Critical role of caspase-8-mediated IL-1 signaling in promoting Th2 responses during asthma pathogenesis

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Allergic asthma is a chronic inflammatory disorder of the airways that affects >300 million people worldwide. The pro-inflammatory cytokines interleukin (IL)-1α and IL-1β have essential roles in the pathogenesis of asthma. However, the mechanisms underlying the production of IL-1 cytokines in allergic asthma remain unclear. In this study, we used a mouse model of ovalbumin-induced asthma to identify a crucial role for caspase-8 in the development of allergic airway inflammation. We further demonstrated that hematopoietic cells have dominant roles in caspase-8-mediated allergic airway inflammation. Caspase-8 was required for the production of IL-1 cytokines to promote Th2 immune response, which promotes the development of pulmonary eosinophilia and inflammation. Thus, our study identifies caspase-8 as a master regulator of IL-1 cytokines that contribute to the pathogenesis of asthma and implicates caspase-8 inhibition as a potential therapeutic strategy for asthmatic patients.

INTRODUCTION

Allergic asthma is a heterogeneous, chronic inflammatory disease characterized by hyperresponsiveness, smooth muscle hypertrophy, obstruction, and infiltration of inflammatory cells in the airway. T lymphocytes, particularly T-helper type 2 (Th2) cells and Th2-type cytokines, such as interleukin (IL)-4, IL-5, and IL-13, are immunological signatures of allergic immune responses to inhaled antigens.[1,2] Accumulating evidence points to the involvement of pro-inflammatory cytokines within the IL-1 family, specifically IL-1α and IL-1β, in the development of pulmonary Th2 immune responses and asthma.[3–5] Patients with asthma often have excessive production of IL-1 cytokines.[6] In addition, administration of active human IL-1β to the mouse trachea induces airway inflammation and tissue remodeling.[7] The involvement of IL-1 in asthmatic diseases is further supported by the observation that genetic disruption of the IL-1 signaling pathway strongly attenuates allergic disease in murine models of asthma.[4,8] Moreover, recent studies revealed that IL-1 cytokines secreted in response to inhaled allergens can instruct pulmonary dendritic cells to induce Th2 responses and directly promote the activation of Th2 cells.[9,10] Collectively, results from these studies suggest that IL-1 is one of the apical signals of the cytokine cascade that drives dendritic cell activation and Th2 immunity in response to inhaled allergens. Despite important advances in understanding the crucial role of IL-1 cytokines in the genesis of allergic asthma, the mechanism of IL-1 production that contributes to inflammatory airway disease remains unknown.

Certain members of the IL-1 family, including IL-1β and IL-18, require proteolytic processing in order to exert their full biological potential.[11,12] These cytokines are generated as inactive pro-forms, cleaved by the cysteine protease caspase-1 within the inflammasome, and are released as bioactive forms by the cell.[13] The nucleotide-binding domain, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3) inflammasome respond to a repertoire of activators and have been implicated in the development of asthma.[14–17] Increased expression of components of the NLRP3 inflammasome has been found in the sputum of patients with asthma compared with healthy controls.[14] However, the precise role of the NLRP3

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inflammasome in pathogenesis of allergic asthma is controversial.[15] Previous studies suggest that *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Caspase-1/11*$^{-/-}$ mice have markedly reduced airway inflammation and expression of pro-inflammatory cytokines compared with wild-type (WT) mice in a model of ovalbumin (OVA)-induced allergic asthma.[16,17] In contrast, others found that the NLRP3 inflammasome or caspase-1/11 does not substantially contribute to either OVA-mediated or house dust mite-mediated allergic asthma.[18–20]

Recently, several studies highlighted novel roles for caspase-8 in the processing of IL-1β and regulating inflammation beyond its role in cell death.[21] Caspase-8 is required for IL-1β production in response to fungal and bacterial infection, and dysregulation of caspase-8 has been associated with cancer and autoinflammatory diseases.[22–26] However, the role of caspase-8 in a chronic inflammatory lung disease is poorly understood.

In this study, we identified that caspase-8 mediates IL-1α and IL-1β production and has a key role in the development of allergic lung inflammation in mice, via a mechanism independent of caspase-1 and 11. We found that caspase-8-mediated IL-1 signaling promotes Th2 immune responses, which contributes to the development of pulmonary eosinophilia and inflammation.

RESULTS

Caspase-8 and Fas-associated protein with death domain have pivotal roles in the OVA-induced pulmonary inflammatory response

Dysregulation of the pro-inflammatory cytokines IL-1α and IL-1β is associated with inflammatory and autoimmune diseases.[27] Caspase-1/-11, cathepsin G, elastase, and proteinase-3 function at the intracellular or extracellular level to process IL-1.[12] We used an OVA/alum-sensitized and OVA-challenged mouse model to analyze the role of these proteinases in promoting infiltration of inflammatory cells into the lungs of mice. The pulmonary infiltration of total inflammatory cells, eosinophils, and neutrophils was comparable between WT mice and mice doubly deficient in caspases-1 and -11 (*Casp1/11*$^{-/-}$), elastase (*Elastase*$^{-/-}$), elastase and proteinase-3 (*Nepr3*$^{-/-}$), and cathepsin G (*Ctsg*$^{-/-}$; **Supplementary Figure S1** online), suggesting that the caspase-1/-11 and the proteinases examined were dispensable for the OVA-induced allergic pulmonary inflammatory response.

