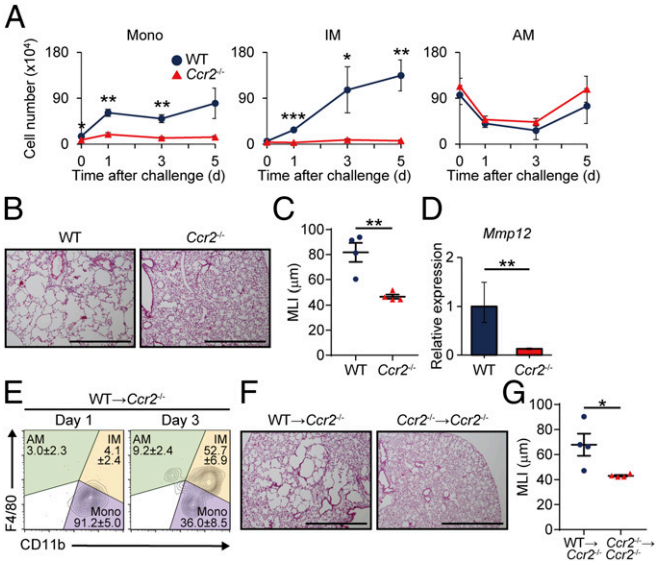

Fig. 2. Monocytes migrate to the lung and differentiate into IMs, leading to emphysema formation. (*A–D*) WT or *Ccr2*$^{-/-}$ BALB/c mice were treated with elastase to elicit emphysema. (*A*) Time course of cell number in each subset is shown (mean ± SEM, $n = 3$ each). (*B* and *C*) On day 21, lung pathology (*B*) and MLI (*C*; mean ± SEM, $n = 4$ each) were examined as in Fig. 1. (Magnification: 40×.) (Scale bars: 500 μm.) (*D*) Relative expression of *Mmp12* mRNA in the lung isolated on day 5 is shown (mean ± SEM, $n = 3$ each), where the value in WT mice is set to 1. (*E–G*) Ly6C^{high} inflammatory monocytes isolated from the bone marrow of WT or *Ccr2*$^{-/-}$ mice were labeled with CellTrace Violet and adoptively transferred to *Ccr2*$^{-/-}$ mice 1 d before elastase instillation. The frequency (percentage) of CellTrace Violet-positive Mono, IM, and AM in the lung at indicated time points is shown in *E* (mean ± SEM, $n = 3$ each). On day 21, lung pathology (*F*) and MLI (*G*; mean ± SEM, $n = 4$ each) were examined. (Magnification: 40×.) (Scale bars: 500 μm.) Data shown are representative of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, measured by an unpaired Student's *t* test.

