

Interferon response factor 3 is essential for house dust mite-induced airway allergy

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Background: Pattern-recognition receptors (PRRs) are critically involved in the pathophysiology of airway allergy, yet most of the signaling pathways downstream of PRRs implicated in allergic airway sensitization remain unknown.

Objective: We sought to study the effects of genetic depletion of interferon response factor (IRF) 3 and IRF7, important transcription factors downstream of various PRRs, in a murine model of house dust mite (HDM)-induced allergic asthma.

Methods: We compared HDM-induced allergic immune responses in IRF3-deficient (IRF3^{-/-}), IRF7^{-/-}, and wild-type mice.

Results: Parameters of airway allergy caused by HDM exposure were strongly attenuated in IRF3^{-/-}, but not IRF7^{-/-}, mice compared with those in wild-type mice. Indeed, in HDM-exposed IRF3^{-/-} mice HDM-specific T_H2 cell responses did not develop. This correlated with impaired maturation and migration of IRF3^{-/-} lung dendritic cells (DCs) on HDM treatment.

Furthermore, adoptive transfer of HDM-loaded DCs indicated that IRF3^{-/-} DCs had an intrinsic defect rendering them unable to migrate and to prime HDM-specific T_H2 responses.

Intriguingly, we also show that DC function and allergic airway sensitization in response to HDM were independent of signaling by type I interferons, the main target genes of IRF3.

Conclusion: Through its role in DC function, IRF3, mainly known as a central activator of antiviral immunity, is essential for the development of T_H2-type responses to airway allergens. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Airway allergy, interferon response factor 3, interferon response factor 7, dendritic cell, type I interferon

Allergic asthma arises from aberrant immune responses against inhaled allergens. It is now well accepted that these responses are mainly initiated by lung dendritic cells (DCs) and orchestrated by subsequent T_H2 lymphocyte differentiation and activation.¹⁻³ Yet the cause of allergic asthma, one of the most prevalent allergic conditions worldwide, remains poorly understood.

Allergic asthma is the result of complex interactions between genetic and environmental factors.⁴ In addition to allergens, ambient air contains a large variety of immunostimulatory molecules that also play a crucial role in the development of asthma.⁵⁻¹⁰ Many of these molecules, notably those derived of microorganisms, activate specific immune receptors collectively referred to as pattern-recognition receptors (PRRs).¹¹ The PRR group encompasses various families of evolutionarily conserved receptors, such as Toll-like receptors (TLRs), NOD-like receptors, or retinoic-acid-inducible gene-I-like receptors. Not only do PRRs ensure activation of antimicrobial responses by the “innate” arm of immunity, they also directly or indirectly modulate adaptive immunity.¹¹

In recent years, clinical and experimental studies have abundantly illustrated the importance of PRRs in the pathophysiology of asthma. Although activation of certain PRRs, such as TLR9, might dampen airway allergy,¹² activation of others, such as TLR4, has more complex or negative outcomes on asthma.^{13,14} Clinical studies showed, for instance, that exposure to house dust endotoxin, a ligand for TLR4, is a significant risk factor for increased asthma prevalence and severity,^{5,7,15,16} although high levels of endotoxin exposure are protective in early life.^{17,18} Additionally, studies on murine models of asthma supported the importance of PRR signaling in allergic airway sensitization. Indeed, mice deficient for TLR4 were protected against allergic sensitization in murine models of ovalbumin (OVA)-induced asthma.^{19,20}

Most murine models of allergic asthma used to rely on sensitization to the experimental allergen OVA, which sometimes cast doubt on their relevance to human allergic asthma. Consequently, murine models of allergic airway sensitization with clinically relevant allergens were recently developed, most of which rely on intranasal administration of extracts of house dust mites (HDM), the main allergen source in allergic asthma. Most notably, these models confirmed the important role played by PRRs in patients with airway allergy. Indeed, mice deficient for TLR4 or for the downstream adaptor protein myeloid differentiation primary response gene 88 (MyD88) are protected against HDM-induced airway allergy.^{21,22} This likely is due to the fact that HDM preparations contain endotoxin and that the HDM

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Abbreviations used

BLN:	Bronchial lymph node
BMDC:	Bone marrow-derived dendritic cell
CFSE:	Carboxyfluorescein succinimidyl ester
DC:	Dendritic cell
HDM:	House dust mite
IFNAR2:	IFN- α/β receptor 2
IRF:	Interferon response factor
MyD88:	Myeloid differentiation primary response gene 88
OVA:	Ovalbumin
PRR:	Pattern recognition receptor
TLR:	Toll-like receptor
TRIF:	TIR domain-containing adaptor inducing IFN- β
WT:	Wild type

antigen Der p 2 displays sequence homology with MD-2, the endotoxin-binding protein of the TLR4 receptor complex.²³ HDM also activates TLR2 without affecting airway allergy.²² Because of its molecular complexity,²⁴ HDM probably also activates other PRRs and pathways implicated in the pathophysiology of airway allergy. For example, β -glucan moieties of HDM activate chemokine secretion by lung epithelial cells through as-yet-unidentified PRRs,²⁵ and some HDM antigens display protease activity that might contribute to its allergenicity.²⁶

PRRs signal through a limited number of evolutionarily conserved pathways.²⁷ For example, all TLRs commonly activate nuclear factor B and activator protein 1 to induce proinflammatory cytokine and chemokine expression. In most cases this requires MyD88 to transduce signal. TLR3, TLR4, TLR7, TLR8, and TLR9 and RIG-I-like receptors also activate the transcription factors interferon response factor (IRF) 3, IRF7, or both, leading notably to type I interferon gene expression. Although the role of MyD88 has been recently documented,^{21,22} the potential involvement of IRF3 and IRF7 in allergen-induced airway sensitization remains unknown. Here we studied the implication of IRF3 and IRF7 in airway allergy by comparing allergy parameters in wild-type (WT), IRF3-deficient (IRF3^{-/-}), and IRF7-deficient (IRF7^{-/-}) mice exposed to HDM. We demonstrate that IRF3, but not IRF7, was required for HDM-induced airway allergy. We show that HDM-specific T_H2 cell responses did not develop in HDM-treated IRF3^{-/-} mice, which was due to impaired DC maturation and migration. Thus this study demonstrates for the first time a requirement for IRF3 in airway allergy.

METHODS**Mice**

IRF3^{-/-}²⁸ and IRF7^{-/-}²⁹ mice were purchased from the RIKEN BioResource Center, and TIR domain-containing adaptor inducing IFN- β (TRIF)^{-/-} mice³⁰ were purchased from the Jackson Laboratory (Bar Harbor, Me). IFN- α/β receptor 2 (IFNAR2)^{-/-} mice³¹ were a gift from Dr M. Aguet (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). WT C57Bl/6 mice were purchased from Harlan Nederland. All transgenic mice were backcrossed in total for at least 16 generations onto a C57Bl/6 background. All mice were bred and housed in institutional specific pathogen-free facilities. Age-paired groups of female animals were used at 8 to 10 weeks of age. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Liege. We also followed the "Guide for the care and use of laboratory animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996).

Reagents

Lyophilized HDM (*Dermatophagoides pteronyssinus*) extract was from Greer Laboratories (Lenoir, NC; endotoxin contents: 0.84 ng/mg; ie, 0.084 ng per intranasal administration). Carboxyfluorescein succinimidyl ester (CFSE) and 4',6'-diamidino-2-phenylindole were from Invitrogen (Carlsbad, Calif). Methacholine and recombinant murine IFN- β 1 were from Sigma-Aldrich (St Louis, Mo). Monensin solution was from eBioscience (San Diego, Calif). Recombinant murine GM-CSF was kindly provided by Dr Kris Thielemans (Medical School of the Vrije Universiteit Brussel, Brussels, Belgium).

Antibodies

Phycoerythrin-conjugated anti-F4/80 (BM8), allophycocyanin-conjugated anti-CD11c (N418), eFluor 450-anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD3e (17A2), and biotinylated anti-CD19 (MB19-1) were from eBioscience. Biotinylated anti-MHC class II (I-Ab; AF6-120.1) and allophycocyanin-conjugated anti-IL-4 (11.B.11) were from BD Biosciences (Mississauga, Ontario, Canada). Fluorescein isothiocyanate-conjugated anti-CD40 (3/23) and anti-CD86 (PO3) were from Serotec (Raleigh, NC). Pacific-Blue-streptavidin was from Molecular Probes Invitrogen. Information on the isotype controls used is provided in Table E1 (available in this article's Online Repository at www.jacionline.org).

Induction of airway allergy

Lightly isoflurane-anesthetized mice received an intranasal instillation of vehicle (LPS-free saline) or HDM extract on days 0, 7, and 14 (100 μ g in 50 μ L). Airway hyperresponsiveness was assessed, and all mice were killed on day 17.