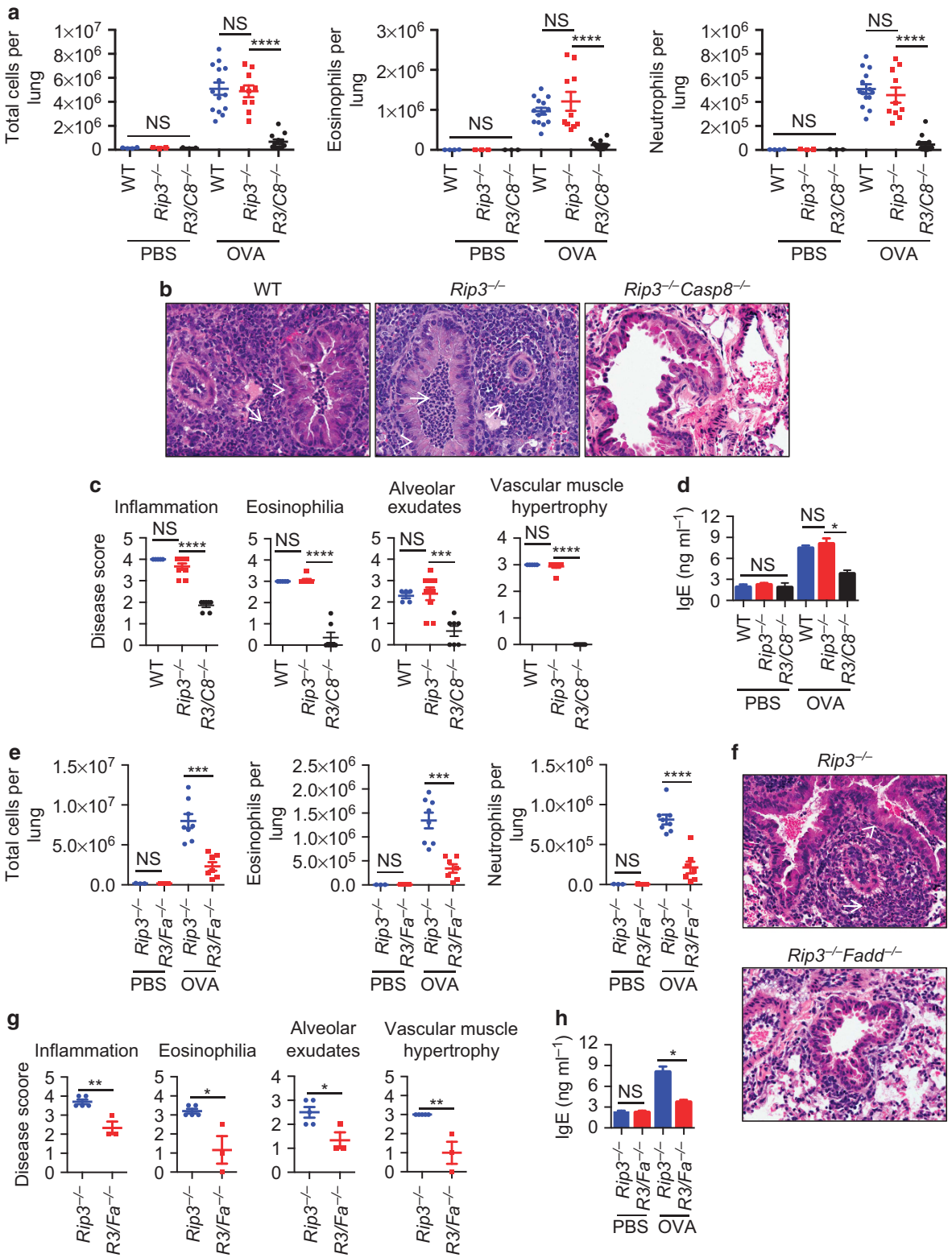
A previous study suggested that inhibition of caspase activity with the use of a general caspase inhibitor can reduce airway inflammation.[28] In addition to caspase-1/-11, caspase-8 is essential for priming and processing of pro-IL-1β.[22,26,29] The embryonic lethality of caspase-8-deficient mice can be rescued by deleting receptor interacting protein kinase-3 (RIP3).[30,31] To determine whether caspase-8 has a role in inflammation during asthma, WT mice and mice deficient in RIP3 (*Rip3*$^{-/-}$) or RIP3 and caspase-8 (*Rip3*$^{-/-}$*Casp8*$^{-/-}$) were sensitized with OVA/alum and challenged with OVA. Genetic deletion of the gene encoding RIP3 did not substantially affect OVA-induced allergic pulmonary inflammation compared with WT mice, whereas *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice had significantly reduced inflammatory cell infiltration in the lung. Markers of asthmatic lung inflammation, such as the numbers of total immune cells, eosinophils, and neutrophils, in the lung of *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice were reduced by 7.5-, 8-, and 11-fold, respectively, compared with those of WT mice (**Figure 1a**). Histopathological analysis of OVA-treated lung tissues showed remarkable peribronchial and perivascular infiltration of eosinophils, alveolar exudates, and vascular muscle hypertrophy in WT and *Rip3*$^{-/-}$ mice but not in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice (**Figure 1b** and **c**). In line with these data, the level of allergy marker IgE was also significantly reduced in the absence of caspase-8 (**Figure 1d**). These results altogether suggested that caspase-8 promoted inflammatory responses in the lung during OVA-induced asthma.

Fas-associated protein with death domain (FADD) is a signaling adapter protein for death receptor that has an important role in apoptosis and necroptosis by recruiting and activating caspase-8. Genomic deletion of FADD is lethal, an effect that can be rescued by genetic deletion of RIP3.[32] Thus, we analyzed the involvement of FADD in OVA-induced allergic pulmonary inflammation response. Consistent with the data from *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice, *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice had substantially reduced pulmonary infiltration of total immune cells, eosinophils, and neutrophils compared with *Rip3*$^{-/-}$ mice (**Figure 1e**). Furthermore, histopathological and enzyme-linked immunosorbent assay (ELISA) analysis revealed that *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice had decreased inflammation, eosinophilia, alveolar exudates, vascular muscle hypertrophy, and IgE production compared with *Rip3*$^{-/-}$ mice (**Figure 1f–h**). The reduced disease burden in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice suggest that caspase-8 and FADD possibly function in the same complex to drive manifestation of allergic asthma.

Differentiation of Th2 cells is impaired in the absence of caspase-8

An exorbitant Th2 immune response and Th2-type cytokines, such as IL-4, IL-5, and IL-13, contribute to the pathogenesis of allergic asthma.[1] Interferon (IFN)-γ and IL-17 also act in conjunction with Th2-type cytokines to maintain allergic airway inflammation.[3] To further investigate the precise role of caspase-8 in OVA-induced Th2 immune responses, we analyzed populations of T cells producing IL-4, IL-17, or IFN-γ in OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. Compared with WT mice, the percentage of CD4^{+} T cells producing IL-4, IFN-γ, or IL-17 did not change substantially in *Rip3*$^{-/-}$ mice (**Figure 2a,b**). In contrast, the percentage of CD4^{+} T cells producing IL-4 was significantly reduced in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice (2.94 ± 0.21% vs. 1.18 ± 0.17%), whereas the percentage of CD4^{+} T cells producing IFN-γ was higher than that in WT and *Rip3*$^{-/-}$ mice (**Figure 2a,b**). The percentage of CD4^{+} T cells producing IL-17 and the percentage of CD8^{+} T cells producing IFN-γ in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice were similar to that in WT and *Rip3*$^{-/-}$ mice (**Figure 2a,b**). Notably, the total number of T cells producing IL-4, IL-17, and IFN-γ was substantially lower in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice than in WT and *Rip3*$^{-/-}$ mice (**Figure 2c**),

suggesting that it is a consequence of less inflammatory cell infiltrates in the lung of protected *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. Furthermore, quantitative reverse transcription PCR and ELISA analysis revealed that the gene expression and production of critical Th2-type cytokines IL-4, IL-5, and IL-13, but not of IFN-γ, were markedly lower in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice than in WT and *Rip3*$^{-/-}$ mice (**Figure 2d,e**). Consistently, the production of IL-4, IL-5, and IL-13 was significantly reduced in the absence of FADD (**Supplementary Figure S2a**), but not in the mice deficient in caspase-1/-11,



elastase, Nepr3, or cathepsin G (**Supplementary Figure S2b**). These results suggest that caspase-8 and FADD have critical roles in the differentiation and activation of Th2 cells, which contribute to OVA-induced allergic airway inflammation.