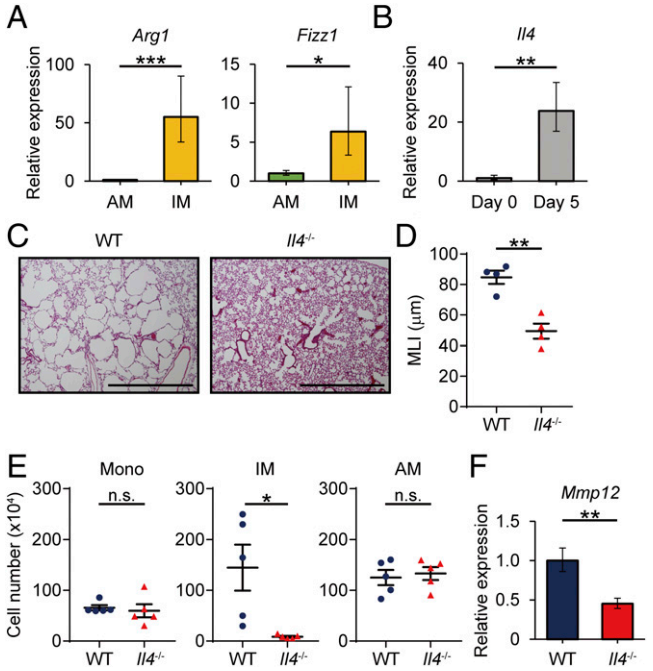

Fig. 3. IL-4 is involved in the generation of IMs and emphysema formation. (*A* and *B*) BALB/c mice were treated with elastase to elicit emphysema. (*A*) Relative expression of indicated mRNAs in AM and IM isolated from the lung on day 5 is shown (mean ± SEM, $n = 4$ each), where the value in AM is set to 1. (*B*) Relative expression of *Il4* mRNAs in the lung isolated on day 0 and day 5 is shown (mean ± SEM, $n = 5$), where the value on day 0 is set to 1. (*C*–*F*) WT or $Il4^{-/-}$ C57BL/6 mice were treated with elastase to elicit emphysema. On day 21, lung pathology (*C*) and MLI (*D*; mean ± SEM, $n = 4$ each) were examined. (Magnification: 40×.) (Scale bars: 500 μm.) Cell numbers of Mono, IM, and AM in the lung isolated on day 5 are shown in *E* (mean ± SEM, $n = 5$ each). Relative expression of *Mmp12* mRNA in the lung isolated on day 5 is shown in *F* (mean ± SEM, $n = 5$ each), where the value in WT mice is set to 1. Data are representative of at least three independent experiments. n.s., not significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, measured by an unpaired Student's *t* test.

Mice Deficient for IL-4 Only in Basophils Fail to Develop Emphysema. To directly assess the role of basophil-derived IL-4 in emphysema formation, we established $Mcpt8^{iCre/+}Il4^{fl/fl}$ mice that are deficient for IL-4 only in basophils by crossing two newly generated mouse strains, $Mcpt8^{iCre/+}$ and $Il4^{fl/fl}$ mice (*SI Appendix*, Fig. S12 *A* and *C*). When $Mcpt8^{iCre/+}$ mice were crossed with Rosa26-loxP-Stop-loxP-YFP (R26-YFP) reporter mice, the Cre recombinase activity was detected as YFP expression in ∼90% of basophils, ∼15% of eosinophils, and ∼8% of neutrophils, while it was negligible in other cell lineages examined, confirming the predominant expression of iCre in basophils (*SI Appendix*, Fig. S12*B*). When stimulated in vitro, basophils isolated from $Mcpt8^{iCre/+}Il4^{fl/fl}$ mice failed to produce IL-4, whereas IL-6 production remained intact, demonstrating an IL-4–specific defect in basophils of these mice (*SI Appendix*, Fig. S12*D*). Of note, when treated with elastase, $Mcpt8^{iCre/+}Il4^{fl/fl}$ but not control $Mcpt8^{iCre/+}$ mice were resistant to emphysema formation (Fig. 5 *A* and *B*), in parallel with impaired IM generation (Fig. 5*C*) and reduced expression of *Mmp12* in the lung (Fig. 5*D*). These results clearly demonstrated that basophil-derived IL-4 plays a crucial role in the generation of MMP-12–producing IMs and the development of emphysema.

Discussion

Previous studies reported that basophils play important roles in Th2-type immune responses, including protective immunity against parasitic infections and allergic inflammation such as atopic dermatitis and asthma (15–17). Even though COPD has been generally considered a non-Th2-type lung disease, we here identified an unexpected and crucial role for basophils and Th2 cytokine IL-4 in triggering emphysema formation at the initiation phase of COPD, despite the fact that basophils account for less than 1% of cellular infiltrates in the affected lung. Basophil-derived IL-4 acts on lung-infiltrating monocytes to promote their differentiation into IMs that in turn produce a pathogenic enzyme, MMP-12, resulting in the destruction of alveolar walls leading to emphysema.

Previous reports suggested that the main producers of MMP-12 are AMs in the lungs of COPD patients and animal models (25–27). Recent studies have shown that the lung contains another subset of macrophages called IMs which reside in the lung parenchyma, unlike AMs present in the luminal side of lung alveoli (24, 32, 33). Owing to their anatomical localization, IMs are much less accessible than AMs, and therefore, IMs might have been missed by previous studies, particularly those using bronchoalveolar lavage. In the present study, we found that monocyte-derived IMs rather than lung-resident AMs are the major producers of MMP-12 in the mouse model. Adoptive transfer experiments clearly demonstrated that monocytes recruited to the lung differentiated into MMP-12–producing IMs but rarely AMs at the early stage of disease process, initiating emphysema formation. It was reported in the elastase-induced COPD model that inhalation of alendronate, a nitrogen-containing bisphosphonate, induces apoptosis of macrophages in the lung, resulting in ameliorated emphysema formation (34). Notably, uptake of alendronate in the lung was predominantly detected in $F4/80^{+}CD11b^{high}CD11c^{low}$ rather than $F4/80^{+}CD11b^{low}CD11c^{high}$ macrophages. As the former cells appear to phenotypically overlap with IMs defined in our study, those observations further support our conclusion that IMs play a crucial

