

T-cell immunoglobulin and mucin domain 1 deficiency eliminates airway hyperreactivity triggered by the recognition of airway cell death

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Background: Studies of asthma have been limited by a poor understanding of how nonallergic environmental exposures, such as air pollution and infection, are translated in the lung into inflammation and wheezing.

Objective: Our goal was to understand the mechanism of nonallergic asthma that leads to airway hyperreactivity (AHR), a cardinal feature of asthma independent of adaptive immunity.

Method: We examined mouse models of experimental asthma in which AHR was induced by respiratory syncytial virus infection or ozone exposure using mice deficient in T-cell immunoglobulin and mucin domain 1 (*TIM1/HAVCR1*), an important asthma susceptibility gene.

Results: *TIM1*^{-/-} mice did not have airways disease when infected with RSV or when repeatedly exposed to ozone, a major component of air pollution. On the other hand, the *TIM1*^{-/-} mice had allergen-induced experimental asthma, as previously shown. The RSV- and ozone-induced pathways were blocked by treatment with caspase inhibitors, indicating an absolute requirement for programmed cell death and apoptosis. TIM-1-expressing, but not TIM-1-deficient, natural killer T cells responded to apoptotic airway epithelial cells by secreting cytokines, which mediated the development of AHR.

Conclusion: We defined a novel pathway in which TIM-1, a receptor for phosphatidylserine expressed by apoptotic cells, drives the development of asthma by sensing and responding to injured and apoptotic airway epithelial cells. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: *TIM-1, natural killer T, apoptosis, asthma*

Until recently, bronchial asthma has been considered a single disease characterized by allergic inflammation and resulting from the actions of allergen-specific T_H2 cells and adaptive immunity. T_H2 cells secrete cytokines, such as IL-4, IL-5, and IL-13, which orchestrate many of the classical features of asthma, including eosinophilic inflammation, reversible airway obstruction, and airway hyperreactivity (AHR). However, although environmental allergens drive the development of pulmonary T_H2 cells that are present in the lungs of many patients with asthma, nonallergic triggers, such as exposure to air pollution or viral infections, are associated with major morbidity and mortality in asthmatic patients. These nonallergic environmental triggers for asthma suggest that asthma is far more heterogeneous than previously thought and that multiple asthma phenotypes exist, some of which are associated with allergy and eosinophils but others that are associated with airway neutrophilia, steroid unresponsiveness, and innate immunity.^{1,2} Unfortunately, the precise mechanisms by which nonallergic innate processes trigger asthma symptoms remain undefined, although a role for airway injury, apoptosis, and oxidative stress has been suggested.³⁻⁵ A consequence of the lack of a satisfactory molecular understanding of nonallergic pathways is that most of the nonallergic forms of asthma lack effective therapies and remain significant clinical challenges.

Genetic analyses support the idea that asthma might involve both allergic and nonallergic processes. For example, the linkage of asthma to polymorphisms in *IL13*, *IL4RA*, and *FCER1* corroborate a role for T_H2-biased allergic inflammation in asthmatic patients. However, the linkage of asthma to genes, such as *ADAM33*,⁶ *ORMDL3*,⁷ and T-cell immunoglobulin and mucin domain 1 (*TIM1* [*HAVCR1*]),⁸ suggests additional pathogenic pathways that might be dependent on innate immunity or defects in the cells present in the airways. Understanding the role of the *TIM1* gene in particular might help to link and provide insight into allergic and nonallergic processes in asthmatic patients. *TIM1* was identified as a susceptibility gene for AHR by using linkage analysis in congenic mice⁹ and was subsequently shown to be a susceptibility gene for atopic diseases, including asthma, in human subjects.^{8,10-12}

Previous studies regarding the function of TIM-1 in regulating immunity have shown that it can function as a costimulatory molecule for T cells (eg, T_H2 cells), although the precise intracellular signaling events that occurred downstream of TIM-1 engagement

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Abbreviations used

AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
α -GalCer:	α -Galactosylceramide
NKT:	Natural killer T
OVA:	Ovalbumin
PtdSer:	Phosphatidylserine
RSV:	Respiratory syncytial virus
TIM:	T-cell immunoglobulin and mucin domain
TUNEL:	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
WT:	Wild-type

are not yet understood.^{9,13,14} Indeed, blockade of TIM-1 with antibody prevented the development of allergen-induced AHR in mice,¹⁵ including humanized mice (mice containing human lymphoid cells).¹⁶ However, these results have been perplexing because *TIMI*^{-/-} mice have been reported to normally have allergen-induced AHR.¹⁷ In addition, TIM-1, which is expressed by activated T cells, natural killer T (NKT) cells, mucosal epithelial cells, hepatocytes, and injured kidney cells, is a receptor for the hepatitis A virus, infection with which is associated with protection against asthma and allergy.^{8,18,19} Furthermore, TIM-1 regulates the severity and progression of hepatitis A virus infection, HIV infection, and cerebral malaria.²⁰⁻²² Moreover, TIM-1 has been shown recently to be a receptor for phosphatidylserine (PtdSer),²³⁻²⁵ a marker of apoptotic cells that serves as an “eat me” signal. Also, the capacity of TIM-1 (as well as TIM-3 and TIM-4) to bind PtdSer appears to allow TIM-1 to enhance entry of enveloped viral particles expressing PtdSer, including Ebola, West Nile virus, and dengue virus.²⁶ Finally, regulatory B cells have been shown to express TIM-1.^{27,28} All of these observations, although supporting an interaction between environmental factors and *TIMI* and an important role for TIM-1 in asthmatic patients, have confounded our understanding of how TIM-1 might regulate asthma.

To better understand how TIM-1 affects the development of asthma, we generated *TIMI*^{-/-} mice, which we examined in several distinct experimental models of asthma. First, the *TIMI*^{-/-} mice had severe allergen-induced AHR similar to that seen in wild-type (WT) mice but did not have AHR when infected with respiratory syncytial virus (RSV). RSV is a very common respiratory tract infection in young children and adults²⁹⁻³¹ and is the most frequent respiratory cause of hospitalization of infants in the United States associated with significant wheezing through processes that are poorly understood.

Second, in a model of asthma associated with air pollution,³² *TIMI*^{-/-} mice exposed repeatedly to ozone did not have AHR. Exposure to ozone, a major component of air pollution, is known to increase lung oxidant stress, which is commonly seen in patients with severe asthma,³³ and to increase asthmatic symptoms in children, even at concentrations of less than the US Environmental Protection Agency standard.³⁴ Furthermore, on days of high ambient ozone concentrations, hospital admissions for asthma are higher,³⁵ presumably because ozone exposure induces AHR associated with airway epithelial cell damage³⁶ and with neutrophils and inflammatory mediators in the airways.^{37,38}

Importantly, the development of both RSV- and ozone-induced AHR was blocked by treatment with caspase inhibitors, whereas

allergen-induced AHR was unaffected. These results indicate that airway injury and apoptosis play critical roles in nonallergic asthma and are sensed by TIM-1-expressing cells in the lungs, which translate airway cell death into AHR and airway inflammation. These results highlight differences between allergic and nonallergic forms of asthma and provide specific mechanisms for the development of nonallergic asthma.

METHODS**Mice**

WT BALB/c ByJ mice on the BALB/c background were purchased from the Jackson Laboratory (Bar Harbor, Me). *Ja18*^{-/-} mice were gifts from M. Taniguchi and T. Nakayama (Chiba University, Chiba-Shi, Japan). The mice were studied at 6 to 8 weeks of age and age matched. All the animal experiments were approved by the Animal Care and Use Committee of Children's Hospital Boston.

Antibodies and reagents

Anti-mouse TIM-1 mAb blocking antibody (3D10)³⁹ was injected 24 hours before ozone exposure. Anti-Fas mAb (Jo2) and caspase inhibitor (Q-VD-OPH) were purchased from BD Biosciences (San Jose, Calif) and SM Biochemicals (Anaheim, Calif), respectively. α -Galactosylceramide (α -GalCer) was synthesized by P. B. Savage (Brigham Young University, Provo, Utah) and used for *in vitro* NKT cell cultures.

Measurement of AHR

Mice were anesthetized with 50 mg/kg pentobarbital and instrumented for the measurement of pulmonary mechanics (Buxco Electronics, Wilmington, NC). Mice were tracheostomized, intubated, and mechanically ventilated at a tidal volume of 0.2 mL and a frequency of 150 breaths/min, as previously described.⁴⁰ Lung resistance was measured in response to increasing doses (0.125–40 mg/mL) of aerosolized acetyl- β -methylcholine chloride methacholine (Sigma-Aldrich, St Louis, Mo).

Statistical tests

Unpaired Student *t* tests or ANOVA were used for comparisons between groups (Prism 4; GraphPad Software, San Diego, Calif). Significance for all statistical tests are shown in figures.

RESULTS**A critical role for TIM-1 in ozone-induced AHR**

Using an experimental model of air pollution-associated asthma in which mice were exposed repeatedly to ozone,³² we found that *TIMI*^{-/-} mice, generated by targeted deletion of *TIMI/HAVCR1* in BALB/c embryonic stem cells (Fig 1, A) did not have ozone-induced AHR and airway inflammation (Fig 1, B–D). The *TIMI*^{-/-} mice had normal numbers of T and B cells in lymphoid organs (see Fig E1 in this article's Online Repository at www.jacionline.org) but did not express TIM-1, for example, on plasmacytoid dendritic cells, activated B cells, and NKT cells, which are known to express TIM-1 (see Fig E2 in this article's Online Repository at www.jacionline.org). Because ozone-induced AHR and airway inflammation are dependent on NKT cells (they do not occur in *CD1d*^{-/-} mice),³² we compared the number of NKT cells in the lungs after air or ozone exposure. The total number of NKT cells in the lungs was increased significantly in WT mice but not in *TIMI*^{-/-} mice (Fig 1, E). To our knowledge, this is the first report of a specific phenotypic defect in *TIMI*^{-/-} mice.