Hematopoietic cells have crucial roles in caspase-8-mediated allergic airway inflammation

To determine the cell type that contributed to preventing the progression of OVA-induced allergic airway inflammation in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice, we generated a series of bone marrow chimeric mice. *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice were lethally irradiated and received donor *Rip3*$^{-/-}$ or *Rip3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow cells for hematopoietic reconstitution. Eight weeks later, the reconstitued mice were sensitized with OVA/alum and challenged with OVA. Notably, the level of inflammatory cell infiltration was significantly higher in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice that received *Rip3*$^{-/-}$ bone marrow cells compared with *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice that received *Rip3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow cells (**Figure 3a**). Moreover, the level of inflammatory cell infiltration was lower in *Rip3*$^{-/-}$ mice that received *Rip3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow cells compared with *Rip3*$^{-/-}$ mice that received *Rip3*$^{-/-}$ bone marrow cells (**Figure 3a**). These analyses suggested that caspase-8 in the hematopoietic compartment contributed to the development of OVA-induced allergic airway disease. We also noticed that *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice that received *Rip3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow cells had less inflammatory infiltrates in the lungs compared with *Rip3*$^{-/-}$ mice that received *Rip3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow cells. This finding argued that caspase-8 in the radioresistant compartment may, in part, contribute to the development of the disease; however, caspase-8 has a more dominant role in the hematopoietic cells in the development of allergic airway inflammation.

In line with the inflammatory cell infiltration data, we found that transfer of *Rip3*$^{-/-}$ bone marrow cells to *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice restored the number of CD4$^+$ T cells producing IL-4 in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice to normal level (**Figure 3b**). By contrast, transfer of *Rip3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow cells to *Rip3*$^{-/-}$ mice markedly reduced the capacity of CD4$^+$ T cells producing IL-4 (**Figure 3b**). These results indicated that the hematopoietic compartment of *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice has a major role in preventing the development of the OVA-induced Th2 immune response.

To examine how caspase-8 controlled the Th2 immune response in OVA-induced allergic asthma, we assessed whether caspase-8 had a T-cell intrinsic role in controlling its polarization. To this end, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ naive CD4$^+$ T cells were subjected to an *in vitro* T-helper cell polarization assay. The capacity of naive CD4$^+$ T cells isolated from *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice to differentiate into Th1 and Th2 cells was comparable (**Supplementary Figure S3**). Next, we asked whether caspase-8 functioned within the dendritic cells to promote Th2 responses. To determine the role of caspase-8 in dendritic cells, we co-cultured dendritic cells from *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice with CD4$^+$ T cells from OVA-primed WT mice for 5 days in the absence or presence of the OVA peptide and analyzed the differentiation of Th2 cells. The number of CD4$^+$ T cells producing IL-4 induced by OVA treatment was significantly lower in WT CD4$^+$ T cells co-cultured with dendritic cells from *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice than in WT CD4$^+$ T cells co-cultured with dendritic cells from *Rip3*$^{-/-}$ mice (**Figure 3c,d**). The role of caspase-8 for Th2 cell differentiation was specific, because the differentiation of CD4$^+$ T cells producing IFN-γ and IL-17 in the presence of dendritic cells from *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice was similar to that in the presence of dendritic cells from *Rip3*$^{-/-}$ mice (**Figure 3c** and **d**). Taken together, these results suggest that caspase-8 activity in dendritic cells is required for balancing Th1/Th2 responses, and that the absence of caspase-8 in dendritic cells result in attenuated Th2 immune response in OVA-treated *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice.

Caspase-8-induced IL-1 production promotes a Th2 immune response during OVA treatment

To identify the molecule regulated by caspase-8 that accounts for the Th2 immune response, we investigated the expression of IL-1 cytokines in OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. The expression of the genes encoding IL-1α and IL-1β was much lower in OVA-treated *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice compared with treated WT and *Rip3*$^{-/-}$ mice (**Figure 4a**), suggesting that IL-1 cytokines regulated by caspase-8 could contribute to the development of a Th2 immune response during OVA-induced allergic airway inflammation. In agreement with a disease-associated role for IL-1 in asthma, the pulmonary infiltration of total inflammatory cells, eosinophils, and neutrophils was significantly lower in OVA-treated *Il1r*$^{-/-}$ mice than in OVA-treated WT mice (**Figure 4b**). The attenuated pulmonary inflammatory response in *Il1r*$^{-/-}$ mice was confirmed by a lower score

Figure 1 OVA-induced allergic pulmonary inflammation is significantly reduced in the absence of caspase-8 or FADD. (**a**) Wild-type (WT) mice, RIP3-deficient (*Rip3*$^{-/-}$) mice, and RIP3-caspase-8 double-knockout (*R3/C8*$^{-/-}$) mice were sensitized with OVA/alum and challenged with OVA. Fluorescence-activated cell sorting (FACS) analysis was performed for pulmonary immune cell infiltration 24 h after the last OVA challenge. (**b**) Representative lung hematoxylin and eosin (H&E) sections from OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. (**c**) Clinical scores of pulmonary disease on the basis of inflammation, eosinophils, alveolar exudates, and vascular muscle hypertrophy. (**d**) IgE level in the lung of OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. (**e**) *Rip3*$^{-/-}$ and RIP3-FADD double-knockout mice (*R3/Fa*$^{-/-}$) were sensitized with OVA/alum and challenged with OVA. FACS analysis was performed for pulmonary immune cell infiltration 24 h after the last OVA challenge. (**f**) Representative lung H&E sections from OVA-treated *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice. (**g**) Clinical scores of pulmonary disease on the basis of inflammation, eosinophils, alveolar exudates, and vascular muscle hypertrophy. (**h**) IgE level in the lung of OVA-treated *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice. Arrow and arrowhead indicate immune cell infiltration and thick airway muscle, respectively. Each symbol indicates an individual mouse and mean ± s.e.m. values are shown. Results are representative of three independent experiments for **a**–**d** and two independent experiments for **e**–**h**. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$; FADD, Fas-associated protein with death domain; NS, not significant; OVA, ovalbumin; RIP3, receptor interacting protein kinase-3.