Measurement of airway hyperresponsiveness

Dynamic airway resistance was measured in anesthetized animals by using a flexiVent small animal ventilator (SCIREQ), as previously described.³² Changes were expressed as percentage change from the baseline measurement (performed after PBS exposure).

Bronchoalveolar lavage and cytology

The trachea was catheterized, and the lungs were washed with 1 mL of ice-cold Mg- and Ca-free PBS containing 0.6 mmol/L EDTA. Cell density in bronchoalveolar lavage fluid was assessed by the use of a hemocytometer. Differential cell counts were performed on cytopsin preparations stained with Diff-Quick (Dade Behring, Eschborn, Germany).

Lung histology

Lungs were fixed in 10% formalin, paraffin embedded, and cut in 5- μ m sections. The extent of peribronchial inflammation was estimated by a score calculated by means of quantification of peribronchial inflammatory cells in lung sections stained with hematoxylin and eosin, as described previously.³³ Mucus production was quantified as the percentage of periodic acid-Schiff-stained goblet cells per total epithelial cells in randomly selected bronchi. Seven randomly selected sections were analyzed per murine lung.

Determination of serum levels of total IgE

Total IgE serum levels were measured by means of ELISA (Bethyl Laboratories, Montgomery, Tex), according to the manufacturer's protocol.

Restimulation of bronchial lymph node cells

Bronchial lymph node (BLN) cells (2×10^5 cells in a 96-well plate) were cultured in Click medium supplemented with 0.5% heat-inactivated murine serum and additives with or without HDM (30 μ g/mL). The proliferation was measured as tritiated thymidine incorporation during the last 16 hours of a 4-day culture. Culture supernatants were assayed for IL-4, IL-5, IL-13, and IFN- γ by means of ELISA (Biosource/Invitrogen).

Assessment of HDM-specific T-cell activation

Mice were intranasally administered either 100 μ g of HDM or vehicle (LPS-free saline). Briefly, 5 days later, BLN cells were labeled with CFSE (5 μ mol/L), washed, and cultured *in vitro* for 5 days in complete Click medium, as reported above. For the last 6 hours, cells were cultured with 10 ng/mL phorbol 12-myristate 13-acetate and 0.5 μ g/mL ionomycin and in the presence of 2 μ mol/L monensin for the last 5 hours of culture. Cells were first extracellularly stained for CD4, CD8, and CD19 and then stained intracellularly for IL-4 and analyzed by means of flow cytometry.

Lung cell preparation

Lungs were lavaged with 1 mL of ice-cold Mg- and Ca-free PBS containing 0.6 mmol/L EDTA. This procedure was repeated 10 times. To obtain single lung cell suspensions, lungs were then perfused with PBS through the right ventricle, minced, and digested for 1 hour at 37°C in 1 mg/mL collagenase A (Roche, Mannheim, Germany) and 0.05 mg/mL DNaseI (Roche) in HBSS. Debris were removed by using 70 μ m diameter cell strainers (BD Falcon).

Flow cytometry and cell sorting

Staining reactions were performed at 4°C. Cells were first incubated with 2.4G2 Fc receptor antibodies to reduce nonspecific binding. Flow cytometry was performed on a FACScanto II (Becton Dickinson, Mountain View, Calif). Lung DCs were sorted on a FACSaria (Becton Dickinson) as CD11c⁺ cells, whereas F4/80⁺ CD11c⁺ alveolar macrophages were excluded.

Cell migration

WT and IRF3^{-/-} lung DCs were pulsed with HDM in a 16-hour culture and then labeled with CFSE (2 μ mol/L) for 10 minutes at 37°C. Cells were washed and instilled into the trachea of naive recipients (4×10^5 cells). Control mice received vehicle. Twenty-four hours later, BLNs were collected, minced, and digested for 20 minutes at 37°C in 1 mg/mL collagenase A (Roche) and 0.05 mg/mL DNaseI (Roche) in HBSS. CFSE⁺ F4/80⁻ CD11c⁺ cells were detected by means of flow cytometry.

Cell viability

Viability of CFSE-labeled lung DCs and bone marrow–derived dendritic cells (BMDCs) before adoptive transfer was assessed by means of 4',6'-diamidino-2-phenylindole staining and subsequent flow cytometric analysis.

T_H2 sensitization induced by intratracheal administration of HDM-pulsed BMDCs

BMDCs were differentiated from the bone marrow of naive mice, as previously described.³² At day 8 of culture, BMDCs were pulsed overnight with 60 μ g/mL HDM extract. Control BMDCs were left unpulsed. At day 9, BMDC supernatants were assayed for IFN- β 1 and IL12p70 by means of ELISA (PBL and R&D, Minneapolis, Minn, respectively). Then 1×10^6 cells were injected into the tracheas of anesthetized naive WT recipients. Ten days later, mice were challenged on 2 consecutive days intranasally with 5 μ g of HDM. Three days after the last challenge, the mice were killed, and airway allergy was evaluated.

Statistical analysis

Data are presented as means $\pm$ SDs. The differences between mean values were estimated by using pairwise Student *t* tests after Anderson-Darling tests for assessment of normality of the data distributions. (In all figures ° and * symbols are used to indicate significant differences observed when comparing control- versus HDM-treated groups and WT versus IRF3^{-/-} HDM-treated groups, respectively [° and **P* < .05, °° and ***P* < .01, and °°° and ****P* < .001].)

RESULTS

IRF3^{-/-} mice, but not IRF7^{-/-} mice, are protected from HDM-induced airway allergy

In WT mice repeated intranasal instillation of HDM induces most of the features of allergic asthma, namely eosinophilic airway inflammation, mucus cell hyperplasia, airway hyperreactivity, and increased serum IgE titers.^{21,22,34} We used a previously described model of acute exposure to HDM,²¹ consisting of intranasal instillation of HDM once a week for 3 weeks. Using this model, we first compared allergic airway sensitization to HDM in WT and IRF3^{-/-} mice.

In contrast to WT mice, after HDM treatment, IRF3^{-/-} mice did not display significantly increased numbers of inflammatory cells in the bronchoalveolar lavage fluid (Fig 1, A). This observation was reflected at the histologic level. Indeed, HDM-treated IRF3^{-/-} mice had reduced peribronchial and perivascular inflammatory cell infiltration compared with WT mice (Fig 1, B, and see Fig E1, A, in this article's Online Repository at www.jacionline.org). Similarly, mucus cell hyperplasia in response to HDM was not detected in HDM-treated IRF3^{-/-} mice (Fig 1, C, and see Fig E1, B). In line with their decreased allergic inflammation, after HDM instillations, IRF3^{-/-} mice also did not have the increased IgE serum levels observed in WT mice (Fig 1, D). Finally, assessment of airway hyperreactivity to methacholine by using invasive measurement of dynamic resistance in mechanically ventilated mice indicated that HDM-treated IRF3^{-/-} mice displayed a significantly decreased responsiveness to methacholine compared with their WT counterparts (Fig 1, E).

We next compared HDM-induced allergic airway inflammation in WT and IRF7^{-/-} mice. In contrast with the marked differences observed between WT and IRF3^{-/-} mice, IRF7^{-/-} mice displayed parameters of allergic airway inflammation similar to those of WT mice in response to HDM exposure (see Fig E2 in this article's Online Repository at www.jacionline.org).

Thus IRF3, but not IRF7, is required for HDM-induced allergic airway inflammation.

IRF3 is required for initiation of HDM-specific T_H2 responses

The quasiabsence of allergic airway inflammation in HDM-exposed IRF3^{-/-} mice suggested that these mice were unable to have T_H2 responses. We therefore assessed for anti-HDM responses in the draining BLNs of HDM-treated WT and IRF3^{-/-} mice.

First, 3 days after the last saline or HDM instillation, BLN cells of WT and IRF3^{-/-} mice were isolated and cultured *in vitro* with or without HDM. BLN cell proliferation was estimated by measurement of tritiated thymidine incorporation. On HDM restimulation, as opposed to BLN cells from HDM-treated WT mice, BLN cells from HDM-treated IRF3^{-/-} mice failed to proliferate significantly more than BLN cells from saline-treated mice (Fig 2, A). ELISA measurement of cytokine concentrations in HDM-restimulated BLN culture supernatants revealed decreased production of IL-4, IL-5, and IL-13, 3 T_H2 cytokines, by BLN cells from HDM-treated IRF3^{-/-} mice (Fig 2, B).