Allergen-induced AHR does not require TIM-1

The requirement for TIM-1 was specific for ozone-induced AHR because both WT and *TIM1*^{-/-} mice sensitized and challenged with ovalbumin (OVA) had allergen-induced AHR with airway eosinophilia (Fig 2, A and B), as previously reported.¹⁷ In this response *TIM1*^{-/-} mice had normal T_H2 and pulmonary NKT cell responses similar to those seen in WT mice (Fig 2, C). Therefore TIM-1 was required for the development of ozone-induced but not OVA-induced AHR.

Ozone exposure induces apoptosis of airway epithelial cells

Because TIM-1 is a receptor for PtdSer and mediates the binding and recognition of apoptotic cells,²³⁻²⁵ we asked whether apoptosis was involved in the ozone-induced AHR response. Indeed, after repeated ozone exposure, increased numbers of apoptotic airway epithelial cells were found in the lung, which were propidium iodide and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) double positive, as previously reported (Fig 3, A).⁴¹ Annexin V⁺ apoptotic airway cells developed after ozone exposure to the same degree in both WT and *TIM1*^{-/-} mice (Fig 3, B). Furthermore, treating ozone-exposed WT mice with an anti-TIM-1 mAb that blocks TIM-1 recognition of PtdSer prevented the development of ozone-induced AHR and airway inflammation (Fig 3, C and D). These results suggest that ozone exposure results in apoptosis of airway epithelial cells and that TIM-1-expressing cells can translate apoptosis into inflammation.

Prevention of apoptosis blocks ozone-induced AHR

We next showed that treatments that block apoptosis surprisingly prevented the development of ozone-induced AHR. Administration of Q-VD-OPH binds caspases, thereby preventing caspase-dependent apoptosis before ozone exposure abolished ozone-induced AHR and airway inflammation (Fig 4, A-C). Treatment with Q-VD-OPH reduced the number of apoptotic airway epithelial cells (Fig 4, D), supporting the idea that apoptosis is required for the development of ozone-induced AHR. In contrast, treatment with Q-VD-OPH had no effect on OVA-induced AHR and airway eosinophilia (see Fig E3 in this article's Online Repository at www.jacionline.org), indicating that apoptosis was required for the development of ozone-induced, but not OVA-induced, AHR.

Damaged and apoptotic cells stimulate NKT cells

Because ozone-induced AHR does not occur in *CD1d*^{-/-} mice and therefore requires the presence of NKT cells,³² we next examined whether TIM-1-expressing NKT cells participated in the response. Apoptotic or healthy mouse airway epithelial cells (MLE12; Fig 5, A) were cocultured with NKT cells from WT or *TIM1*^{-/-} mice. We found that apoptotic, but not healthy, airway epithelial cells activated WT NKT cells when cocultured *in vitro* with a suboptimal dose of α -GalCer (1 ng/mL), as demonstrated by production of IL-4 and IL-13 (Fig 5, B, and see Fig E4 in this article's Online Repository at www.jacionline.org). However, apoptotic airway epithelial cells did not activate NKT cells from *TIM1*^{-/-} mice, although both WT and *TIM1*^{-/-} NKT cells could be activated by an optimal dose of α -GalCer (100 ng/mL) to produce both IL-4 and IL-13. This TIM-1-dependent response to

apoptotic cells required the recognition of PtdSer because it was blocked by addition of Annexin V (Fig 5, B), which binds PtdSer, thus preventing the recruitment of TIM-1 in the apoptotic cell-NKT cell synapse.³⁹ Moreover, human NKT cells were also activated by apoptotic but not healthy human type II epithelial cells (A549) and produced significant levels of IL-4 after coculture with apoptotic cells *in vitro* (Fig 5, C and D). Therefore TIM-1 delivers a costimulatory signal to NKT cells, resulting in cytokine production in both mice and human subjects.

TIM-1 expression by NKT cells is required for ozone-induced AHR

We next examined the *in vivo* role of TIM-1-expressing NKT cells in the development of ozone-induced AHR. *J α 18*^{-/-} mice, which lack NKT cells, did not have ozone-induced AHR,³² but the response in *J α 18*^{-/-} mice was fully reconstituted by adoptive transfer of NKT cells from WT but not *TIM1*^{-/-} mice (Fig 6, A-C). Moreover, in the lungs of WT mice exposed to ozone, the number of NKT cells producing IL-4 (Fig 6, D, left panel), IL-4 plus IL-13 (Fig 6, D, middle panel), or IL-17 (Fig 6, D, right panel) increased significantly, and this was greatly reduced in the *TIM1*^{-/-} mice. Together, these observations indicate that apoptotic cells in the airways activate TIM-1-expressing NKT cells, which then drive the development of AHR and airway inflammation.

Infection with RSV induces AHR in WT, but not *TIM1*^{-/-}, mice

We next examined the role of TIM-1 in another innate model of asthma induced with RSV infection. RSV is an important cause of serious respiratory illness and wheezing in young children and adults²⁹⁻³¹ through processes that are poorly understood. Unexpectedly, *TIM1*^{-/-} mice did not have RSV-induced AHR, whereas WT mice had robust AHR (Fig 7, A and B). Furthermore, TIM-1-expressing NKT cells were required for the development of RSV-induced AHR because it did not occur in NKT cell-deficient *CD1d*^{-/-} mice (Fig 7, C and D). RSV infection in WT mice was associated with a significant increase in the number of NKT cells in bronchoalveolar lavage (BAL) fluid (Fig 7, E). RSV infection also resulted in an increase in the number of apoptotic cells in the lungs (Fig 7, F), as previously reported.⁴²⁻⁴⁴ Moreover, treatment with Q-VD-OPH blocked the induction of AHR by RSV (Fig 7, G and H), indicating that apoptosis was also required for RSV-induced AHR. Furthermore, adoptive transfer of NKT cells from WT, but not *TIM1*^{-/-}, mice reconstituted RSV-induced AHR and airway inflammation (Fig 7, I and J). Together, these results strongly support an important role for TIM-1-expressing NKT cells in recognizing apoptotic airway epithelial cells and in translating programmed cell death into airway inflammation and AHR (Fig 8).

DISCUSSION

In these experiments we demonstrated an important role for *TIM1*, an important asthma susceptibility gene, in which NKT cells responding to apoptotic airway cells combine in a novel pathway that triggers the development of AHR. This innate TIM-1 pathway clarifies several previously unexplained elements of nonallergic asthma that do not fit with the prevailing T_H2 paradigm of asthma, such as its association with lung injury, oxidative stress, and airway neutrophilia. We showed that