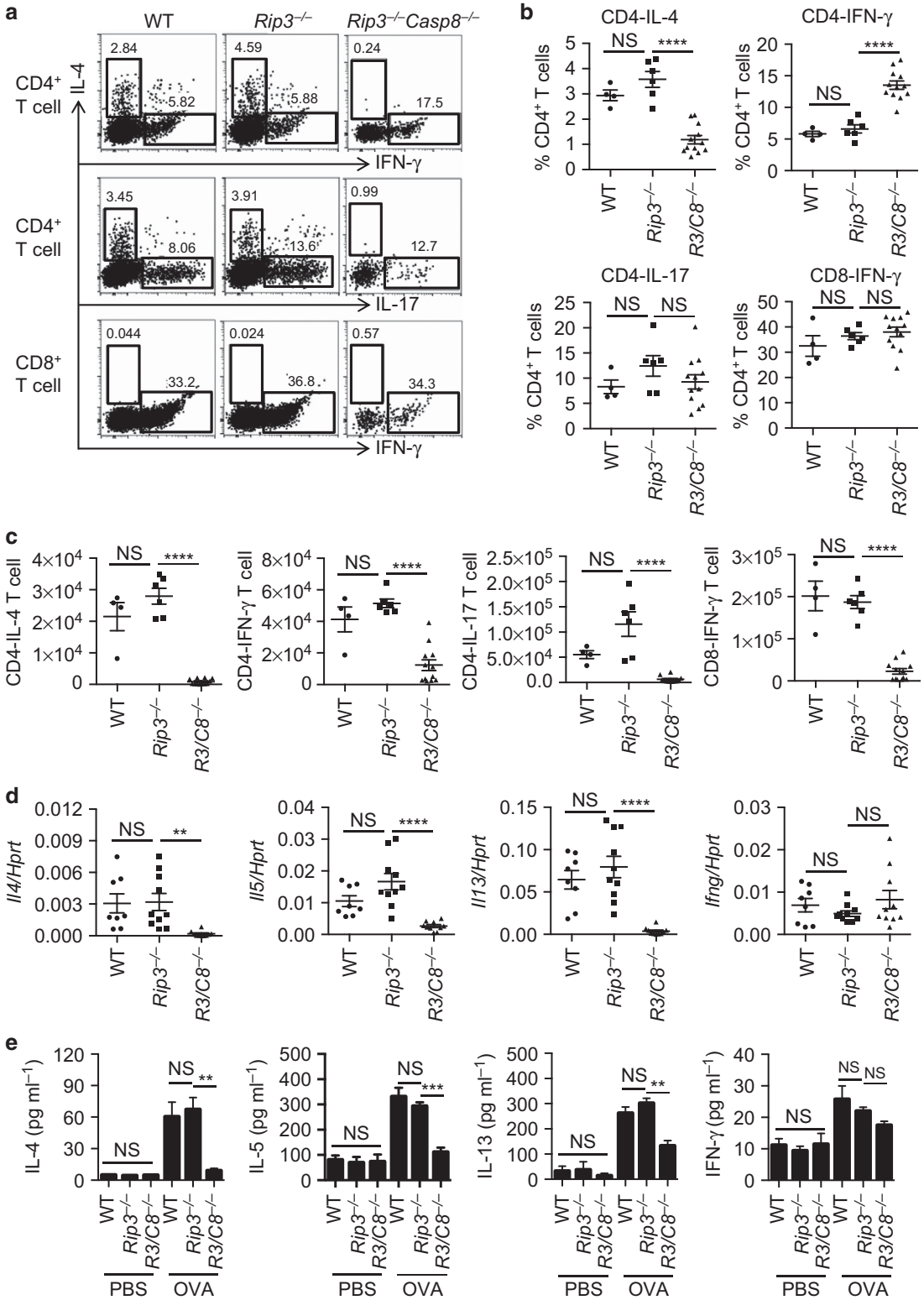


Figure 2 Caspase-8 deficiency markedly attenuates Th2 immune response in the allergic asthma model. (**a**) T cells from OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice were analyzed for IL-4, IFN-γ, and IL-17 expression by intracellular cytokine staining. (**b**) Percentage of T cells producing IL-4, IFN-γ, and IL-17 relative to total CD4^{+} or CD8^{+} T cells in **a**. (**c**) The total number of T cells producing IL-4, IFN-γ, and IL-17 in **a**. (**d**) Reverse transcription PCR analysis of *Il4*, *Il5*, *Il13*, and *Ifng* in OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. (**e**) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in OVA-treated WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. Each symbol indicates an individual mouse and mean ± s.e.m. values are shown. Results are representative of three independent experiments. $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$; IFN-γ, interferon-γ; IL, interleukin; NS, not significant; OVA, ovalbumin; WT, wild type.