Second, to more directly assess HDM-specific T_H2 responses, BLN cells from WT and IRF3^{-/-} mice exposed to a single saline or HDM treatment were isolated, labeled with CFSE, and restimulated *in vitro* with HDM. Cell viability was high after CFSE labeling and not significantly different between WT and IRF3^{-/-} cells (data

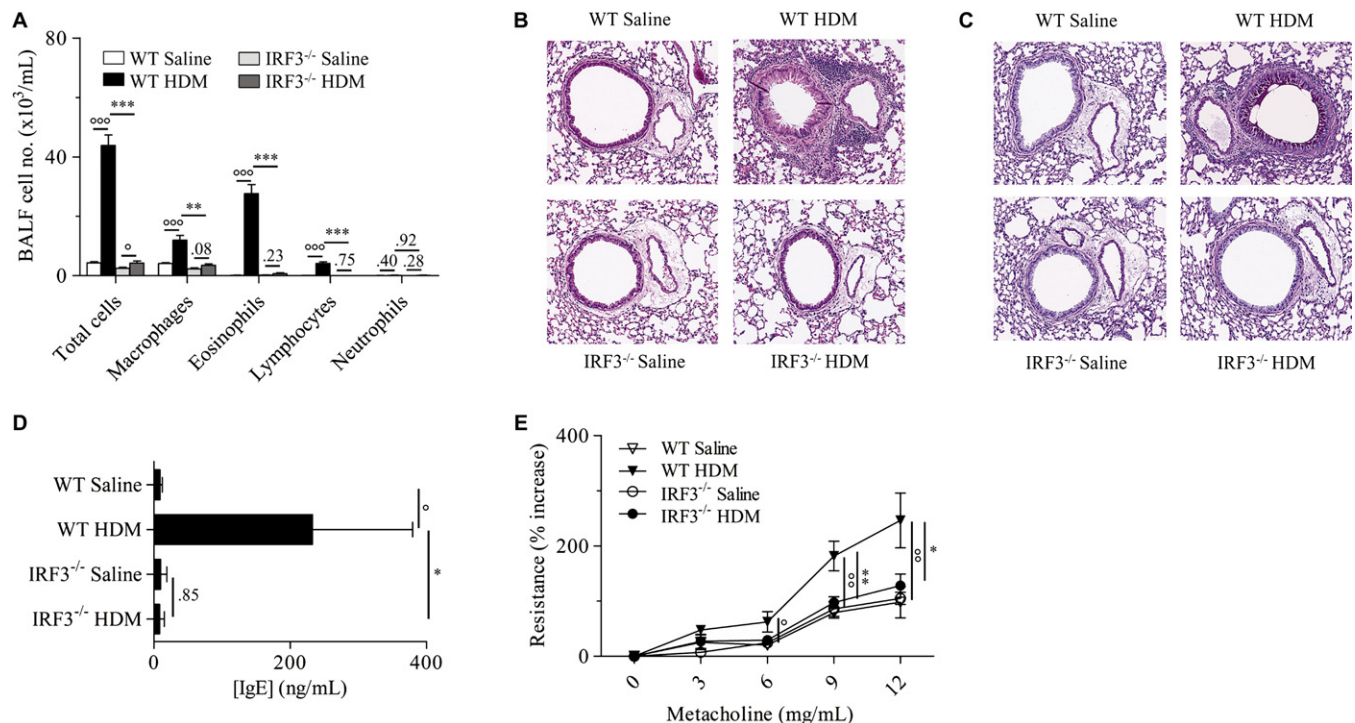


FIG 1. IRF3^{-/-} mice are protected from HDM-induced airway allergy. WT and IRF3^{-/-} mice were treated once a week for 3 consecutive weeks with saline or HDM. **A**, Total and differential immune cell counts in bronchoalveolar lavage fluid (BALF). **B** and **C**, Hematoxylin and eosin (Fig 1, B) and periodic acid-Schiff (Fig 1, C) staining of lung sections (original magnification $\times 100$). **D**, ELISA measurement of serum IgE levels. **E**, Measurement of dynamic airway resistance ($n = 6$). Comparable results were obtained in 3 independent experiments.

not shown). Unstimulated cells did not proliferate (data not shown), nor did CD8⁺ or CD19⁺ cells proliferate in response to HDM (data not shown). In contrast, CD4⁺ T cells from HDM-treated WT mice proliferated strongly in response to HDM (Fig 2, C and D). In comparison, CD4⁺ T cells from HDM-treated IRF3^{-/-} mice proliferated significantly less (Fig 2, C and D) and also were less numerous in terms of expressing IL-4 (Fig 2, E).

Taken together, these results demonstrate that HDM-specific T_H2 responses were impaired in IRF3^{-/-} mice.

Impaired maturation and migration of DCs in IRF3^{-/-} mice exposed to HDM

The inability of IRF3^{-/-} mice to mount normal HDM-specific T_H2 responses might have been indicative either of a proliferation defect of IRF3^{-/-} T cells or of deficient antigen presentation to T cells. Proliferation in response to CD3 and CD28 stimulation was comparable between WT and IRF3^{-/-} T cells (see Fig E3 and the Methods section in this article's Online Repository at www.jacionline.org). We therefore assessed for the maturation of lung DCs and for their migration to the BLNs in HDM-treated WT and IRF3^{-/-} mice.

Study of surface expression of maturation markers revealed that lung DCs (identified as CD11c⁺ cells after exclusion of F4/80⁺ CD11c⁺ alveolar macrophages³²) from IRF3^{-/-} mice displayed a more immature phenotype than did their WT counterparts after HDM treatment, as evidenced by their reduced surface expression of MHC class II, CD40, and CD86 18 hours after treatment (Fig 3, A, and see Fig E4 in this article's Online Repository at www.jacionline.org).

We next assessed for the recruitment of DCs to the BLNs of HDM-treated WT and IRF3^{-/-} mice. We observed that, in contrast to WT mice, DC numbers did not increase in the BLNs of IRF3^{-/-} mice 24 hours after HDM treatment (Fig 3, B). This suggested impaired migration of DCs in IRF3^{-/-} mice. This impairment could be due either to a DC-intrinsic defect or to deficient instruction by other cells. Lung DCs from WT and IRF3^{-/-} mice were purified by means of cell sorting (>94% purity), cultured overnight in the presence of HDM, labeled with CFSE, and injected into the trachea of recipient syngeneic WT mice to test these alternatives. Survival after CFSE labeling was high and not significantly different for IRF3^{-/-} and WT DCs (data not shown). Fig 3, C, shows that lung DCs from IRF3^{-/-} mice migrated significantly less to the BLNs than did their WT counterparts. Because recipient mice all were on a WT background, these results suggest that the defect in the induction of allergic airway sensitization in IRF3^{-/-} mice might lie in intrinsically impaired DC maturation and migration.

IRF3^{-/-} DCs do not induce airway allergy

We next performed DC transfer experiments to test whether a DC-intrinsic defect was sufficient to explain protection of IRF3^{-/-} mice against HDM-induced airway allergy. Because lung DCs are too scarce to perform large-scale experiments, we used BMDCs differentiated from the bone marrow of WT and IRF3^{-/-} mice in the transfer experiments. This model is relevant because it was recently shown that BMDCs display a phenotype very similar to that of lung DCs on allergenic challenge of the airways.³⁵ Differentiation into BMDCs and BMDC survival were