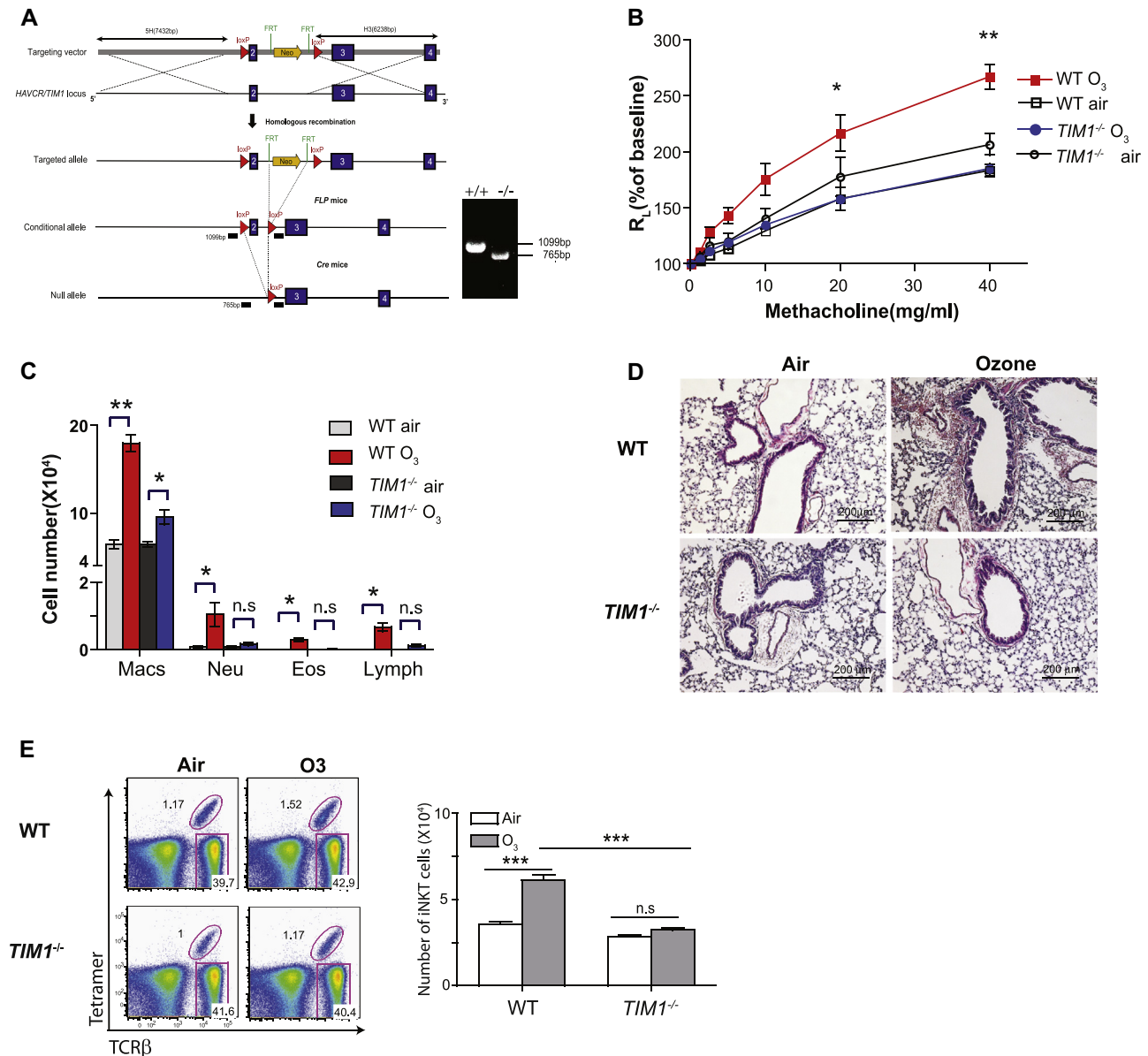


FIG 1. TIM-1 is required for the development of ozone-induced AHR. **A**, Strategy for disruption of the *TIM1* gene. Deletion of the *TIM1* gene was confirmed by using PCR. **B**, Results represent changes in lung resistance in wild-type or *TIM1*^{-/-} mice after air or ozone exposure. * $P < .05$ and ** $P < .01$ compared with the air-exposed group (2-way ANOVA). **C**, Inflammatory cell numbers in BAL fluid of mice from Fig 1, B (cells per lung). * $P < .05$ and ** $P < .01$ compared with the air-exposed group (2-tailed *t* test). **D**, Representative lung sections stained with hematoxylin and eosin (x10 magnification). **E**, Percentage of tetramer-positive T-cell receptor (TCR) β^+ NKT cells in the lungs after exposure to either air or ozone (left) and total number of NKT cells (right). *** $P < .001$ (WT, air- vs ozone-exposed group) or *** $P < .001$ (WT vs *TIM1*^{-/-} mice after ozone exposure; 2-tailed *t* test). Data are representative of at least 3 independent experiments with 3 to 5 mice per group. n.s., Not significant.

TIM-1-expressing NKT cells responded to apoptotic, but not healthy, airway epithelial cells, translating excessive airway cell death into airway inflammation and AHR in the absence of adaptive immunity. These studies are important because they explain a specific function for TIM-1, a receptor for PtdSer, in mediating the development of airways disease and provide a specific molecular mechanism for asthma triggered by lung injury. Together, these studies of a TIM-1/apoptotic cell-dependent pathway provide an important new dimension in our understanding of the heterogeneity and complexity of asthma.

In the normal lung apoptotic cells are rarely observed, presumably because the removal of apoptotic cells by alveolar macrophages, a process called efferocytosis, is extremely efficient. However, after repeated exposure to ozone, hyperoxia, or infection or exposure to particulates, large numbers of airway epithelial cells can undergo apoptosis and overwhelm the normal clearance mechanisms. Apoptotic cells appeared in the lungs of both WT and *TIM1*^{-/-} mice after exposure to ozone, but only NKT cells that expressed TIM-1 could recognize and translate apoptosis into AHR. Therefore we suggest that TIM-1, expressed by airway NKT cells, senses apoptosis,

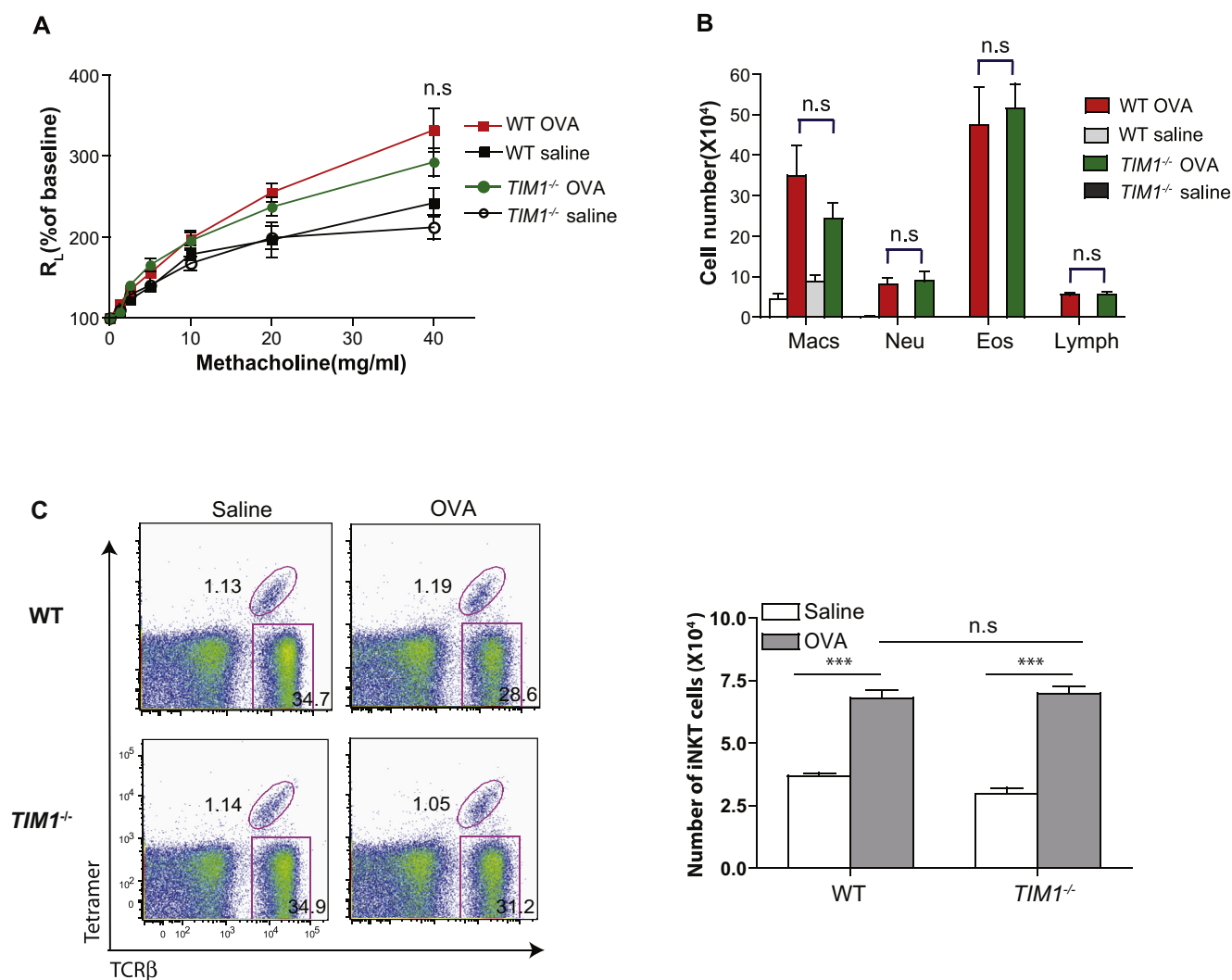


FIG 2. TIM-1 is not required for the development of OVA-induced AHR. **A**, WT and $TIM1^{-/-}$ mice have AHR equivalently after sensitization and challenge with OVA. The graph represents the changes in lung resistance (R_L). The $TIM1^{-/-}$ OVA group was compared with the WT OVA group (2-way ANOVA). **B**, Inflammatory cell numbers in BAL fluid after OVA challenge (cells per lung). The $TIM1^{-/-}$ OVA group was compared with the WT OVA group (2-tailed *t* test). **C**, The number of NKT cells was calculated as in Fig 1, G. Tetramer-positive T-cell receptor (TCR) β^+ NKT cells after saline or OVA challenge (left) are shown, and graphs represent the total number of NKT cells in each group. ****P* < .001 (WT) or ****P* < .001 $TIM1^{-/-}$, saline vs OVA group; not significant (WT vs $TIM1^{-/-}$ mice after OVA challenge; 2-tailed *t* test). Data are representative of at least 3 independent experiments with 3 to 5 mice per group. n.s., Not significant.

mediates the activation of both mouse and human NKT cells, and triggers the development of inflammation and AHR. This process develops in the context of lung injury with apoptosis but not during allergen-induced AHR, explaining why $TIM1^{-/-}$ mice have allergen-induced but not ozone-induced AHR. Thus although both allergen-induced and ozone-induced AHR require the presence of NKT cells,^{32,40} the activation of NKT cells during allergen challenge is independent of TIM-1 and was not affected by treatments that interfered with apoptosis. These observations highlight a fundamental difference between the pathologic pathways that lead to allergic versus nonallergic asthma.

Although apoptosis has not been thought to play a prominent role in asthmatic patients, increased numbers of apoptotic cells have been observed in the lungs of these patients, particularly in patients with severe asthma. In such patients the number of

apoptotic cells was 10-fold greater than in subjects with mild or no asthma.⁴⁵ In asthmatic children apoptotic epithelial cell numbers were also noted to be greater than in nonasthmatic control subjects, as was basement membrane thickening.³³ Although impaired removal of apoptotic cells by alveolar macrophages is thought to contribute to chronic airway inflammation in patients with cystic fibrosis and chronic obstructive pulmonary disease,⁴⁶ the precise mechanisms by which apoptosis leads to airway inflammation or AHR has been previously unknown. Because NKT cells are normally present in the lungs but are increased in number in the lungs of many, although not all, asthmatic patients, particularly those with severe disease,⁴⁷ the colocalization of and interaction between apoptotic cells and NKT cells is likely to occur in the lungs of asthmatic patients after exposure to many different environmental agents that are known to cause epithelial