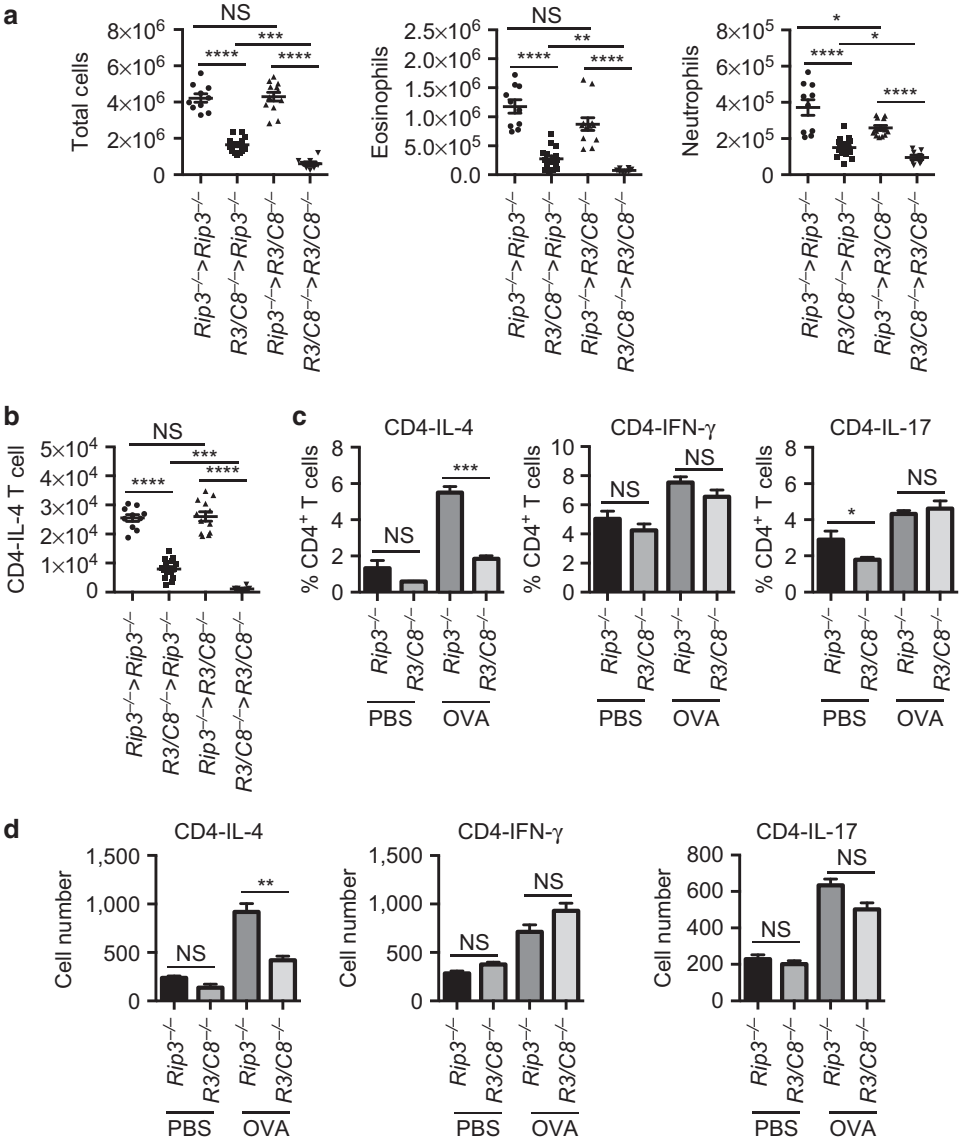


Figure 3 Contribution of hematopoietic and radioresistant cells to the protected phenotype of *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice in response to OVA administration. (**a** and **b**) Bone marrow chimeric mice (> indicates bone marrow donor cells transferred to recipient mice) were sensitized with OVA/alum and challenged with OVA. Pulmonary immune cell infiltration (**a**) and CD4$^+$ T cells producing IL-4 (**b**) were analyzed by fluorescence-activated cell sorting 24 h after the last OVA challenge. (**c** and **d**) Dendritic cells sorted from *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice and co-cultured with OVA-primed CD4$^+$ T cells in the presence or absence of the OVA peptide (1 μg ml^{-1}) *in vitro* for 5 days. The proliferated T cells were stimulated with phorbol myristate acetate and ionomycin, and analyzed for cytokine production. The percentage of total CD4$^+$ T cells (**c**) and total CD4$^+$ T cells producing IL-4, IFN-γ, and IL-17 (**d**) are shown. Results are pooled from two independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$; IFN-γ, interferon-γ; IL, interleukin; NS, not significant; OVA, ovalbumin.

of pulmonary disease severity indicated by reduced lung inflammation, alveolar exudates, and vascular muscle hypertrophy (**Figure 4c**). Consistently, the production of Th2-type cytokines and IgE was significantly reduced in *Il1r*$^{-/-}$ mice compared with WT mice after OVA treatment (**Figure 4d** and **e**).

To address whether the impaired IL-1 signaling is specific for *Rip3*$^{-/-}$*Casp8*$^{-/-}$ or *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice during asthma pathogenesis, we analyzed the production of IL-1α and IL-1β in WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, *Rip3*$^{-/-}$*Fadd*$^{-/-}$, *caspase-1/11*$^{-/-}$, *Elastase*$^{-/-}$, *Nepr3*$^{-/-}$, and *Ctsg*$^{-/-}$ mice after OVA treatment. Notably, reduced production of IL-1α and IL-1β was only observed in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice, but not in WT, *Rip3*$^{-/-}$, *caspase-1/11*$^{-/-}$, *Elastase*$^{-/-}$, *Nepr3*$^{-/-}$, or *Ctsg*$^{-/-}$ mice (**Figure 4f,g**), suggesting reduced IL-1 production in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ or *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice as the mechanism for attenuated pulmonary inflammation. In contrast, the production of another IL-1 family member, IL-18, was comparable between WT and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice after OVA treatment (**Supplementary Figure S4a**). Furthermore, *Il18*$^{-/-}$ mice did not exhibit a significant change