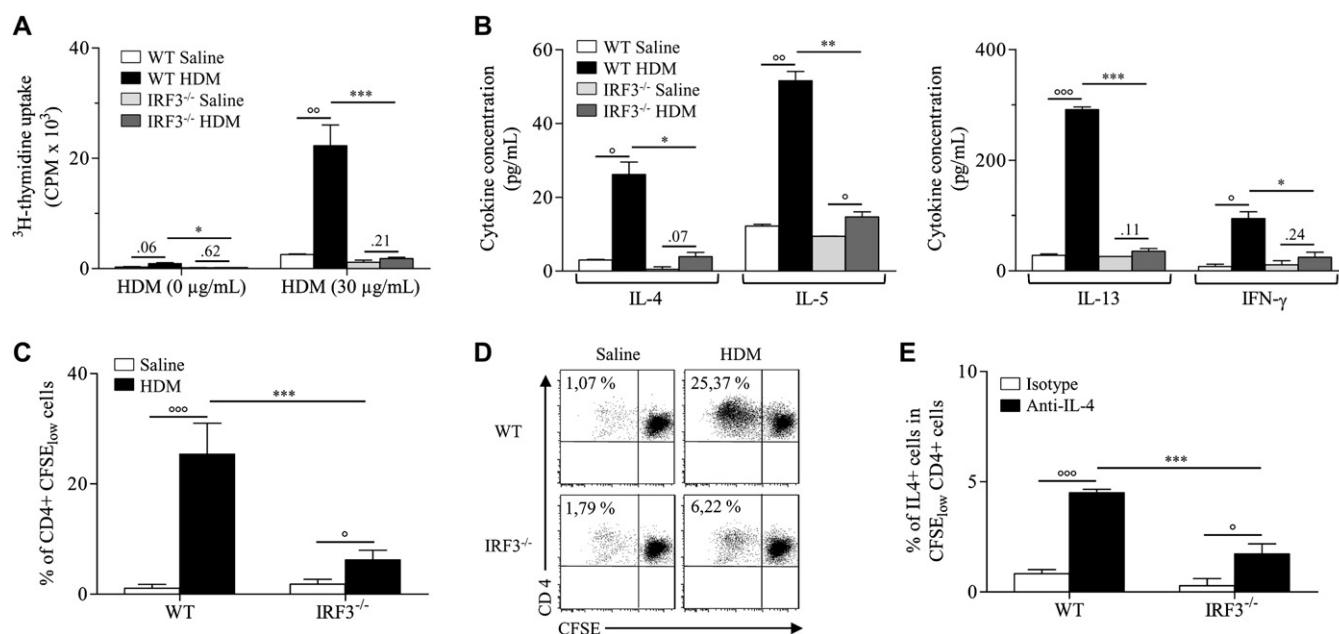


FIG 2. IRF3^{-/-} mice display impaired TH2 cytokine secretion and HDM-specific TH2 cell differentiation in response to HDM. **A**, Proliferation of BLN cells from saline- or HDM-treated WT and IRF3^{-/-} mice after *in vitro* HDM stimulation. **B**, ELISA measurement of IL-4, IL-5, IL-13, and IFN- γ in the supernatants of HDM-stimulated BLN cells. **C**, Percentages among CD4⁺ BLN cells of activated CD4⁺ T cells that proliferated in response to HDM (identified as CD4⁺ CFSE_{low} cells). **D**, Representative histograms of samples compared in Fig 2, C. **E**, Percentages of IL-4⁺ cells among CD4⁺ CFSE_{low} cells (n = 4). Comparable results were obtained in 3 independent experiments.

similar for bone marrow precursors from WT and IRF3^{-/-} mice (data not shown).

Consistent with the observation that lung DCs from IRF3^{-/-} mice have an intrinsic maturation defect, BMDCs from IRF3^{-/-} mice, compared with WT BMDCs, displayed a less mature phenotype after overnight HDM stimulation *in vitro* (Fig 4, A, and see Fig E5 in this article's Online Repository at www.jacionline.org). They also secreted lower amounts of IL-12p70 (Fig 4, B), a marker for their activation.³²

Adoptive transfer of antigen-loaded WT BMDCs induces allergic airway inflammation on subsequent exposure to aerosolized antigen.³⁶ We adapted this protocol, loading BMDCs with HDM and evoking allergic airway inflammation by means of a daily intranasal instillation of low-dose HDM for 2 days. This regimen of HDM exposure did not induce significant airway inflammation in mice transferred with control unloaded BMDCs. However, in WT mice that received HDM-loaded WT BMDCs, HDM exposure induced airway eosinophilia (Fig 5, A), peribronchial and perivascular inflammatory cell infiltration (Fig 5, B, and see Fig E6, A, in this article's Online Repository at www.jacionline.org), and mucus cell hyperplasia (Fig 5, C, and see Fig E6, B). In contrast, these parameters were much attenuated in the same conditions when WT mice were transferred with HDM-loaded BMDCs derived from IRF3^{-/-} mice (Fig 5, A-C, and see Fig E6). In addition, BLN cell proliferation and TH2 cytokine production were reduced in BLN cells from recipient WT mice that received HDM-loaded IRF3^{-/-} BMDCs compared with that seen in mice that received WT HDM-loaded BMDCs (Fig 5, D and E). Thus IRF3^{-/-} DCs are intrinsically unable to induce allergic airway sensitization to HDM.

Importantly, IRF3^{-/-} mice did not appear to display deficiencies leading to impaired TH2 responses in processes other

than those related to antigen presentation. Indeed, IRF3^{-/-} mice that received HDM-loaded WT BMDCs displayed normal parameters of airway allergy (see Fig E7 and the Methods section in this article's Online Repository at www.jacionline.org). Furthermore, the deficit of IRF3^{-/-} DCs in inducing TH2 responses to HDM was specifically dependent on impaired IRF3 activation by HDM. Indeed, IRF3^{-/-} BMDCs stimulated with an adjuvant that does not signal through IRF3, namely Pam3CSK4, a synthetic lipoprotein ligand of TLR2, were able to induce normal TH2 responses to OVA in recipient WT mice (see Fig E8 and the Methods section in this article's Online Repository at www.jacionline.org).

HDM-induced DC maturation and airway allergy are independent of type I interferon signaling

IRF3 is a central inducer of type I interferon gene expression,^{37,38} and type I interferons were reported to play an important autocrine role in the maturation of antigen-presenting cells in response to activation of certain PRRs.³⁹ We therefore examined whether the defects observed in IRF3^{-/-} DCs were due to impaired type I interferon expression.

Even though IRF3^{-/-} BMDCs displayed impaired IFN- β 1 secretion in response to HDM (Fig 6, A), their incubation with recombinant IFN- β 1 in concentrations similar to those secreted by WT BMDCs was not sufficient to compensate for their maturation defect (Fig 6, B, and see Fig E9 in this article's Online Repository at www.jacionline.org). Also, IFN- β 1 complementation at higher concentrations of 1 and 5 ng/mL was not sufficient either (data not shown). IRF3 therefore seems to promote DC maturation through type I interferon-independent mechanisms.

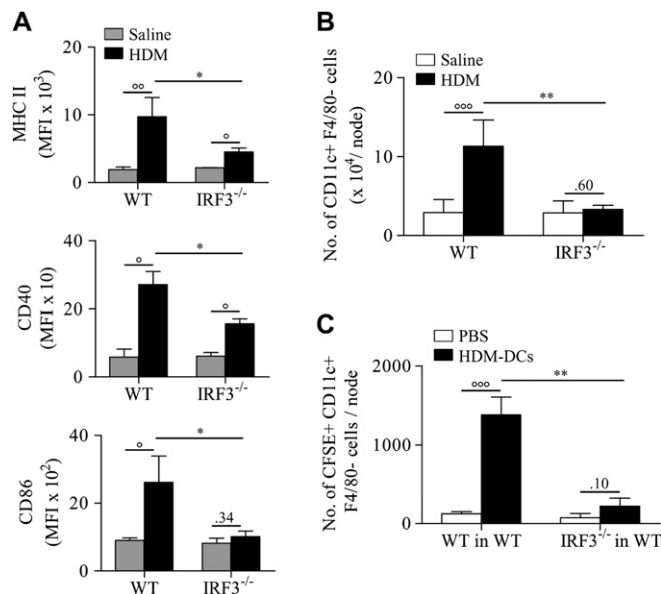


FIG 3. Immature phenotype and deficient migration of lung DCs from IRF3^{-/-} mice on HDM exposure. **A**, Expression of MHC class II, CD40, and CD86 on lung DCs of saline- or HDM-treated WT and IRF3^{-/-} mice. **B**, Number of DCs in the BLNs of saline- and HDM-treated WT and IRF3^{-/-} mice. **C**, Number of CFSE⁺ DCs in the BLNs of WT mice transferred with PBS- or HDM-treated lung DCs labeled with CFSE (n = 4). Comparable results were obtained in 3 independent experiments. *MFI*, Mean fluorescence intensity.

In agreement with this observation, mice deficient for chain 2 of the type I interferon receptor (IFNAR2^{-/-} mice) displayed similar airway allergy parameters as WT mice in response to HDM (see Fig E10, A-F, in this article's Online Repository at www.jacionline.org). DCs in IFNAR2^{-/-} mice migrated normally to the BLNs in response to HDM treatment (see Fig E10, G). Furthermore, BLN cells from IFNAR2^{-/-} mice proliferated and expressed T_H2 cytokines normally in response to HDM restimulation (see Fig E10, H and I). These results thus unambiguously indicate that the migratory and proallergic potential of DCs in response to HDM exposure are dependent on IRF3 independently of type I IFN signaling.

The IRF3-dependent proallergic effects of HDM are independent of TLR4 signaling through TRIF

HDM might activate TLR4 through its endotoxin content or mimicry of MD-2,²³ and TLR4 might activate IRF3 through a TRIF-dependent, MyD88-independent signaling pathway.⁴⁰ Thus we finally sought to determine the potential contribution of TLR4-mediated IRF3 activation to HDM-induced airway allergy.