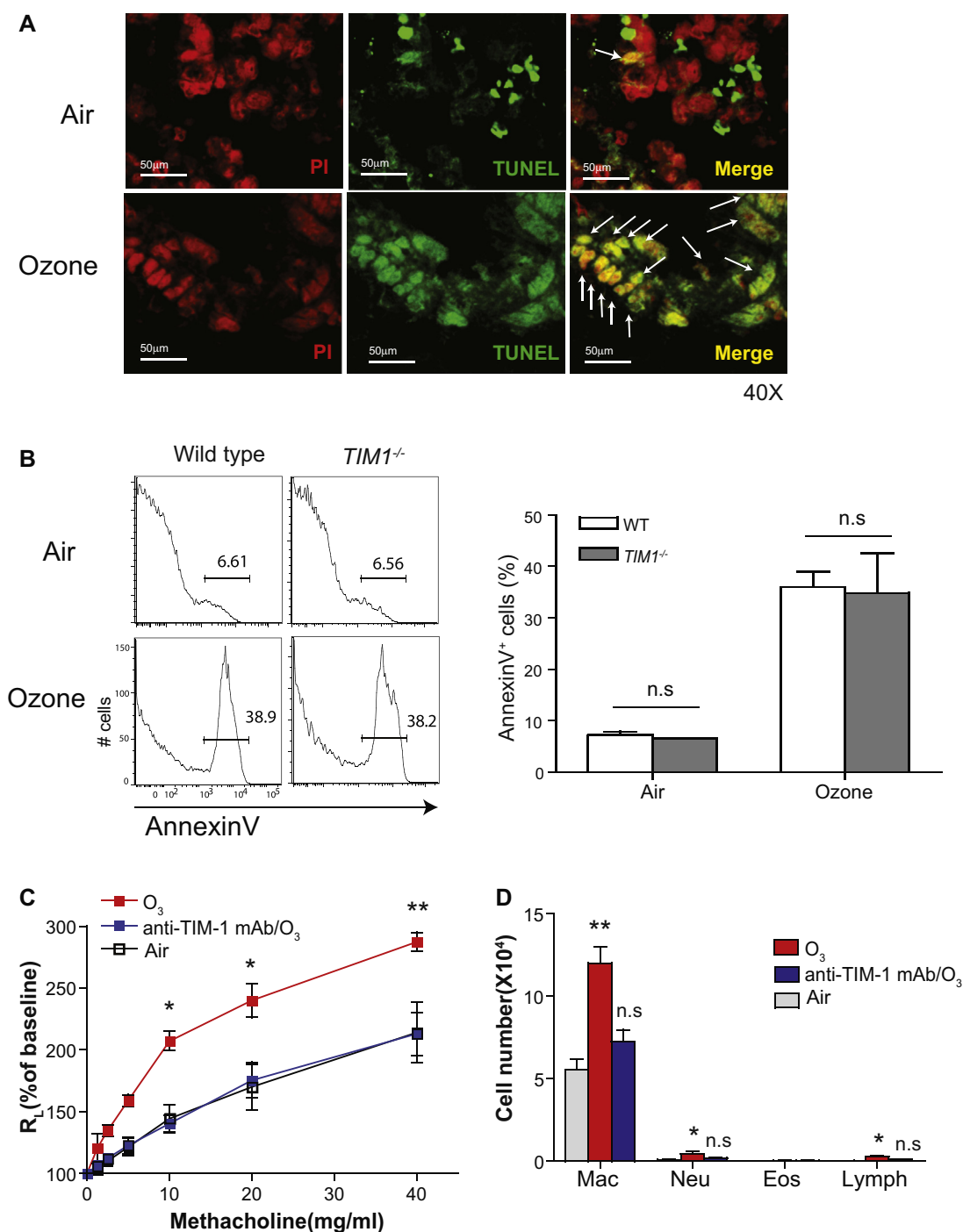


FIG 3. Oxidative stress induces apoptosis in the airway. **A**, WT mice were exposed to air (*top*) or ozone (*bottom*) as in Fig 1, and lungs were taken 24 hours after the last ozone exposure for immunofluorescence staining: propidium iodide (PI; nuclear) staining is shown in red, and TUNEL staining is shown in green. Arrows indicate the apoptotic cells (yellow; original magnification $\times 40$). Data are representative of 2 independent experiments with 3 to 4 mice in each group. **B**, WT or *TIM1*^{-/-} mice were exposed to air (*top*) or ozone (*bottom*), and apoptotic epithelial cells (CD45⁻ cells) were stained with Annexin V–fluorescein isothiocyanate (*left panel*). The graph represents the percentage of Annexin V⁺ cells in each group. *TIM1*^{-/-} mice were compared with WT mice (not significant, 2-tailed *t* test). **C**, WT mice were exposed to air or ozone, and anti-TIM-1 (3D10) mAb was injected 24 hours before the first ozone exposure. Results represent the changes in lung resistance (*R_L*). **P* < .05 and ***P* < .01 compared with the air-exposed group (2-way ANOVA). **D**, Inflammatory cell numbers in BAL fluid of mice from Fig 1, A (cells per lung). **P* < .05 and ***P* < .01 compared with air-exposed mice (2-tailed *t* test). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils; *n.s.*, Not significant.

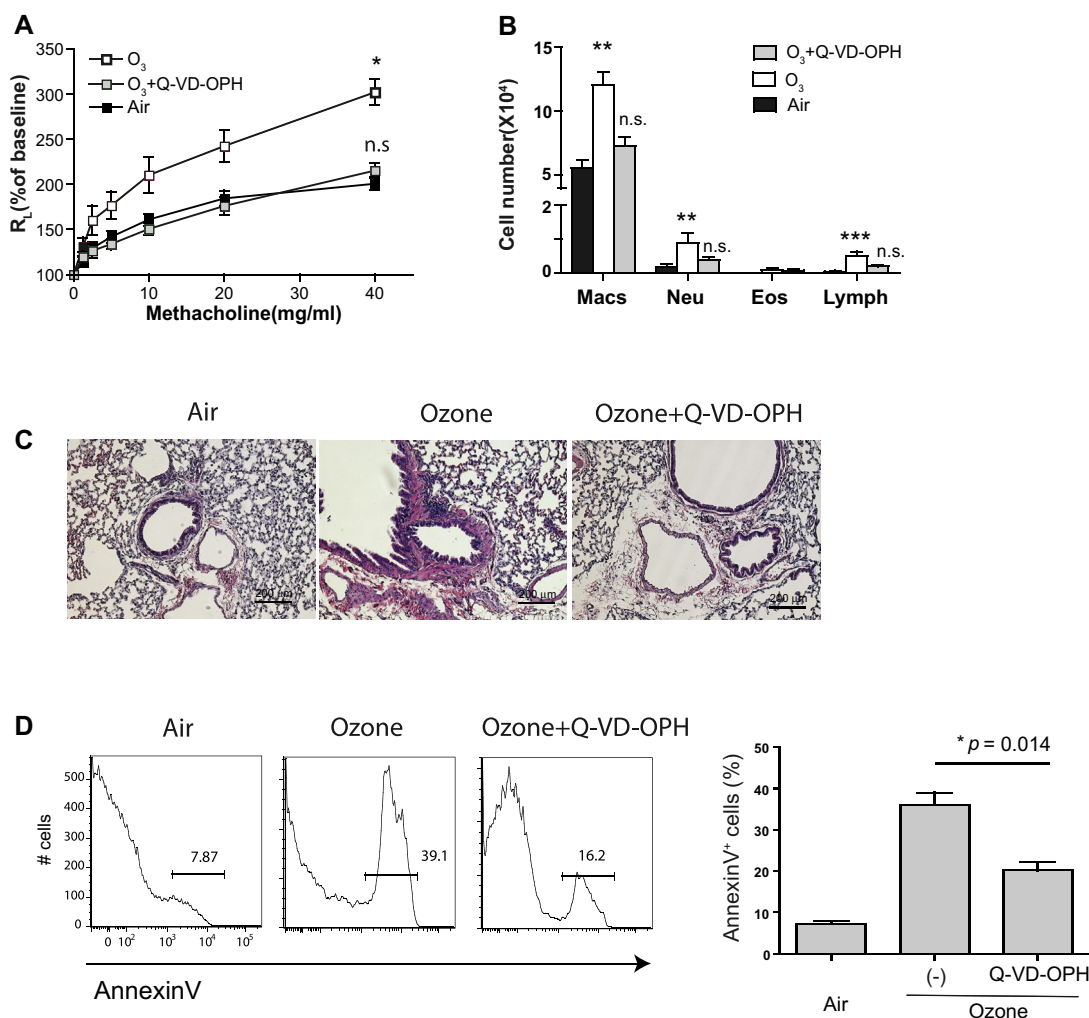


FIG 4. Apoptotic cells were critical for the development of ozone-induced AHR. **A**, Q-VD-OPH (2 mg/kg) was injected 24 hours before every ozone exposure, and AHR was measured 6 days after first exposure. * $P < .05$ compared with the air-exposed group (2-way ANOVA). **B**, Inflammatory cell numbers in BAL fluid (cells per lung). ** $P < .01$ and *** $P < .001$ compared with the air-exposed group (2-tailed t test). *Mac*, Macrophage; *Neu*, neutrophils; *n.s.*, not significant. **C**, Lung sections from the mice shown in Fig 4, A, stained with hematoxylin and eosin (original magnification $\times 10$). **D**, Apoptotic airway epithelial cells (left) and the percentage of Annexin V⁺ cells in each group (right). Q-VD-OPH-treated mice were compared with ozone-exposed mice. * $P < .05$ (2-tailed t test). Data are representative of 2 independent experiments with 3 to 5 mice.

cell apoptosis. Our studies now suggest that apoptosis is a critical trigger for the development of asthma associated with lung injury from viral infection or oxidative stress. This mechanism of asthma might be considered a specific “asthma endotype,” which is defined as an asthma subtype driven by a specific molecular-biological pathway.⁴⁸

In our studies we found that TIM-1-dependent AHR was observed not only with ozone exposure but also with RSV infection. Until now, the conventional wisdom has been that RSV infection causes wheezing by enhancing T_H2 immunity, which then causes “allergic” asthma.^{49,50} This seems an unlikely pathway because RSV infection very rapidly causes wheezing regardless of the presence of allergy. On the other hand, RSV infection induces airway epithelial cell apoptosis,⁴³ which we suggest initiates the activation of TIM-1-expressing NKT cells and leads to AHR. Moreover, it is tempting to speculate that the TIM-1/

apoptosis/NKT cell axis might be generalizable to a number of other situations in which AHR occurs in association with airway injury and epithelial cell apoptosis, such as during rhinovirus infection⁵¹ or after oxidative stress or ischemia-reperfusion injury.