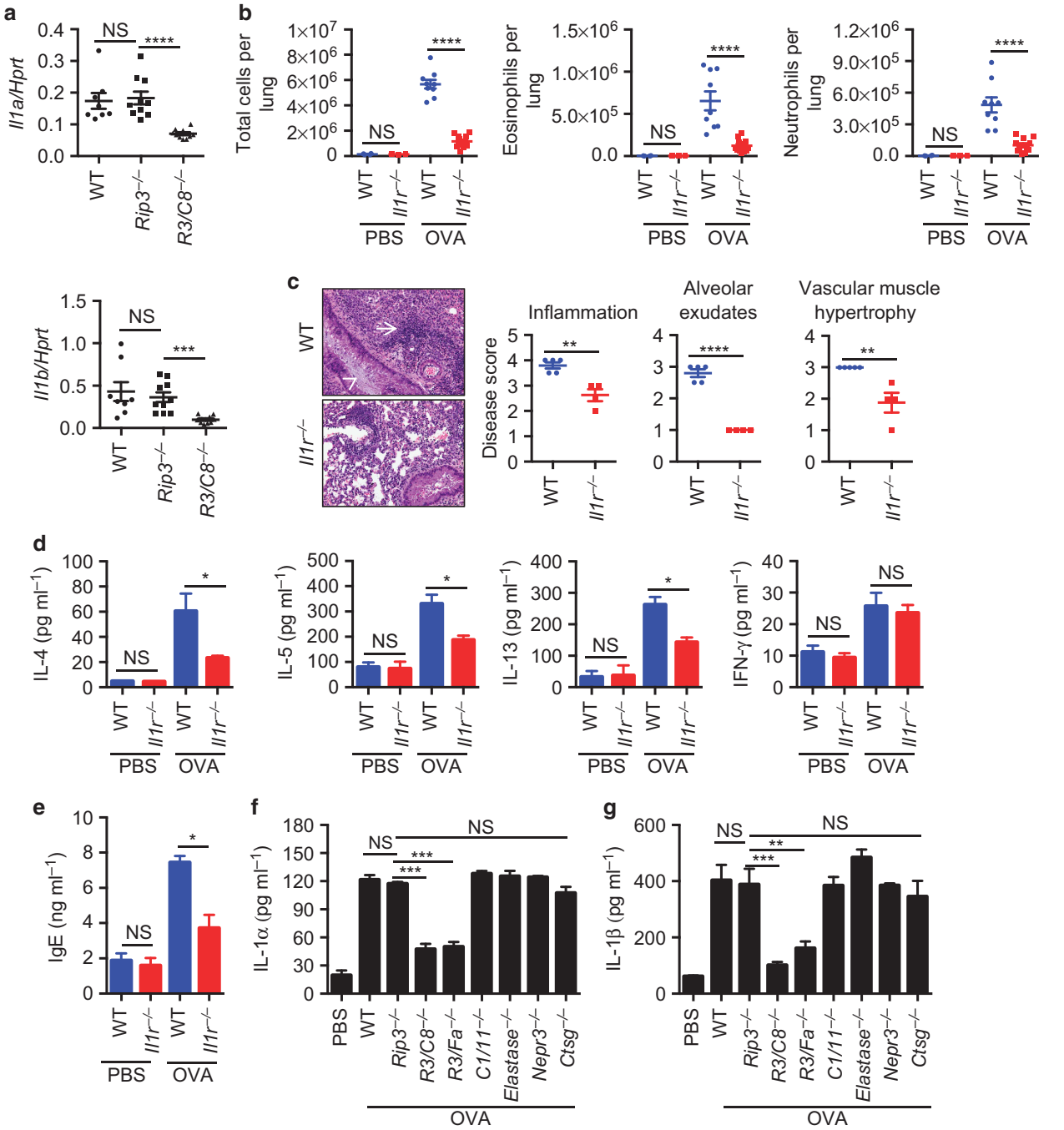


Figure 4 Caspase-8-mediated IL-1 signaling is crucial for OVA-induced allergic pulmonary inflammation. (**a**) The expression of *Il1a* and *Il1b* in lung tissue from OVA-treated *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$ *Casp8*$^{-/-}$ mice were analyzed by qRT-PCR. (**b**) WT and IL-1R mutant mice (*Il1r*$^{-/-}$) were sensitized with OVA/alum and challenged with OVA. Fluorescence-activated cell sorting analysis was performed for pulmonary immune cell infiltration 24 h after the last OVA challenge. (**c**) Representative lung hematoxylin and eosin sections from OVA-treated WT and *Il1r*$^{-/-}$ mice (left), and clinical scores of pulmonary disease on the basis of inflammation, alveolar exudates, and vascular muscle hypertrophy (right). (**d** and **e**) Cytokines (**d**) and IgE (**e**) in the supernatants of the lungs collected from OVA/alum-sensitized and OVA-challenged (OVA), or PBS-treated WT and *Il1r*$^{-/-}$ mice. (**f** and **g**) IL-1α (**f**) and IL-1β (**g**) in the supernatants of the lungs collected from OVA/alum-sensitized and OVA-challenged (OVA) WT, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$ *Casp8*$^{-/-}$ (*R3/C8*$^{-/-}$), *Rip3*$^{-/-}$ *Fadd*$^{-/-}$ (*F3/Fa*$^{-/-}$), *Caspase-1/11*$^{-/-}$ (*C1/11*$^{-/-}$), *Elastase*$^{-/-}$, *Nepr3*$^{-/-}$, and *Ctsg*$^{-/-}$ mice or PBS-treated WT mice. Arrow and arrowhead indicate immune cell infiltration and thick airway muscle, respectively. Data represent mean ± s.e.m. Results are representative of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$; IL, interleukin; NS, not significant; PBS, phosphate-buffered saline; OVA, ovalbumin; WT, wild type.

in airway inflammation compared with WT mice in response to OVA treatment (**Supplementary Figure S4b**), indicating that IL-1/IL-1R axis is the critical signaling axis in caspase-8-mediated allergic airway inflammation. IL-1 induced secretion of IL-33, thymic stromal lymphopoietin (TSLP), and IL-25 from epithelial cells largely contributes to the development of