To this end, we compared HDM-induced airway allergy in WT and TRIF^{-/-} mice, in which TLR4 signaling to IRF3 is disrupted. Indeed, we could not use TLR4^{-/-} mice for this purpose because TLR4-dependent MyD88 signaling is required for HDM to induce airway allergy.^{21,22} We observed that TRIF^{-/-} mice had parameters of airway allergy in response to HDM similar to those of their WT counterparts (see Fig E11, A-F, in this article's Online Repository at www.jacionline.org). DCs in TRIF^{-/-} mice were also recruited normally to the BLNs in response to HDM treatment (see Fig E11, G). Finally, BLN cells from TRIF^{-/-} mice proliferated and expressed T_H2 cytokines normally in response to HDM

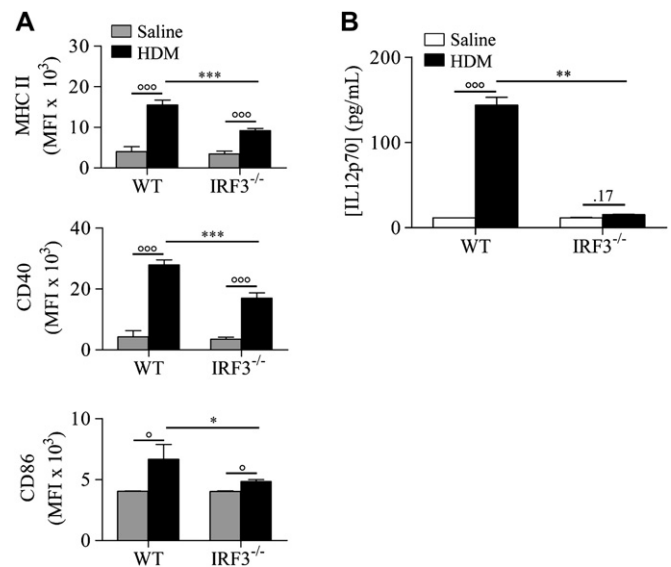


FIG 4. Deficient maturation of IRF3^{-/-} BMDCs exposed to HDM *in vitro*. **A**, Expression of MHC class II, CD40, and CD86 on saline- or HDM-treated WT and IRF3^{-/-} BMDCs. **B**, ELISA measurement of IL12p70 secretion in the supernatants of BMDC cultures (n = 3). Comparable results were obtained in 3 independent experiments. *MFI*, Mean fluorescence intensity.

restimulation (see Fig E11, H and I). These results thus rule out that TLR4 and endotoxin play a significant role in the IRF3-dependent effects of HDM on the proallergic functions of DCs.

DISCUSSION

Accumulating evidence points to a role for PRRs in the pathophysiology of airway allergy. Here we identify IRF3, a transcription factor downstream of numerous PRRs, as a central player in allergic airway sensitization to HDM. Indeed, we demonstrate that IRF3^{-/-} mice are unable to develop allergic airway inflammation in a murine model of HDM-induced allergic asthma. We further show that DC function is intrinsically impaired in the absence of IRF3. This is reflected by a less mature phenotype of DCs, as well as by their impaired migration to the lung draining lymph nodes on HDM exposure in IRF3^{-/-} mice compared with that seen in WT mice. IRF3^{-/-} DCs being unable to display a mature phenotype and migrate to the BLNs, they do not induce the HDM-specific T_H2 responses that sustain airway allergy. Thus our results present solid evidence that IRF3 is required for lung DCs to evoke T_H2-mediated airway allergy in response to allergen exposure.

To date, studies in murine models of allergic asthma using the clinically relevant allergen HDM have concurred in demonstrating the role of TLR4 and MyD88 in the development of allergic airway disease.^{21,22} Our results indicate that IRF3 also is required for HDM-induced allergic airway sensitization. Intriguingly, although TLR4 signaling through MyD88 is required for airway allergy,^{21,22} TRIF^{-/-} mice had airway allergy, and their DCs behaved normally in response to HDM. As a result, endotoxin and TLR4 signaling do not appear to play a significant role in the IRF3-dependent mechanisms of HDM-induced airway allergy. IRF3 being cell-intrinsically required for DC function, this observation is in agreement with reports indicating that TLR4 expression on DCs is dispensable for HDM-induced airway allergy.²¹ Thus HDM acts through multiple PRR signaling pathways to

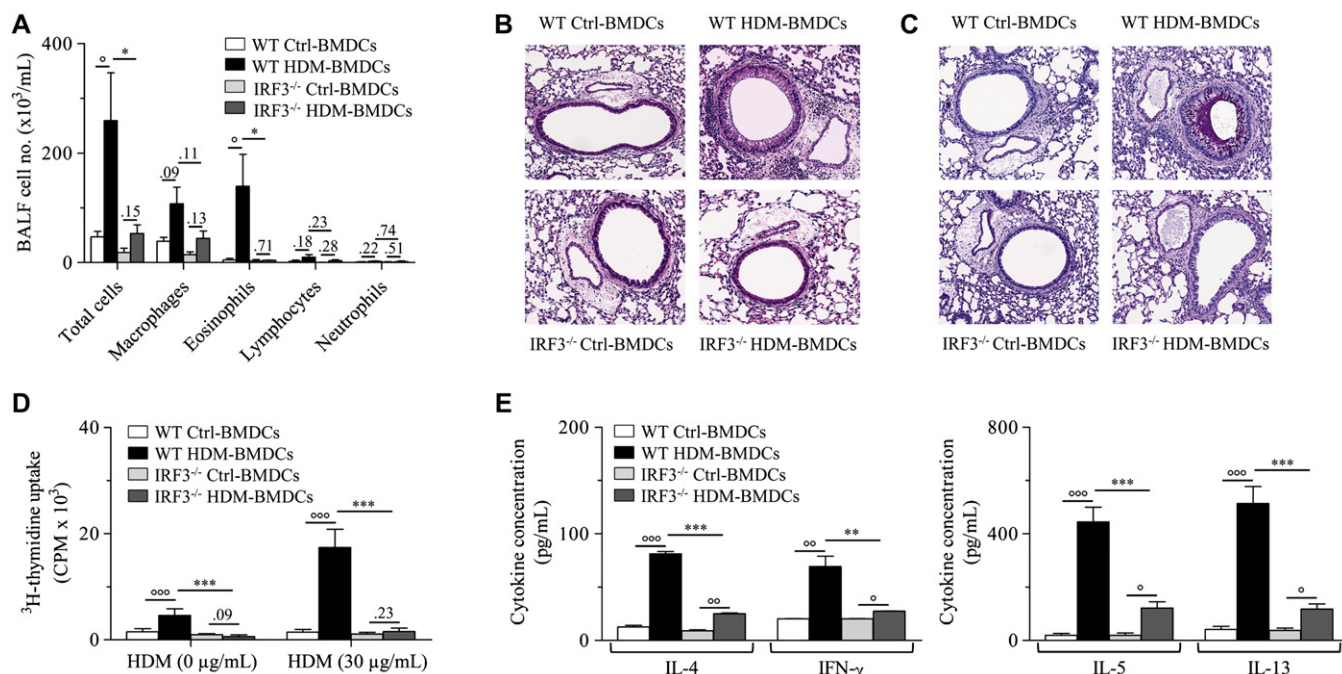


FIG 5. HDM-loaded IRF3^{-/-} DCs do not induce airway allergy. WT mice received intratracheal saline (*Ctrl-BMDCs*) or HDM (*HDM-BMDCs*)-loaded WT or IRF3^{-/-} BMDCs and were challenged with intranasal HDM. **A**, Total and differential immune cell counts in the bronchoalveolar lavage fluid (BALF). **B** and **C**, Hematoxylin and eosin (**B**) and periodic acid-Schiff (**C**) staining of lung sections (original magnification $\times 100$). **D**, BLN cell proliferation in response to *in vitro* HDM stimulation. **E**, ELISA for IL-4, IL-5, IL-13, and IFN- γ on culture supernatants of HDM-stimulated BLN cells ($n = 6$). Comparable results were obtained in 3 independent experiments.