Our current study greatly extends our previous observation that treatment of mice with anti-Fas mAb induced airway apoptosis and resulted in AHR through a process that involved TIM-1.³⁹ Although the previous anti-Fas mAb study suggested a role for apoptosis in patients with lung disease, we now definitively show an essential and nonredundant role for TIM-1, using *TIM1*^{-/-} mice, in 2 clinically relevant models of asthma in which mice were exposed to common environmental agents (eg, viral infection or ozone) causing airway apoptosis and NKT cell activation. Moreover, we demonstrated that both mouse and human NKT cells respond to apoptotic airway epithelial cells, suggesting that TIM-1-expressing NKT cells might indeed regulate human nonallergic asthma. Together, these studies

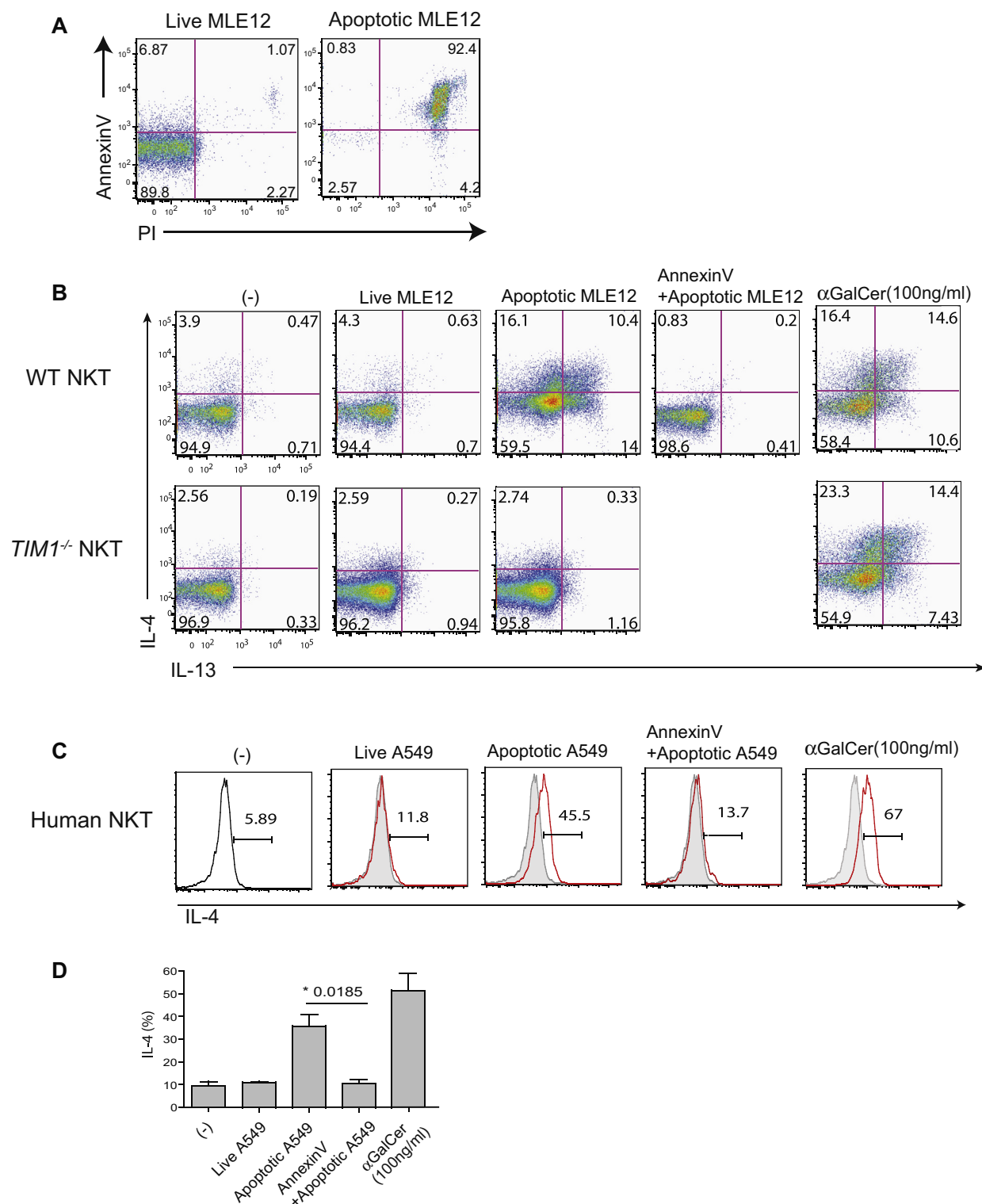


FIG 5. Apoptotic cells activate NKT cells through TIM-1. **A**, Apoptosis of a mouse airway epithelial cell line (MLE12) after hydrogen peroxide treatment was examined by using Annexin V and propidium iodide staining. **B**, WT or *TIM1*^{-/-} NKT cells were cultured with a suboptimal dose of α-GalCer (1 ng/mL; [(-)]), an optimal dose of α-GalCer (100 ng/mL), live MLE12 cells plus suboptimal α-GalCer (1 ng/mL), apoptotic MLE12 cells plus suboptimal α-GalCer (1 ng/mL), or apoptotic MLE12 cells plus suboptimal α-GalCer (1 ng/mL) plus Annexin V (10 μg/mL) for 48 hours. Data are representative of at least 3 independent experiments. **C**, Human NKT cells were cultured with a suboptimal dose of α-GalCer (1 ng/mL [(-)]), an optimal dose of α-GalCer (100 ng/mL), live A549 cells plus suboptimal α-GalCer (1 ng/mL), apoptotic A549 cells plus suboptimal α-GalCer (1 ng/mL), or apoptotic A549 cells plus suboptimal α-GalCer (1 ng/mL) plus Annexin V (10 μg/mL) for 48 hours. NKT cells were then stained for intracellular cytokines. Data are representative of 3 independent experiments. **D**, The percentage of IL-4-producing NKT cells from all 3 experiments was calculated and graphed.

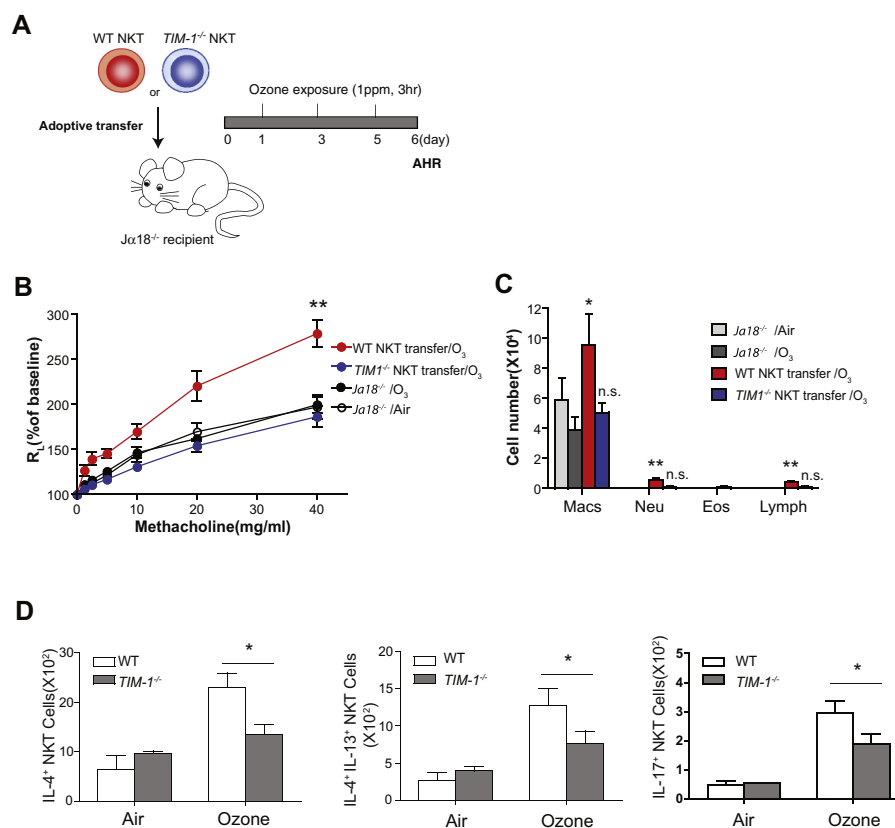


FIG 6. TIM-1 expression by NKT cells is required for ozone-induced AHR. **A**, Schematic diagram of adoptive transfer of WT or *TIM1*^{-/-} NKT cells. **B**, After repeated exposure to ozone, AHR was measured. ***P* < .01 compared with ozone-treated *Jα18*^{-/-} mice (2-way ANOVA). **C**, Inflammatory cells in BAL fluid (cells per lung). Mac, Macrophage; Neu, neutrophils; n.s., not significant. **P* < .05 and ***P* < .01 compared with ozone-treated *Jα18*^{-/-} mice (2-tailed *t* test). **D**, WT or *TIM1*^{-/-} mice were exposed to ozone as in Fig 1, A. Lung cells were prepared and stimulated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Cytokines produced from NKT cells were stained for intracellular IL-4, IL-13, and IL-17. **P* < .05, *TIM1*^{-/-} mice compared with ozone-treated WT mice (2-tailed *t* test). Data are representative of at least 2 independent experiments.

conclusively demonstrate the importance of an axis involving TIM-1, apoptosis, and recognition of PtdSer in patients with nonallergic asthma. Finally, because the *TIM1* gene has been shown to be extremely polymorphic, these results could explain how *TIM1* functions as an important asthma susceptibility gene: TIM-1 variants might differentially bind PtdSer and apoptotic cells, resulting in differential activation of NKT cells in different subjects.