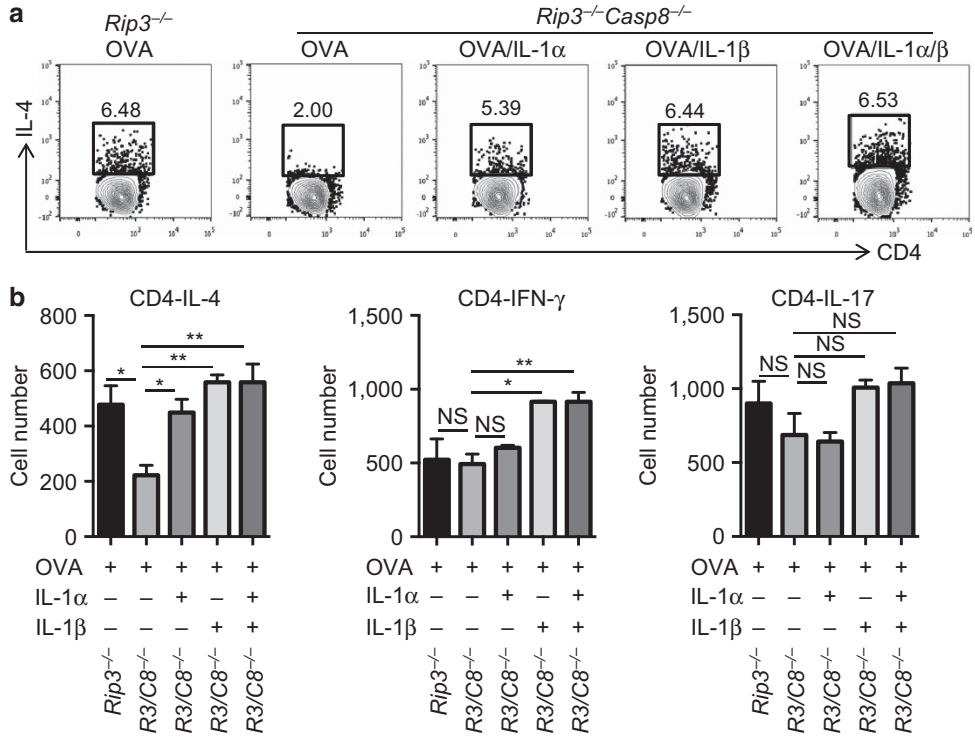


Figure 5 The activity of *Rip3*$^{-/-}$*Casp8*$^{-/-}$ dendritic cells for promoting Th2 cell differentiation is restored by exogenous application of IL-1α or IL-β. Dendritic cells sorted from naive *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice and co-cultured with OVA-primed CD4$^+$ T cells with or without the exogenous application of IL-1α (40 ng ml^{-1}), IL-1β (40 ng ml^{-1}), or both in the presence of the OVA peptide (1 μg ml^{-1}) for 5 days. The proliferated T cells were stimulated with phorbol myristate acetate and ionomycin, and analyzed by fluorescence-activated cell sorting for cytokine production. (**a**) Representative plots of CD4$^+$ T cells producing IL-4. (**b**) Total CD4$^+$ T cells producing IL-4, IFN-γ, and IL-17. Results are representative of three independent experiments. *$P<0.05$; **$P<0.01$; IFN-γ, interferon-γ; IL, interleukin; NS, not significant; OVA, ovalbumin.

allergic airway inflammation.[4] To determine whether caspase-8 deficiency results in reduced IL-33, TSLP, and IL-25 production, we analyzed the gene expression of *Il33*, *Tslp*, and *Il25* in WT, *Rip3*$^{-/-}$, and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice after OVA treatment and found that the expression of *Il33*, but not *Tslp* or *Il25*, was significantly reduced in the absence of caspase-8 (**Supplementary Figure S5a**). Furthermore, the expression of chemokine (KC) that is essential for immune cell infiltration including neutrophils was also remarkably reduced in *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice (**Supplementary Figure S5b**).

To confirm the contribution of IL-1 cytokines in driving a Th2 immune response, we co-cultured *Rip3*$^{-/-}$*Casp8*$^{-/-}$ dendritic cells and CD4$^+$ T cells in the presence or absence of IL-1 cytokines to investigate whether IL-1 cytokines can rescue dendritic cells from *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice to promote the differentiation of Th2 cells in response to OVA treatment. Remarkably, the reduced differentiation of Th2 cells in dendritic cells from *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice was rescued by the addition of exogenous recombinant IL-1α and/or IL-1β (**Figure 5a,b**). In line with previous reports,[33] we found that IL-1β promoted IFN-γ activation by T cells in response to OVA treatment (**Figure 5b**). In contrast, exogenous application of IL-1α and/or IL-1β had a modest effect on the differentiation of Th17 cells (**Figure 5b**). Taken together, our results indicate that caspase-8 controls Th2 immune response by driving the production of IL-1α and IL-1β (**Supplementary Figure S6**).

DISCUSSION

Caspase-8 is a central component of the cell death and inflammatory pathways.[34] Mounting evidence has demonstrated an involvement of caspase-8 in the proteolytic processing of IL-1β via caspase-1-dependent or -independent mechanisms.[21] A number of studies have shown that caspase-8-mediated production of IL-1 cytokines has protective roles in the host defense against infection by pathogens.[22,23] Here we showed that caspase-8 was required for IL-1 production in asthmatic mice, which had a detrimental role by promoting the development of an allergic lung disease. The severity of allergic airway inflammation and production of multiple IL-1 cytokines were markedly reduced in the absence of caspase-8 in OVA-induced mice, indicating that the inflammatory activity of caspase-8 largely contributed to disease development. Although the transcriptional level of the genes encoding IL-1α and IL-1β was reduced in OVA-treated *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice, it is possible that caspase-8 also contributes to the proteolytic processing of IL-1β.

IL-1R signaling and its function within epithelial cells are crucial for the development of allergic disease via the release of the cytokines IL-33, granulocyte–macrophage

colony-stimulating factor (GM-CSF), TSLP, and IL-25.[4,35] Our study showed both IL-1α and IL-1β contributed to the OVA-induced Th2 immune response and allergic airway inflammation, and the production of IL-1 cytokines from hematopoietic cells had more important roles in caspase-8-mediated allergic airway inflammation. These data collectively suggested that IL-1α and IL-1β could have overlapping roles driving progression of asthmatic diseases and function in an autocrine loop acting on IL-1R to promote cytokine production. The role of NLRP3 inflammasome and caspase-1-mediated IL-1 signaling in allergic airway disease is controversial.[16,19,20] In line with the report from Allen *et al* and Bruchard *et al*, we did not observe a positive role of caspase-1/-11 in OVA-induced pulmonary inflammation. Thus, the dispensable role of NLRP3 inflammasome or caspase-1-mediated IL-1β in the development of allergic airway disease seen in some studies could be attributed to a redundant function between IL-1α and IL-1β.[19] Although the role of caspase-8 seems to be dominant in the hematopoietic compartment, we also observed a partial role for caspase-8 in radioresistant cells. The modest effect of caspase-8 in the radioresistant compartment in the development of an asthmatic disease might be attributed to a caspase-8-dependent function on the production of IL-1 cytokines from lung epithelial cells.[4]

IL-1α and IL-1β are pleiotropic pro-inflammatory cytokines that have numerous roles in inflammatory and autoimmune diseases. In adaptive immunity, IL-1 cytokines have fundamental roles in Th1 and Th17 cell activation and differentiation in humans and mice.[33] Surprisingly, we observed that IL-1 promoted the differentiation of Th2 cells in response to OVA treatment, but not in differentiation of Th1 or Th17 cells, indicating a context-specific role for IL-1 in priming Th2 immune responses. The capacity of IL-1 cytokines driving diverse T-helper cell differentiation is directly related to the cytokine milieu and the genetic background of the host.[36] For instance, the Th1-inducing pro-inflammatory cytokine IL-18 promotes Th2 cell differentiation in mice infected with *Leishmania major*.[37] Indeed, our study uncovered a novel role for caspase-8-mediated IL-1 cytokines in promoting Th2 cell differentation during OVA-induced allergic airway inflammation.

Asthma is a complex disease; over 50 cytokines have been identified to be involved in its pathogenesis, including T-cell-derived cytokines, pro-inflammatory cytokines, growth factors, chemokines, and anti-inflammatory cytokines.[3] The clinical outcomes of inhibiting specific cytokines with blocking antibodies in the treatment of asthma have been disappointing, probably because of the redundant effects of disease-associated cytokines.[38] A useful future therapeutic approach might be to block multiple cytokines that are upstream in the cytokine cascade that drives the development of asthma. Importantly, human recombinant IL-1 receptor antagonist (anakinra) has been shown to be effective for asthma treatment.[39] Our results showed that caspase-8-mediated IL-1 cytokines promoted Th2 immune response to induce asthma and demonstrated that caspase-8 was a key in regulating multiple IL-1 cytokines in this process. Overall, our data raise the possibility that inhibition of caspase-8 might be beneficial in the treatment of asthma.