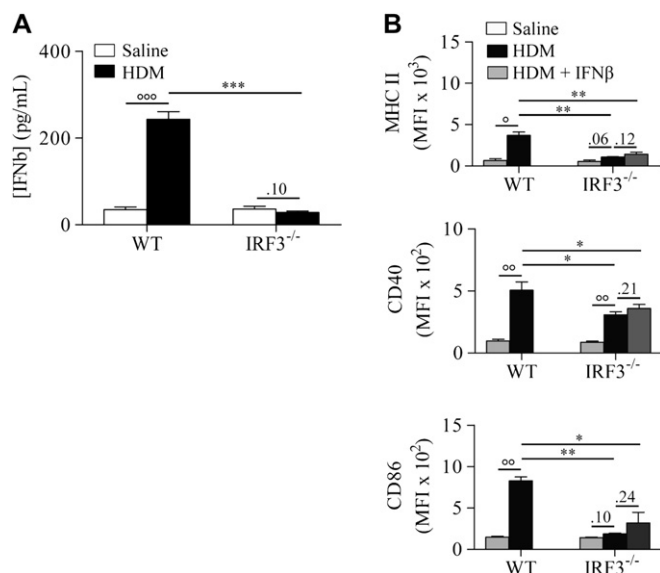


FIG 6. Type I interferon-independent maturation of DCs in response to HDM. **A**, ELISA measurement of IFN- β secretion in the supernatants of saline- or HDM-treated WT and IRF3^{-/-} BMDCs. **B**, Expression of MHC class II, CD40, and CD86 on WT and IRF3^{-/-} BMDCs treated with saline, HDM, or HDM and 250 pg/mL recombinant IFN- β ($n = 3$). Comparable results were obtained in 3 independent experiments. MFI, Mean fluorescence intensity.

induce airway allergy. The identification of the PRRs triggered by HDM to activate IRF3 in DCs will, however, require further experimentation.

Studies on TLR4 and MyD88 have suggested that these proteins are required for the instruction of DCs to induce adaptive T_H2 responses. In the study by Hammad et al,²¹ a correlation was found between impaired TLR4 signaling in lung radio-resistant cells, reduced airway allergy, and deficient lung expression of proinflammatory and proallergic cytokines, such as thymic stromal lymphopoietin and IL-33. To explain these results, the authors argued that it was likely that deficient instruction of lung DCs by HDM-induced cytokine clues from the lung environment did not allow for proper induction of allergic adaptive immune responses. On the other hand, Phipps et al²² observed that in response to HDM exposure *in vivo* lung DCs from TLR4- or MyD88-deficient mice did not display a fully mature phenotype, and migrated less than their WT counterparts to the lung draining lymph nodes. Thus these studies tend to support a role for TLR4 and MyD88 signaling in instructing DCs to mature, migrate, and promote airway allergy. Similarly, in our study IRF3 deficiency also affected DC maturation and migration to the draining lymph nodes. However, although the study of Hammad et al²¹ indicated that TLR4 and MyD88 signaling are not required in DCs themselves for allergic sensitization, our observations showed that TLR4-independent activation of IRF3 in DCs themselves is required to allow their maturation and migration in response to HDM. Taken together, these observations suggest that HDM targets DCs and other lung cells simultaneously but through different pathways to induce proallergic activity of lung DCs.

IRF3 is a master regulator of type I interferon gene expression.^{37,38} Autocrine type I interferon signaling is required for TLR-induced upregulation of costimulatory molecules in certain antigen-presenting cells^{39,41} but not in conventional DCs.⁴² In

line with this last observation, complementation of IRF3^{-/-} BMDCs with type I interferons did not restore their maturation in response to HDM stimulation. Furthermore, IFNAR2^{-/-} mice displayed normal DC migration and allergic airway sensitization in response to HDM. It is therefore likely that HDM induces airway allergy independently of type I interferon signaling. This hypothesis is further supported by the absence of any role for IRF7 in airway allergy in our model. Thus these results indicate that DC maturation and migration in response to HDM require IRF3-dependent but type I interferon-independent DC-intrinsic mechanisms. In addition to type I interferons, IRF3 regulates the expression of numerous other genes potentially implicated in the pathophysiology of airway allergy. These include cytokines, such as thymic stromal lymphopoietin, IL-6, IL12p35, CCL3, CCL5, CXCL10, and type III interferons.⁴³⁻⁴⁷ Identification of the effectors downstream of IRF3 in DCs in airway allergy will thus require further investigation.

Clinical evidence points to a synergism between allergic sensitization and viral respiratory tract infections in inducing severe asthma exacerbations.⁴⁸⁻⁵¹ IRF3 is a central orchestrator of antiviral responses to common respiratory tract viruses.^{38,52} Consequently, viral respiratory tract infections might represent episodes during which IRF3 is activated by both viral products and allergens in the airways of allergic subjects. Synergism might therefore exist during this period of life to promote proallergic functions of lung DCs and contribute to asthma exacerbations. This hypothesis is currently under investigation in our laboratory.

In conclusion, this study provides experimental evidence that IRF3 signaling is an essential component of allergic airway sensitization to clinically relevant allergens. In doing so, it implicates IRF3 in allergy and a T_H2-mediated disease for the first time. It furthermore indicates that IRF3 is required for proallergic DC functions through type I interferon-independent mechanisms.

We thank Professor T. Taniguchi (Graduate School of Medicine and Faculty of Medicine University of Tokyo, Tokyo, Japan) and RIKEN BioResource Center for providing IRF3^{-/-} and IRF7^{-/-} mice and Dr M. Aguet (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland) for providing IFNAR2^{-/-} mice. We also thank Sandra Ormenese and the Cell Imaging and Flow Cytometry GIGA Technological Platform for FACS analyses, Drs Pierre-Vincent Drion and Gilles Gaudray for animal management, and Valérie Conrath, Luc Duwez, Raja Fares, Cédric François, Fabrice Olivier, Julien Parisi, Fabienne Perin, and Ilham Sbati for excellent technical and secretarial assistance.

Key Messages

- IRF3 is required for experimental HDM-induced airway allergy.
- IRF3 is required for the proallergic functions of lung DCs.

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METHODS

Sensitivity of ELISAs

Sensitivities were as follows: IgE, 0.1 ng/mL; IL-4, less than 5 pg/mL; IL-5, less than 3 pg/mL; IL-13, less than 2 pg/mL; IFN- γ , 1 pg/mL; IFN- β 1, 15.6 pg/ml; and IL12p70, less than 2.5 pg/mL.

T-cell stimulation

T cells were purified from BLNs by using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and assessed for purity by staining for CD3 ϵ followed by flow cytometry. Cells (2×10^5 cells) were then cultured with anti-CD28 antibodies (5 μ g/mL; 37.51, eBioscience) in RPMI supplemented with 10% heat-inactivated FCS and additives into 96 well plates coated with anti-CD3 antibodies (10 μ g/mL; 145-2C11, eBioscience). Controls were cultured in uncoated wells without anti-CD28 antibodies. The proliferation was measured as tritiated thymidine incorporation during the last 16 hours of a 2-day culture.

T_H2 sensitization and airway allergy induced by means of intratracheal administration of HDM-pulsed BMDCs

At day 8 of culture, WT BMDCs were pulsed overnight with 60 μ g/mL HDM extract. BMDCs (1×10^6) were injected into the tracheas of

anesthetized naive WT and IRF3^{-/-} recipients. Ten days later, mice received again 1×10^6 HDM-loaded WT BMDCs and 5 μ g of HDM to evoke airway allergy. Three days after the challenge, mice were killed, and airway allergy was evaluated.

T_H2 responses induced by means of intratracheal administration of Pam3CSK4-stimulated OVA-pulsed BMDCs

At day 8 of culture, WT and IRF3^{-/-} BMDCs were pulsed overnight with 125 μ g/mL LPS-free OVA (Endograde Ovalbumin; endotoxin content <1 EU/mg, Hyglos, Regensburg, Germany) in the presence of 0.3 μ g/mL LPS-free Pam3CSK4 (Invivogen, San Diego, Calif). BMDCs (1×10^6) were injected into the tracheas of anesthetized naive WT recipients. Five days later, BLN cells were isolated and cultured in Click medium supplemented with 0.5% heat-inactivated murine serum and additives, with or without OVA (50 μ g/mL). The proliferation was measured as tritiated thymidine incorporation during the last 16 hours of a 4-day culture. Culture supernatants were assayed for IL-4, IL-5, IL-13, and IFN- γ by means of ELISA (Biosource/Invitrogen).

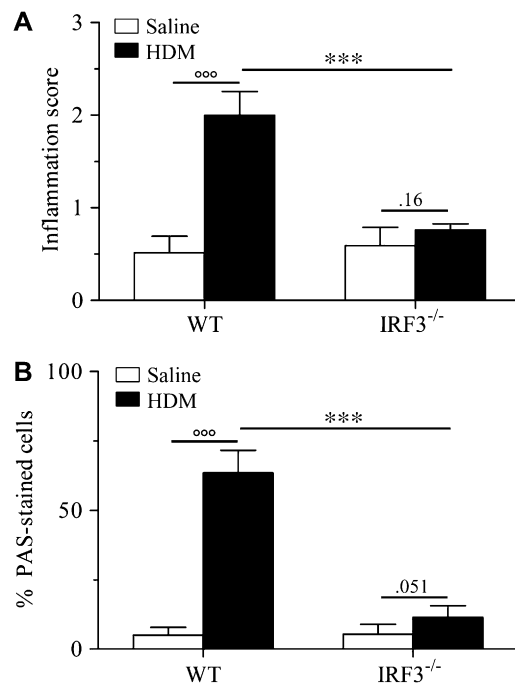


FIG E1. IRF3^{-/-} mice are protected from HDM-induced allergic airway inflammation and mucus cell hyperplasia. **A**, Inflammatory scores estimated from hematoxylin and eosin staining of lung sections. **B**, Percentage of periodic acid–Schiff (PAS)-stained goblet cells per total epithelial cells (n = 6). Comparable results were obtained in 3 independent experiments.