Although apoptosis is considered classically as a “silent death” and tolerogenic, it has become apparent that apoptosis in some situations can be highly immunogenic, eliciting strong immune responses.^{52,53} However, the immunogenicity of cell death depends on many factors, including the characteristics and activation state of the dying cell, what is released from the cell, how quickly the apoptotic cell is removed, and where the apoptotic events occur.⁵³ We suggest that in the lungs apoptosis can increase during viral infection, toxic exposures, or after ischemia-reperfusion injury⁵⁴⁻⁵⁶ and might be recognized by TIM-1-expressing NKT cells, when present. We demonstrated that ozone exposure and RSV infection increased epithelial cell apoptosis, which was required along with NKT cell activation for the development of AHR. Nonetheless, it is possible that additional molecular mechanisms that develop in stressed and injured epithelial cells during RSV infection and ozone exposure, such as secretion

of ATP, exposure of calreticulin at the cell surface, and release of HMGB1 protein, might contribute to the pulmonary inflammatory process.^{52,53}

In summary, we identified a novel innate inflammatory pathway in the lungs triggered by the recognition of apoptotic cells through TIM-1 on NKT cells that results in the development of AHR. These results indicate that lung inflammation and AHR are much more complex than suggested by the T_H2 paradigm and that additional pathways and innate cell types beyond T_H2 cells can lead to asthma.¹ Although T_H2 cells, eosinophils, mast cells, and adaptive immunity play important roles in asthma, additional innate pathways, such as this TIM-1/apoptosis/NKT cell axis, can lead to AHR and asthma. Although each of these pathways can function independently of the others, it is likely that in a given subject, several or all of these pathways might be functional, either separately or synergistically, depending on environmental exposures at a given time. Moreover, it is possible that by developing methods that target the TIM-1/apoptosis pathway, better and more effective therapies for asthmatic patients might result.

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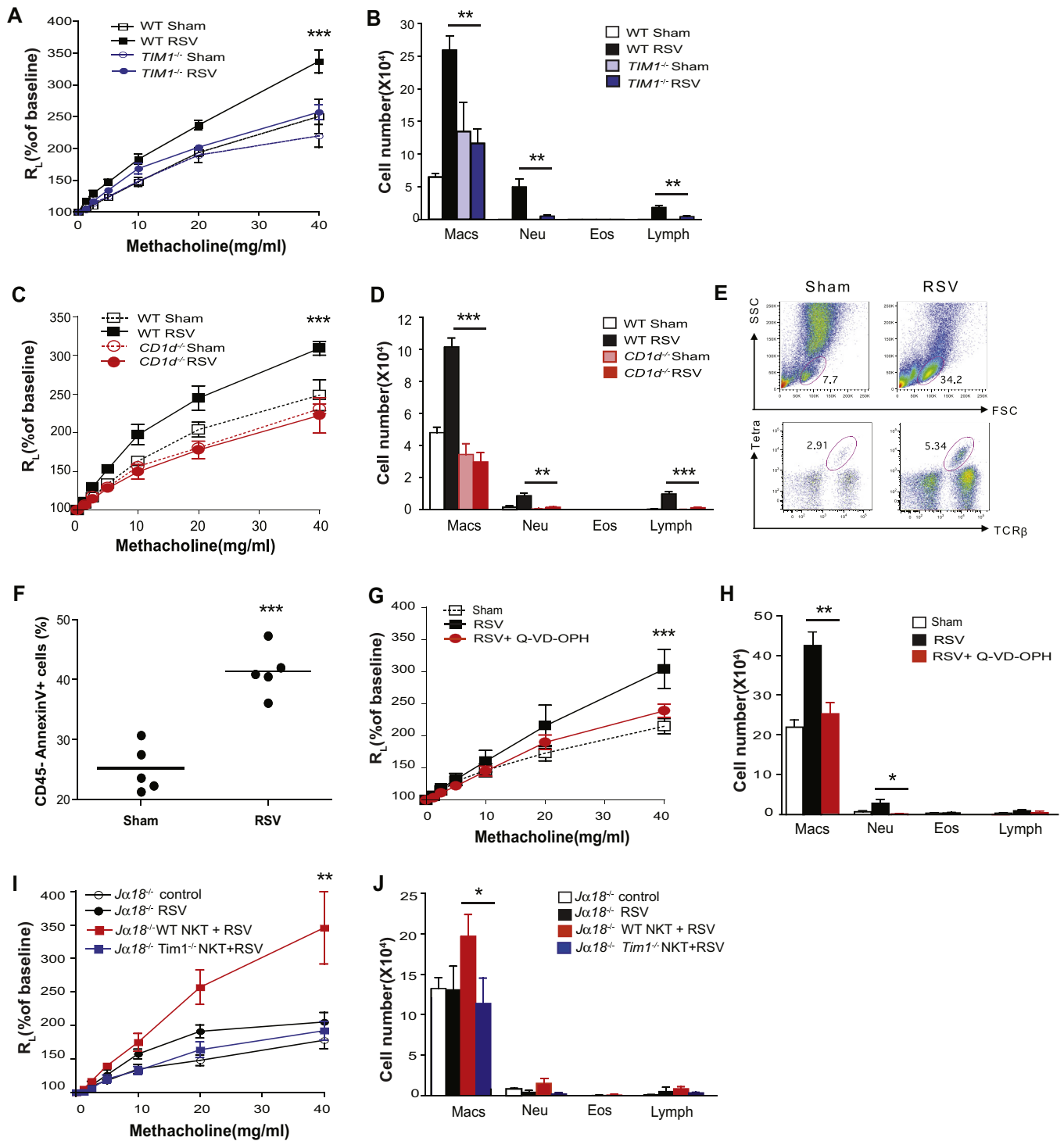


FIG 7. RSV infection induces AHR in a TIM-1-dependent manner. **A**, WT or $TIM1^{-/-}$ mice were infected with RSV or sham infected, and lung resistance (R_L) was measured on day 6 after infection. *** P < .001 compared with the sham-infected group (2-way ANOVA). **B**, BAL fluid was taken from the mice in Fig 7, A, and assessed for inflammatory cells (cells per lung). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils. ** P < .01, WT vs $TIM1^{-/-}$ mice infected with RSV (2-tailed t test). **C**, WT or $CD1d^{-/-}$ mice were infected with RSV or sham infected, and lung resistance (R_L) was measured. *** P < .001, WT vs $CD1d^{-/-}$ mice infected with RSV (2-way ANOVA). **D**, BAL fluid was assessed for inflammatory cells. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils. ** P < .01 and *** P < .001, WT RSV-infected group compared with $CD1d^{-/-}$ RSV-infected group (2-tailed t test). **E**, Tetramer-positive T-cell receptor (TCR) β^+ NKT cells in BAL fluid after sham (left panels) or RSV (right panels) infection. FSC, Forward scatter; SSC, side scatter. **F**, The graph represents the percentage of Annexin V $^+$ cells. *** P < .001 compared with the sham infected group (2-tailed t test). **G**, Q-VD-OPH (2 mg/kg) was injected

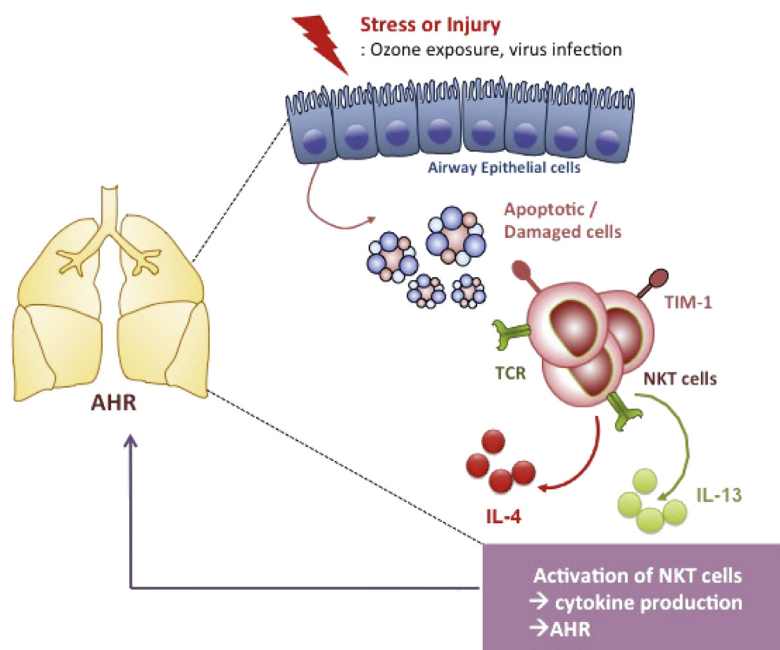


FIG 8. Schematic diagram of the role of TIM-1 in patients with nonallergic forms of asthma. In some forms of asthma (eg, associated with air pollution and RSV infection), apoptotic airway epithelial cells activate NKT cells through TIM-1 recognition of dead cells, and these in turn activate NKT cells, which induce airway inflammation and AHR. TCR, T-cell receptor.

Key messages

- This study defines a novel disease mechanism in which *TIM1*, a receptor for PtdSer exposed on apoptotic cells and an important atopy susceptibility gene, drives the development of nonallergic asthma.
- This pathway results in AHR, a cardinal feature of asthma, and is initiated by the activation of NKT cells that sense and respond to lung injury and the death of airway epithelial cells through TIM-1.
- RSV infection or ozone exposure induce apoptosis or damage of lung cells, which then stimulate NKT cells to produce cytokines that are critical for the development of AHR.