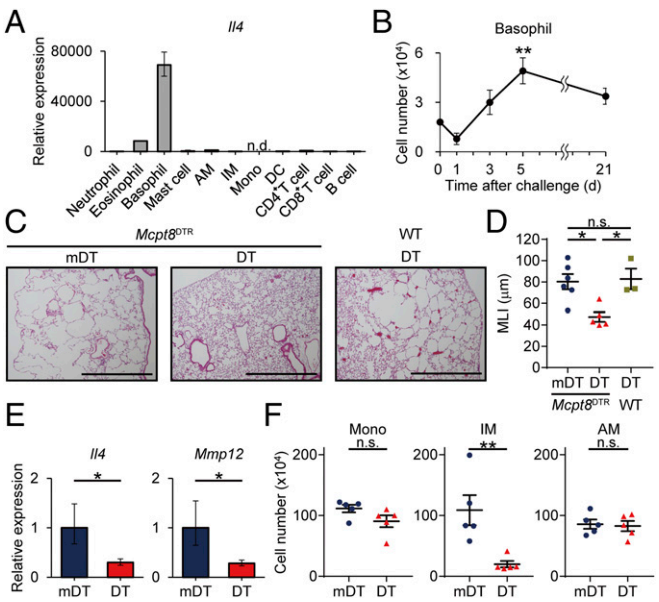

Fig. 4. Basophils are the major producer of IL-4 in the affected lung and crucial for IM generation and emphysema formation. (*A*) BALB/c mice were treated with elastase to elicit emphysema. Relative expressions of *Il4* mRNA in indicated cell lineages isolated from the lung on day 5 are shown (mean ± SEM, $n = 3$ each), where the value in neutrophils is set to 1. n.d., not detectable. (*B*) Cell numbers of basophils isolated from the lung of elastase-treated BALB/c mice at indicated time points are shown (mean ± SEM, $n = 3$–5 each). (*C*–*F*) WT or $Mcpt8^{DTR}$ BALB/c mice were pretreated with DT or inactive mDT 1 d before elastase instillation. On day 21, lung pathology (*C*) and MLI (*D*; mean ± SEM, $n = 3$–6 each) were examined. (Magnification: 40×.) (Scale bars: 500 μm.) Relative expression of *Il4* and *Mmp12* mRNAs in the lung isolated on day 5 is shown in *E* (mean ± SEM, $n = 4$ each), where the value in mDT-treated mice is set to 1. Cell numbers of Mono, IM, and AM in the lung isolated on day 5 are shown in *F* (mean ± SEM, $n = 5$ each). Data shown are representative of at least three independent experiments. n.s., not significant, $^*P < 0.05$, $^{**}P < 0.01$, measured by an unpaired Student's *t* test or one-way ANOVA with post hoc Tukey HSD test.

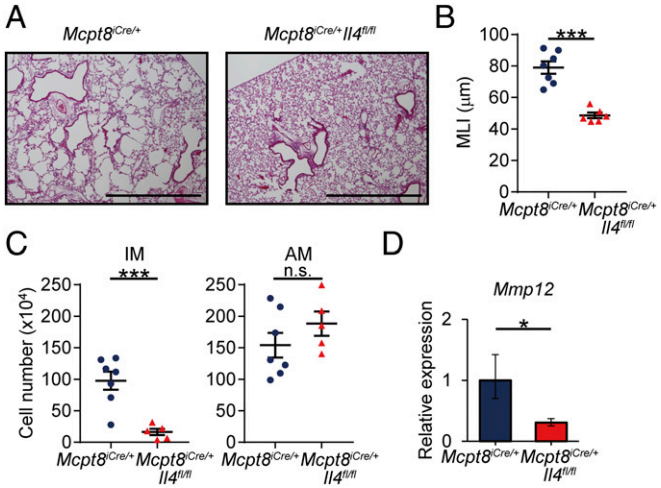

Fig. 5. Mice deficient for IL-4 only in basophils fail to develop emphysema. *Mcpt8*$^{iCre/+}$*Il4*$^{fl/fl}$ and control *Mcpt8*$^{iCre/+}$ mice were treated with elastase (0.9 U) to elicit emphysema. Lung pathology (*A*) and MLI (*B*; mean ± SEM, $n = 6$–7) were examined on day 21. (Magnification: 40×.) (Scale bars: 500 μm.) Cell numbers of IM and AM in the lung isolated on day 5 are shown in *C* (mean ± SEM, $n = 5$–7 each). Relative expression of *Mmp12* mRNAs in the lung isolated on day 5 is shown in *D* (mean ± SEM, $n = 5$–7 each), where the value in *Mcpt8*$^{iCre/+}$ mice is set to 1. Data are representative of at least three independent experiments. n.s., not significant, $*P < 0.05$, $***P < 0.001$, measured by an unpaired Student's *t* test.