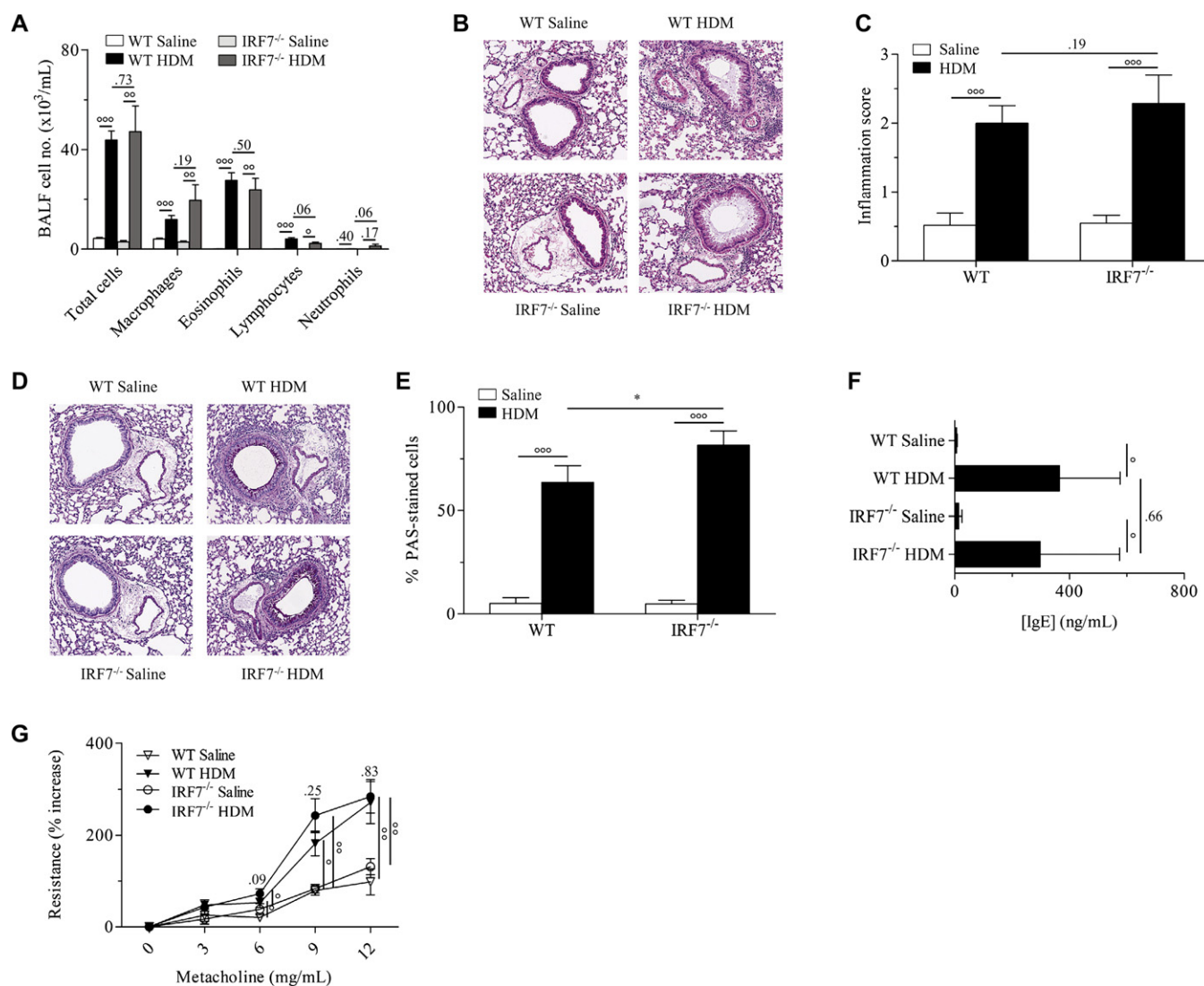


FIG E2. IRF7^{-/-} mice are not protected from HDM-induced airway allergy. WT and IRF7^{-/-} mice were treated once a week for 3 consecutive weeks with saline or HDM. **A**, Total and differential immune cell counts in bronchoalveolar lavage fluid (BALF). **B** and **C**, Representative section (original magnification $\times 100$; Fig E2, B) and inflammatory scores (Fig E2, C) of hematoxylin and eosin-stained lung sections. **D** and **E**, Representative staining (original magnification $\times 100$; Fig E2, D) and percentage (Fig E2, E) of periodic acid-Schiff-stained goblet cells per total epithelial cells in randomly selected bronchi. **F**, ELISA measurement of serum IgE levels. **G**, Measurement of dynamic airway resistance ($n = 6$). Comparable results were obtained in 3 independent experiments.

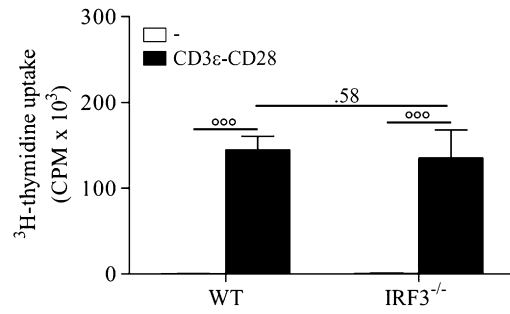


FIG E3. WT and IRF3 $^{-/-}$ T lymphocytes have similar proliferation potential. Proliferation of T cells purified from BLNs of WT and IRF3 $^{-/-}$ mice (>95% purity) after stimulation with anti-CD28 and anti-CD3 antibodies is shown.

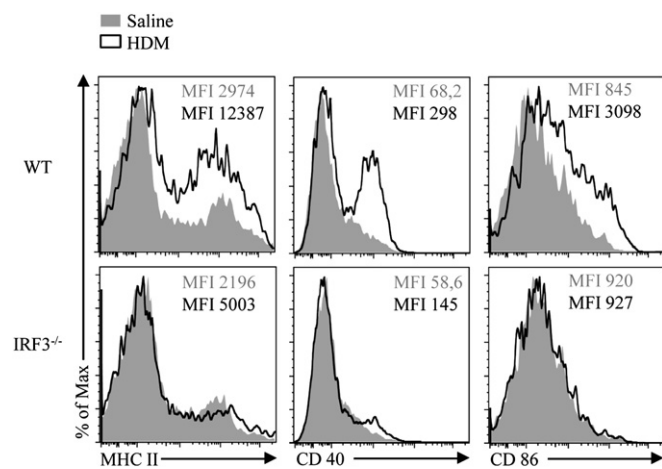


FIG E4. Representative histograms of the expression of MHC class II, CD40, and CD86 on lung DCs of saline- or HDM-treated WT and IRF3^{-/-} mice 18 hours after treatment. *Inserts* indicate the mean fluorescence intensity of the signal for each marker in saline-treated (*gray font*) and HDM-treated (*black font*) mice.

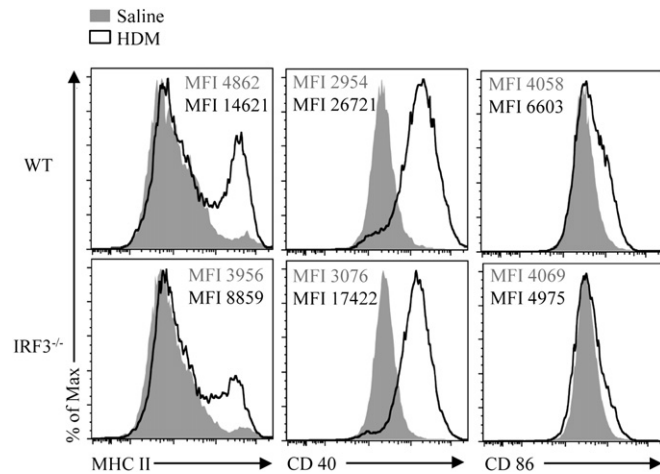


FIG E5. Representative histograms of the expression of MHC class II, CD40, and CD86 on saline- or HDM-treated WT and IRF3^{-/-} BMDCs after overnight treatment. *Inserts* indicate the mean fluorescence intensity of the signal for each marker in saline-treated (*gray font*) and HDM-treated (*black font*) BMDCs.