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before RSV infection, and AHR was measured. *** $P < .001$, compared with the RSV-infected group (2-way ANOVA). **H**, BAL fluid was taken from the mice in Fig 7, **G**, and assessed for inflammatory cells. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils. * $P < .05$ and ** $P < .01$, RSV vs RSV plus Q-VD-OPH group (2-tailed *t* test). **I**, WT or *TIM1*^{-/-} NKT cells (5×10^5 cells per mouse) were transferred into *Jα18*^{-/-} recipients before RSV infection, and AHR was measured. ** $P < .01$, *Jα18*^{-/-} mice with WT NKT cells compared with *Jα18*^{-/-} mice with *TIM1*^{-/-} NKT cells (2-way ANOVA). **J**, BAL fluid was assessed for inflammatory cells. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils. * $P < .05$, *Jα18*^{-/-} mice transferred with WT NKT cells compared with *Jα18*^{-/-} mice transferred with *TIM1*^{-/-} NKT cells (2-tailed *t* test). Data are representative of at least 2 independent experiments with 4 to 6 mice in each group.

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METHODS

Generation of BALB/c *TIM1*^{-/-} mice

A *TIM1/HAVCR1* conditional targeting construct was generated by first inserting a neomycin resistance cassette flanked by FRT sites between exons 2 and 3 of *TIM1/HAVCR1* and then by flanking exon 2, which includes the start codon and signal peptide and the neomycin resistance cassette with loxP sites (Fig 1, C). The *TIM1/HAVCR1* exon 1 contains the transcriptional start and 5' noncoding sequence and is 10.9 kb upstream of exon 2, which encodes the start codon and signal peptide. The 5' and 3' arms of the construct were generated by means of PCR amplification of BALB/c genomic DNA and were 7.3 and 6.4 kb, respectively. BALB/c embryonic stem cells were then electroporated with the targeting vector and homologous recombinants selected by means of genomic DNA digestion and Southern blots with 5' and 3' probes. Recombinant targeted embryonic stem cell clones were microinjected into blastocysts to generate chimeras, which were mated with BALB/c mice. Mice with the *TIM1*-targeted gene were first crossed to BALB/c Flp recombinase mice to remove the Neo resistance gene and then crossed to BALB/c ROSA26 KI Cre deleter mice. After confirming the deletion of exon 2 of *TIM1* (and the removal of the genes for the FLP and Cre recombinases), mice homozygous for the deleted *TIM1* gene were interbred to obtain the BALB/c *TIM1* deleted homozygous mice. In *TIM1*^{-/-} mice excision of exon 2 was confirmed by using PCR of genomic DNA with a primer pair spanning the excised region, resulting in a 765-bp product in knockout mice and a 1099-bp product in WT BALB/c mice (Fig 1, C). The primer pair was as follows: 5' TAAAAGTAGCTGCTC-TAGGAA 3' and 5' AGCTCGTACAAATATATGTCC 3'.

Ozone exposure

Mice were placed in a Plexiglas exposure chamber and exposed to ozone (1 ppm) or air. Ozone exposure was repeated every other day (days 0, 2, and 4) for 3 hours to induce AHR by using invasive measurement of airway resistance. The ozone exposure was monitored, as previously described.^{E1} Q-VD-OPH (2 mg/kg; SM Biochemicals) was injected intraperitoneally 24 hours before every ozone exposure to block apoptosis. AHR was measured 24 hours after the last ozone exposure.

OVA-induced AHR

Mice were sensitized with 100 µg of OVA (Sigma-Aldrich) administered intraperitoneally in 2 mg of alum at day 0, as previously described.^{E2} After sensitization, mice were exposed to intranasal OVA (50 µg) or saline for 3 consecutive days (days 7, 8, and 9). AHR was assessed on the day after the last OVA challenge.

RSV-induced AHR

Human RSV strain A2 (American Type Culture Collection, Manassas, Va) was propagated in Hep-2 cells maintained in Eagle minimum essential medium containing 5% heat-inactivated FBS. The infected cells were harvested after 3 to 4 days, cells were lysed sonically, and the virus was separated from cellular debris under endotoxin-free conditions by using centrifugation, as described previously.^{E3} Titers were determined by using a standard plaque assay on Hep-2 cells.^{E5} Mice were inoculated after achievement of light anesthesia (isoflurane) by means of intranasal instillation of 10⁶ plaque-forming units of purified virus in 75 µL of endotoxin-free PBS. Sham-infected animals were inoculated with lysed Hep-2 cells under identical conditions.

BAL fluid and cytospin preparations

After measurement of AHR, mouse lungs were lavaged twice with 1 mL of PBS containing 2% FCS, and the fluid was pooled. Cells in the lavage fluid were counted with a hemocytometer, and BAL cell differential counts were determined on slide preparations stained with Diff-Quik (Dade Behring, Deerfield, Ill). At least 200 cells were counted by using conventional morphologic criteria.

Lung tissue preparation

After perfusion with PBS and 2% FCS, lung tissue was minced and digested with 1.6 mg/mL collagenase type 4 (Worthington, Lakewood, NJ) and 0.1%

DNase I (fraction IX, Sigma) at 37°C for 1 hour. The resulting lung cell suspension was then lysed for red blood cells and stained for fluorescence-activated cell sorting analysis. Some lungs were fixed in 10% neutral buffered formalin (pH 7.4) for histologic analysis and TUNEL assay.

TUNEL assay

Genomic DNA fragments were labeled with digoxigenin-dNTP and detected with fluorescein-conjugated anti-digoxigenin mAb (ApoTag fluorescein *in situ* apoptosis detection kit, Millipore), according to the manufacturer's instructions. Samples were analyzed on a Zeiss Axiophot microscope with a Spot camera (Diagnostic Instruments, Sterling Heights, Mich).

Flow cytometry

Single-cell suspensions were preincubated with anti-FcγR blocking mAb (2.4G2) and washed before staining. Hematopoietic cells were identified by gating on cells stained with phycoerythrin-Texas red-conjugated anti-mouse CD45 mAb (Invitrogen). Allophycocyanin-conjugated T-cell receptor β and phycoerythrin-conjugated CD1d tetramers were loaded with PBS57 (National Institutes of Health/National Institutes of Allergy and Infectious Diseases MHC Tetramer Core Facility, Atlanta, Ga) to identify NKT cells. Unloaded CD1d tetramers were used as a negative control. For intracellular staining, cells were permeabilized (Cytofix/Cytoperm kit, BD Biosciences) and incubated with fluorescein isothiocyanate-conjugated anti-IL-4 and phycoerythrin-conjugated anti-IL-13. Cells were collected on a FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo 8.3.3 software (Tree Star, Ashland, Ore).

Adoptive transfer of NKT cells

NKT cells were purified from splenocytes of WT BALB/c or *TIM1*^{-/-} mice by using magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.^{E2} Purity of NKT cells was greater than 90%. Purified NKT cells (5 × 10⁵) were adoptively transferred into *Ja18*^{-/-} mice by means of intratracheal injection 1 day before the first exposure to ozone or RSV infection.

Culture and *in vitro* stimulation of NKT cells

NKT cells were purified from splenocytes of WT BALB/c or *TIM1*^{-/-} mice, as previously described.^{E5} Purified NKT cells were cultured with bone marrow-derived dendritic cells in the presence of IL-15. Cells were harvested and rested overnight in the absence of bone marrow-derived dendritic cells or IL-15 before being used for *in vitro* cell stimulation experiments. Rested NKT cells (1 × 10⁵) were stimulated with a mouse airway epithelial cell line (MLE12) or apoptotic MLE12 cells (2 × 10⁴) in the presence of 1 ng/mL α-GalCer. Human NKT cells isolated from PBMCs, as previously described,^{E6} were cocultured with apoptotic type II epithelial cells (A549) *in vitro*. Apoptosis in the MLE12 cell line and in the A549 cell line was induced by exposure to hydrogen peroxide (0.5 mmol/L) for 6 hours. Forty-eight hours after coculture, cells were restimulated with phorbol 12-myristate 13-acetate (1 ng/mL) and ionomycin (500 ng/mL) for 4 hours and stained for intracellular cytokines. Annexin V (10 µg/mL) was added to some cultures to block PtdSer binding to TIM-1. GolgiStop (BD Biosciences) was added at the beginning of restimulation to inhibit cytokine secretion to inhibit cytokine secretion.

ELISA

Supernatants of cell cultures were collected 48 hours after coculture. Mouse IL-4, IL-13, and IFN-γ levels were measured by means of ELISA, as previously described.^{E2}