role in emphysema development. Moreover, cigarette smoke reportedly increased the accumulation of CX3CR1^{+}CD11b^{+} mononuclear phagocytes that are spatially confined to the lung interstitium, and mice deficient for CX3CR1 showed protection from emphysema formation (35), suggesting the important role of IMs in smoke-induced COPD as well. Intriguingly, gene expression profiling in lung samples isolated from COPD patients revealed that IMs display a higher proinflammatory signature than AMs (36), suggesting the possible role of IMs rather than AMs in the pathogenesis of human COPD as identified in the mouse model.

It has been suggested in the elastase-induced COPD model that only 20% of parenchymal tissue loss after elastase administration is caused directly by the enzyme, while the remaining 80% results from the host inflammatory response (37). Based on our observations in the present study, we assume that the basophil-derived IL-4/monocyte–derived IM/MMP-12 axis plays a key role in this host inflammatory response leading to emphysema formation. Even though the exact mechanism underlying basophil activation in vivo remains to be clarified, our experiments in vitro suggested that elastase released from neutrophils recruited to the lung at the very early phase stimulates basophils to secrete IL-4, which in turn promotes the generation of pathogenic MMP-12–producing IMs (*SI Appendix*, Fig. S13 *A* and *B*). It has been reported that elastin fragments created by elastolytic enzymes such as MMP-12 are chemotactic for monocytes (38). This may drive a vicious cycle in the progression of emphysema formation, where monocyte-derived IMs produce MMP-12, which in turn degrades elastin in alveolar walls to release elastin fragments, leading to recruitment of more monocytes as a source of MMP-12–producing IMs.

The infiltration of basophils in the affected lung has not been reported yet in smoke-induced animal models of COPD as far as we could determine. Notably, immunohistochemical analysis of lung tissue sections prepared from COPD patients detected focal infiltration of basophils (39), even though its functional significance remains to be investigated. Human basophils have been shown to lack the expression of protease-activated receptors and hence do not secrete IL-4 when incubated with proteases such as trypsin and thrombin (40). We found that human basophils secreted IL-4 in response to stimulation with human neutrophil elastase, and human basophil-derived IL-4 acted on human monocyte-derived macrophages to up-regulate *MMP12* expression (*SI Appendix*, Fig. S13 *C* and *D*). Single-nucleotide polymorphisms in the *MMP12* gene are reportedly associated with an increased or reduced risk of COPD in adult smoker cohorts (41–43). Taken together, the basophil-derived IL-4/monocyte–derived IM/MMP-12 axis identified in the mouse model could be involved in the pathogenesis of human emphysema as well. To further address this issue, it might be necessary to analyze lung samples isolated from individuals at the very early but not late stage of COPD, even before the clinical diagnosis of COPD. We found in the elastase-induced COPD model that basophil depletion soon after the elastase instillation impaired emphysema formation, whereas that on day 3 or later failed to do so (*SI Appendix*, Fig. S14), indicating the role of basophils at the very early but not late stage of emphysema formation.

In conclusion, we demonstrated here previously unappreciated roles for basophils, IL-4, and IMs in the development of emphysema that have generally been considered a consequence of a non-Th2-type response. IL-4 secreted by lung-infiltrating basophils promotes the differentiation of lung-infiltrating monocytes into pathogenic MMP-12–producing IMs, leading to emphysema formation. Basophils may have been overlooked or underestimated in previous studies on emphysema because of their small number in the affected lung. Our findings also emphasized that the analysis of IMs in addition to AMs is important for studies on lung disorders, even though IMs are less accessible. Further studies of the basophil–IM–MMP-12 axis may help develop novel therapeutics to slow emphysema progression.

Materials and Methods

Further details are available in *SI Appendix, SI Materials and Methods*.