METHODS

Animals. *Rip3*$^{-/-}$,[40] *Rip3*$^{-/-}$*Casp8*$^{-/-}$,[30] *Rip3*$^{-/-}$*Fadd*$^{-/-}$,[32] *Il1r*$^{-/-}$,[41] *Il18*$^{-/-}$,[42] and *Casp1/11*$^{-/-}$ (ref. 43) mice were generated as previously described. Mice were kept in specific pathogen-free conditions in the Animal Resource Center at St Jude Children's Research Hospital (St Jude). Animal studies were conducted according to the protocols approved by the St Jude Institutional Animal Care and Use Committee.

OVA/alum-induced allergic pulmonary inflammation. OVA/alum sensitization and OVA challenge were performed as previously described.[44] Briefly, WT and mutant mice were immunized with 100 μg of OVA (Sigma-Aldrich, St. Louis, MO, A5503) in Imject Alum (Thermo Scientific, Waltham, MA, 77161) at days 0 and 12, followed by intranasal challenge with 50 μg of OVA in phosphate-buffered saline at days 20, 21, 22, and 23. Twenty-four hours after the last challenge, mice were analyzed for inflammatory cell infiltration and antigen-specific T-cell response by flow cytometry.

Histopathologic studies. The inferior lobes of the right lungs were fixed in formalin, and 5-μm sections were stained with hematoxylin and eosin and examined. The severity of lung disease was scored on the basis of the presence of inflammation, eosinophils, vascular muscle hypertrophy, and alveolar exudates by a pathologist blinded to the experimental groups.

Isolation of lung inflammatory cells and fluorescence-activated cell sorting analysis. The left lungs of OVA-induced mice were processed by mincing and then passed through cell strainers. After Percoll density gradient centrifugation, the total number of cells per lung was determined. The single-cell suspension was blocked with the blocking buffer (BioLegend, San Diego, CA, 101320) and stained with various antibodies for flow cytometry analysis (netrophils, CD11b$^+$ Ly-6G$^+$; eosinophils, CD11b$^+$ SiglecF$^+$). For intracelluar cytokine staining, cells were stimulated with phorbol myristate acetate (100 ng ml^{-1}) and ionomycin (1 μg ml^{-1}) in culture media with monensin (eBioscience, San Diego, CA, 00-4505-51). After 4 h, cells were washed, blocked with blocking buffer, and stained with the cell surface markers FITC-anti-mouse CD4 (RM4-5, BioLegend, 100510) and Pacific blue-anti-mouse CD8 (53-6.7, eBioscience, 48-0081-82) for 20 min, washed and fixed in 1% paraformaldehyde for 10 min, permeabilized in permeabilization buffer (eBioscience, 00-8333-56) for 5 min, and stained with specific cytokine antibodies APC-anti-IL-4 (11B11, eBioscience, 17-7041-82), PE-anti-IL-17 (ebio17B7, eBioscience, 12-7177-81), and Percp-anti-IFN-γ (ΞMG1.2, eBioscience, 45-7311-82) for 20 min. Dead cells were excluded by staining with the 7-AAD viability solution (BioLegend, 420402). Data were acquired on a BD FACS Calibur flow cytometer (San Jose, CA) and analyzed by the FlowJo software (Ashland, OR).

Bone marrow chimeric mice. *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice were lethally irradiated with a dose of 1,000 rad and transplanted with 5×10^6 whole bone marrow cells from indicated donor mice by retro-orbital injection. Reconstitution was assessed after 6 weeks. After 8 weeks, reconstituted mice were sensitized with OVA/alum and challenged with OVA.

***In vitro* differentiation of T-helper cells**. CD4$^+$ T cells were sorted from lymph node cells of WT mice 24 h after the last challenge with OVA. Dendritic cells (CD11c$^+$MHCII$^+$) were sorted from the spleens of naive *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice. Sorted dendritic cells and CD4$^+$ T cells were co-cultured in a 96-well plate in the absence or presence of 1 μg ml^{-1} of OVA peptide 323–339. After 5 days, cells were stimulated with phorbol myristate acetate and ionomycin for 4 h and populations of Th1, Th2, and Th17 cells were analyzed by flow cytometry as described above.

Cell polarization assay for Th1 and Th2 cells. Naive $CD4^+$ T cells ($CD4^+CD25^-CD44^-CD62L^+$) were sorted from lymph nodes of *Rip3*$^{-/-}$ and *Rip3*$^{-/-}$ *Casp8*$^{-/-}$ mice. WT splenocytes were irradiated for antigen-presenting cells. Naive $CD4^+$ T cells and irridated WT antigen-presenting cells were co-cultured in the presence of various cytokines and antibodies for T-helper cell differentiation. For Th1 cells, anti-CD3 antibody (2 μg ml^{-1}), anti-CD28 antibody (2 μg ml^{-1}), anti-IL-4 antibody (10 μg ml^{-1}), IL-2 (100 μg ml^{-1}), and IL-12 (0.5 ng ml^{-1}) were used. For Th2 cells, anti-CD3 antibody (2 μg ml^{-1}), anti-CD28 antibody (2 μg ml^{-1}), anti-IFN-γ antibody (10 μg ml^{-1}), IL-2 (100 μg ml^{-1}), and IL-4 (10 ng ml^{-1}) were used. On day 5, cells were stimulated with phorbol myristate acetate and ionomycin for 4 h, and Th1 and Th2 cell populations were analyzed by flow cytometry as described above.

Real-time quantitative PCR. Total RNA was isolated from lung tissues using Trizol (Invitrogen, Carlsbad, CA) and followed by purification with RNeasy Kit (Qiagen, Valencia, CA). Complemntary DNA was reverse transcribed using Superscript III (Invitrogen). Real-time quantitative PCR was performed on the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster city, CA). **Supplementary Table S1** lists the primer sequences.

ELISA. Supernatants from lung homogenates were analyzed for cytokine and chemokine release using ELISA kit (BioLegend ELISA MAX standard or Millipore multiplex assay, Billerica, MA) following the manufacturer's instructions.

Statistical analyses. Data are given as mean ± s.e.m. Statistical analyses were performed using the nonparametric Mann–Whitney test. P-values ≤ 0.05 were considered significant.

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

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DISCLOSURE

The authors declared no conflict of interest.

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