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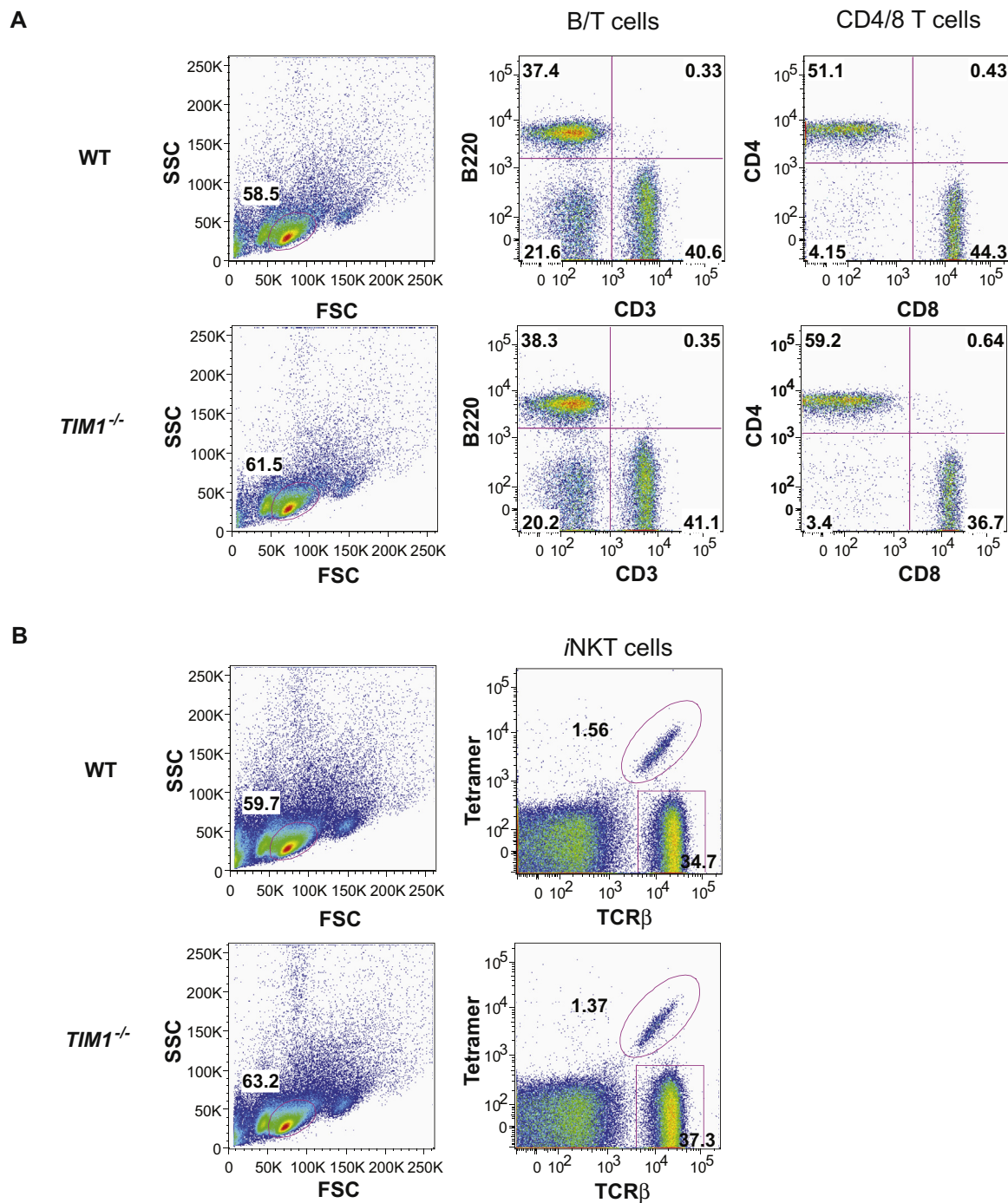


FIG E1. WT or *TIM1*^{-/-} mice have similar numbers of immune cells. **A**, B and T cells (CD4/CD8). **B**, NKT cells from WT or *TIM1*^{-/-} mice. FSC, Forward scatter; SSC, side scatter; TCR, T-cell receptor.

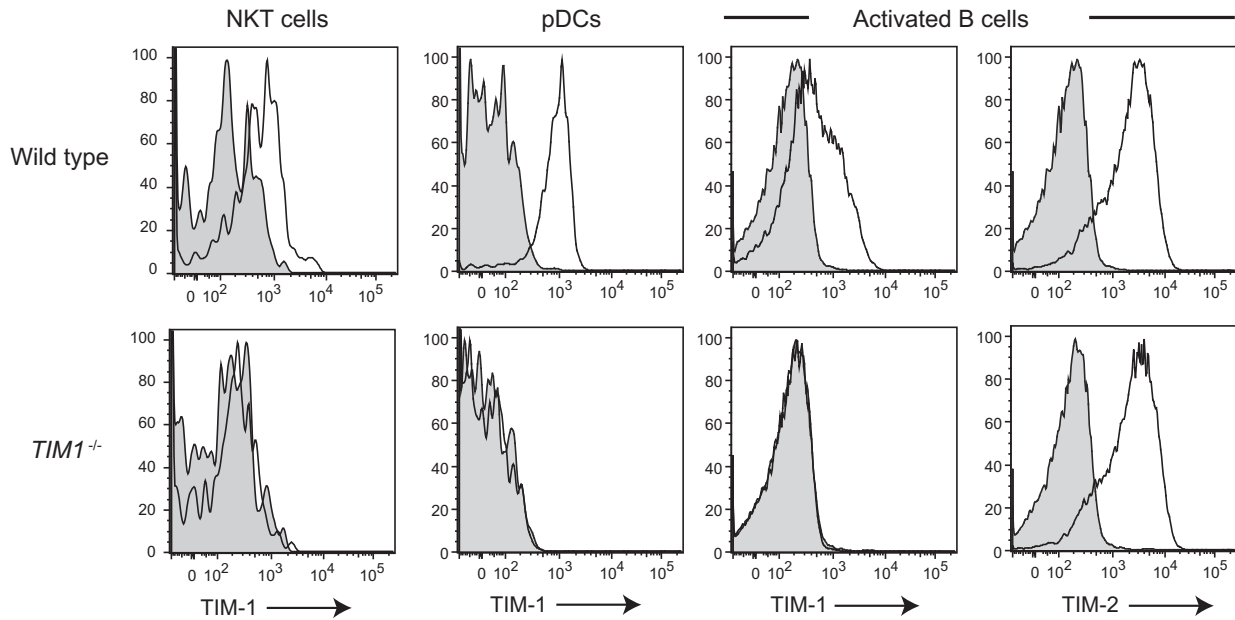


FIG E2. Plasmacytoid dendritic cells (*pDCs*) and activated B cells from *TIM1*^{-/-} mice do not express TIM-1, as demonstrated by using flow cytometry. However, TIM-2 is expressed normally on *TIM1*^{-/-} pDCs and B cells, confirming the specific absence of TIM-1 expression in *TIM1*^{-/-} mice.

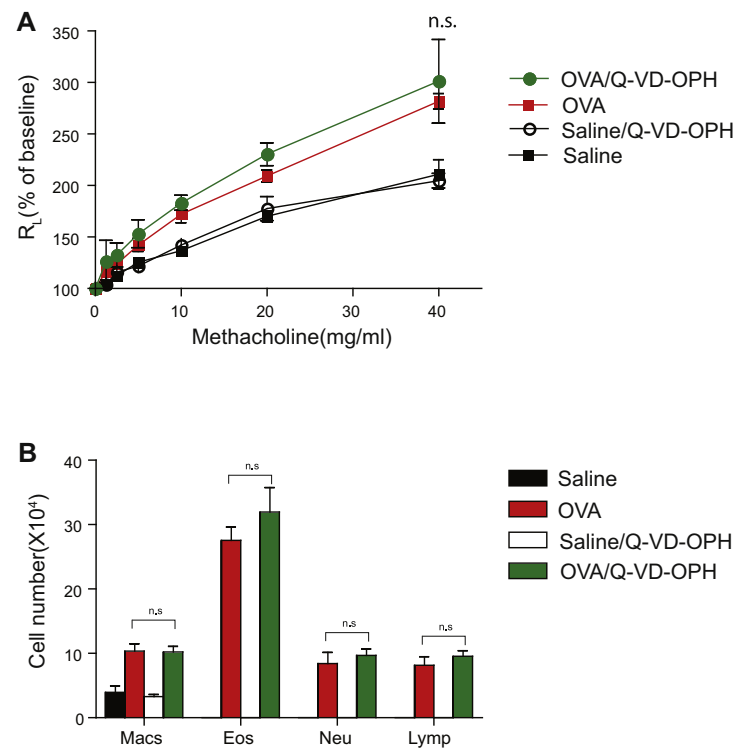


FIG E3. Q-VD-OPH (2 mg/kg) injected 24 hours before OVA challenge does not affect OVA-induced AHR. **A**, The graph represents changes in lung resistance (R_L). **B**, Inflammatory cells in BAL fluid. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils.

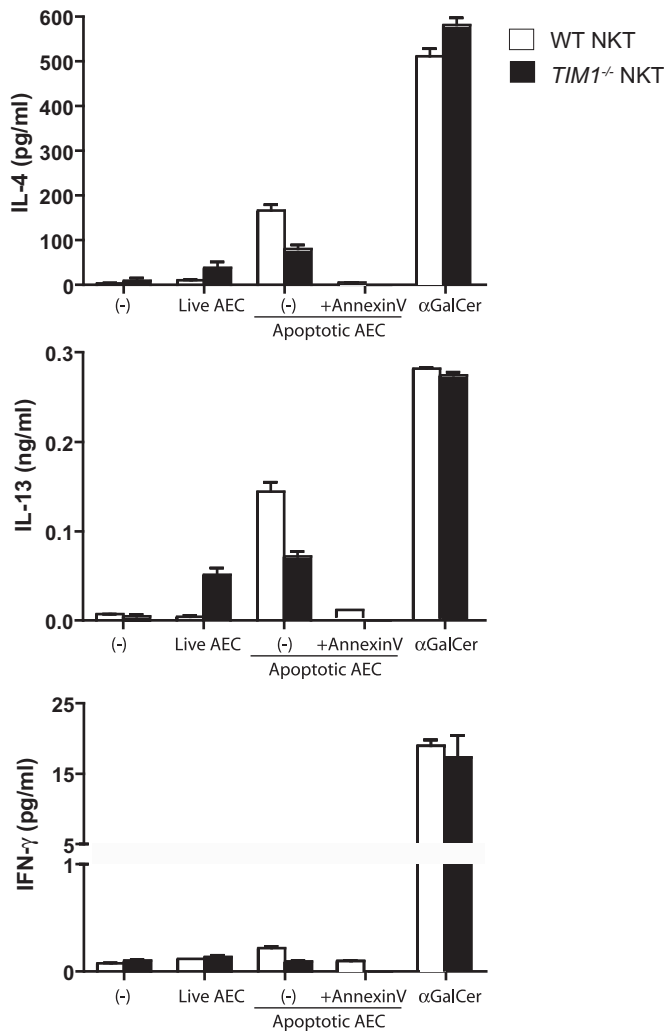


FIG E4. WT or *TIM1*^{-/-} NKT cells were incubated with apoptotic MLE12 or live MLE12 cells (as in Fig 5, B). Culture supernatants were analyzed for IL-4, IL-13, and IFN- γ production by using ELISA. AEC, Airway epithelial cells.