Mice. BALB/c and C57BL/6 mice were purchased from Sankyo Laboratory Service Corporation. *Mmp12*$^{-/-}$ C57BL/6 mice (44) and Rosa26-loxP-Stop-loxP-YFP reporter C57BL/6 mice (45) were purchased from The Jackson Laboratory. *Mcpt8*^{DTR} BALB/c mice (29), *Ccr2*$^{-/-}$ BALB/c mice [provided by N. Mukaida (Kanazawa University, Kanazawa, Japan) and W. A. Kuziel (Daiichi Sankyo Group, Edison, NJ)] (28), G4 C57BL/6 mice (46), and *Il4*$^{-/-}$ C57BL/6 mice (47) were as described previously. Mice were maintained under specific pathogen-free conditions in our animal facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University (No. A2017263).

Human Subjects. All human studies conformed to the Declaration of Helsinki and were approved by the ethical committee on human research of the Tokyo Medical and Dental University (No. M2015-559). Written informed consent was obtained from blood donors.

Elastase-Induced Murine Model of COPD. Female mice at the age of 6–10 wk were intranasally treated with 0.6 U (in BALB/c) or 0.9 U (in C57BL/6) porcine pancreatic elastase (Sigma-Aldrich) in 30 μL of PBS (34) or PBS alone under anesthesia with ketamine (Daiichi Sankyo) and xylazine (Bayer Health Care). In some experiments, mice were intragastrically treated with a selective MMP-12 inhibitor, MMP408 (100 μg each in 200 μL of PBS; Calbiochem) (22, 48), or PBS alone twice a day for seven consecutive days starting 2 d after the elastase instillation.

Flow Cytometric Analysis and Cell Sorting. Lungs were perfused with PBS-containing EDTA through the right ventricle, and isolated lungs were minced with scissors, followed by incubation for 1 h in RPMI medium 1640 (Nacalai Tesque) containing collagenase type I (1 mg/mL; Sigma-Aldrich) and DNase I type II (60 U/mL; Sigma-Aldrich) at 37 °C under agitation conditions (23). Digested lung tissues were passed through wire mesh to obtain a single-cell suspension. After lysis of red blood cells, cells were incubated with anti-CD16/32 mAb (2.4G2) and normal rat serum for 30 min to prevent the nonspecific binding of antibodies. Cells were then stained with indicated combination of antibodies and analyzed with FACS Canto II (BD Biosciences). Dead cells were excluded by staining with propidium iodide. Each cell lineage was defined as follows: T cells (CD3ε^{+}), B cells (CD19^{+}), neutrophils (Ly6G^{high}), eosinophils (Ly6G^{low}Siglec-F^{+}FSC^{low}SSC^{high}), basophils (CD45^{int}CD49b^{+}CD200R3^{+}), mast cells (c-kit^{+}CD200R3^{+}), alveolar macrophages (F4/80^{+}CD11b^{int}CD11c^{+}), interstitial macrophages (F4/80^{+}CD11b^{+}CD11c^{+}), monocytes (F4/80^{low}CD11b^{+}CD11c^{int}), and dendritic

cells ($CD11c^+F4/80^{low}$MHC class II^+). Data were analyzed with FlowJo software (TreeStar). For monocyte transfer experiments, inflammatory monocytes were isolated from bone marrow cells by sorting $CD11b^+Ly6C^{high}Ly6G^-$ cells with FACS Aria II (BD Biosciences), labeled with CellTrace Violet (Life Technologies), and adoptively transferred to recipient mice (2×10^6 cells per mouse).

Ablation of Basophils in Mice. For the DT-mediated basophil depletion, $Mcpt8^{DTR}$ mice were i.v. treated with DT (750 ng in 150 μL of PBS; Sigma-Aldrich) or its inactive mutant [Glu^{52}]-DT (mDT, 750 ng in 150 μL of PBS; Sigma-Aldrich) 1 d before the elastase instillation (29). For the antibody-mediated depletion, mice were intraperitoneally treated with rat anti-CD200R3 antibody (Clone, Ba103; 50 μg in 100 μL PBS) (30, 31) or control rat IgG (50 μg in 100 μL PBS; Jackson ImmunoResearch) 1 d before the elastase instillation.

Statistical Analysis. All statistical analyses were performed with the GraphPad Prism (version 7.04; GraphPad Software). A P value of less than 0.05 was considered statistically significant. Comparisons between two groups were performed using an unpaired Student's t test. Comparisons between multiple treatment groups and a control group were performed using one-way ANOVA with a post hoc Tukey honestly significant difference (HSD) test.

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