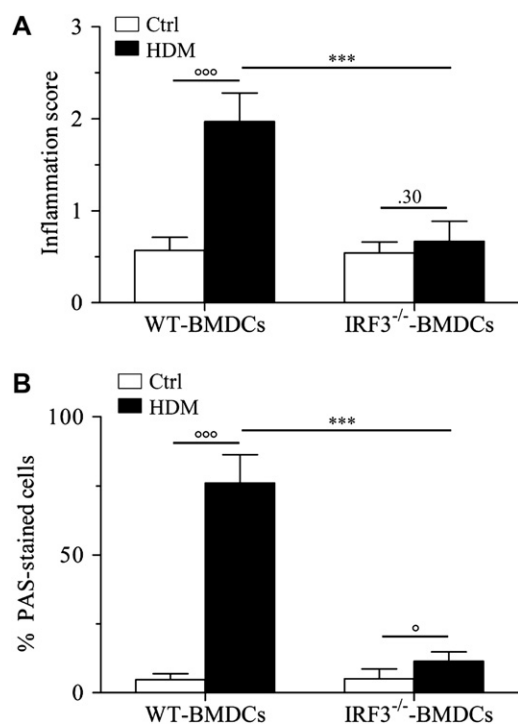


FIG E6. HDM-loaded IRF3^{-/-} DCs do not induce allergic airway inflammation and mucus cell hyperplasia. WT mice received intratracheal saline (*Ctrl-BMDCs*) or HDM (*HDM-BMDCs*)-loaded WT or IRF3^{-/-} BMDCs and were challenged with intranasal HDM. **A**, Inflammatory scores estimated from hematoxylin and eosin staining of lung sections. **B**, Percentage of periodic acid Schiff (PAS)-stained goblet cells per total epithelial cells ($n = 6$). Comparable results were obtained in 3 independent experiments.

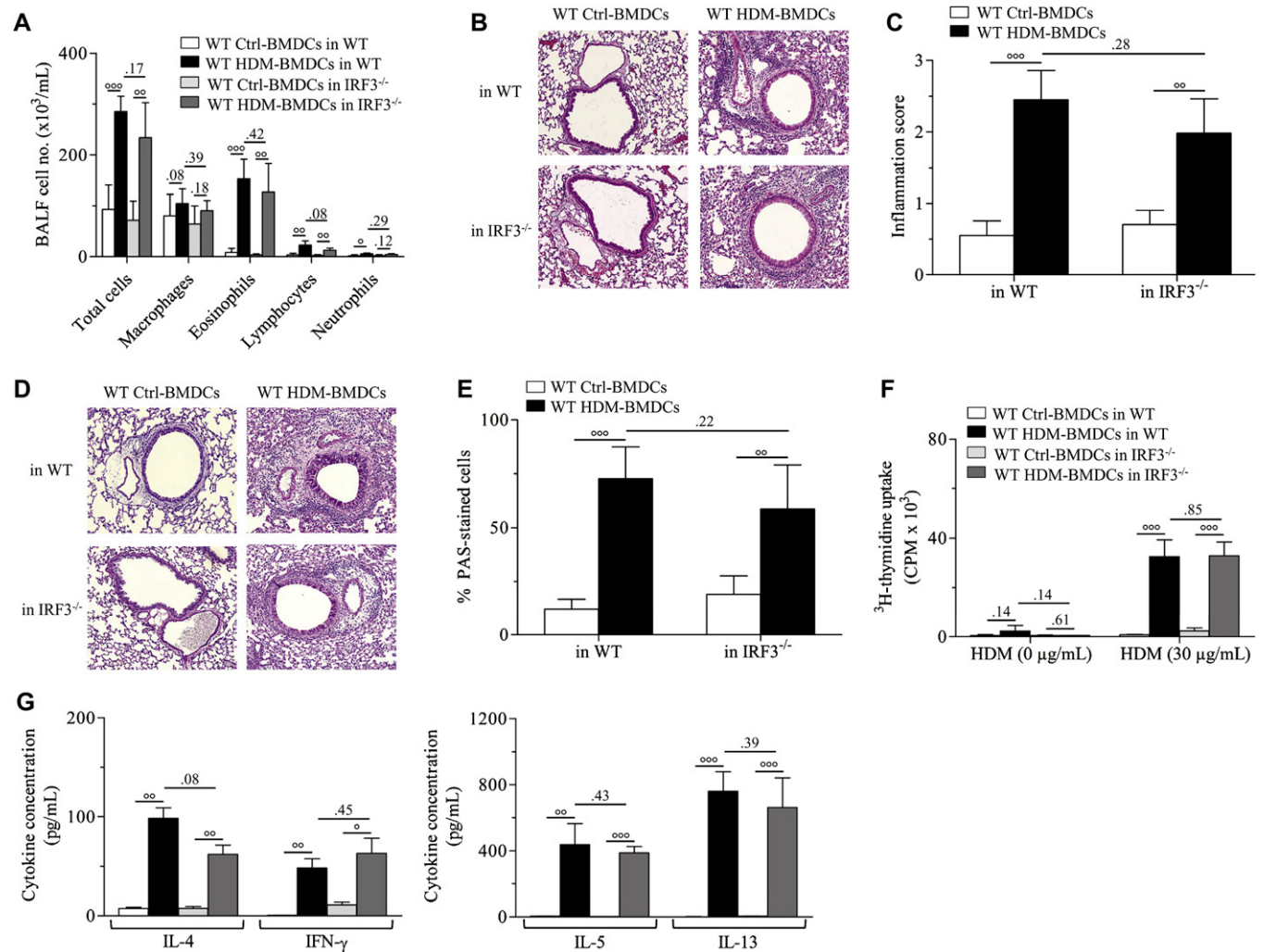


FIG E7. IRF3^{-/-} mice display normal airway allergy after transfer of WT HDM-loaded DCs. WT and IRF3^{-/-} mice received intratracheal saline (Ctrl-BMDCs) or HDM (HDM-BMDCs)-loaded WT BMDCs and were challenged with control or HDM-WT BMDCs and a low dose of HDM. **A**, Total and differential immune cell counts in the bronchoalveolar lavage fluid (BALF). **B** and **C**, Representative section (original magnification $\times 100$; Fig E7, B) and inflammatory scores (Fig E7, C) of hematoxylin and eosin staining of lung sections. **D** and **E**, Representative staining (original magnification $\times 100$; Fig E7, D) and percentage (Fig E7, E) of periodic acid Schiff-stained goblet cells per total epithelial cells in randomly selected bronchi. **F**, BLN cell proliferation in response to *in vitro* HDM stimulation. **G**, ELISA for IL-4, IL-5, IL-13, and IFN- γ on culture supernatants of HDM-stimulated BLN cells ($n = 4$). Comparable results were obtained in 2 independent experiments.

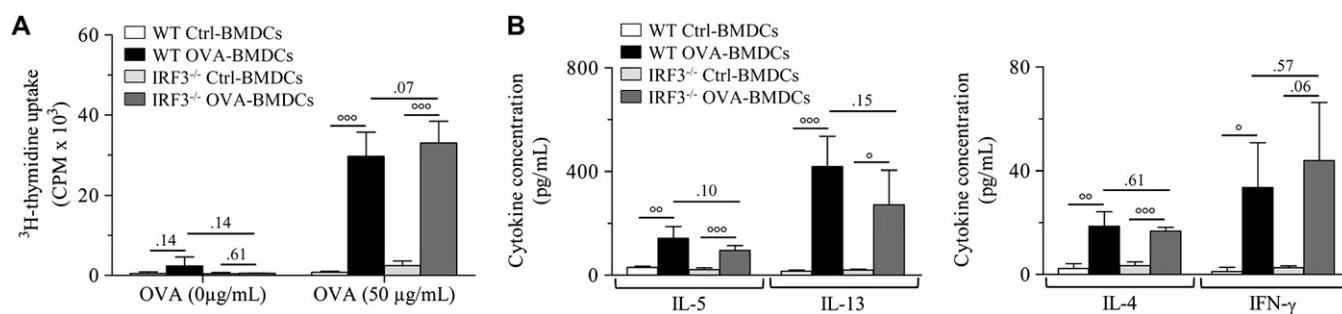


FIG E8. IRF3^{-/-} DCs induce normal T_H2 responses when stimulated with an IRF3-independent adjuvant. WT and IRF3^{-/-} BMDCs were loaded (*OVA-BMDCs*) or not (*Ctrl-BMDCs*) with OVA and stimulated with Pam3CSK4 before intratracheal administration to WT recipient mice. **A**, BLN cell proliferation in response to *in vitro* OVA stimulation. **B**, ELISA for IL-4, IL-5, IL-13, and IFN- γ on culture supernatants of OVA-stimulated BLN cells (n = 4). Comparable results were obtained in 2 independent experiments.

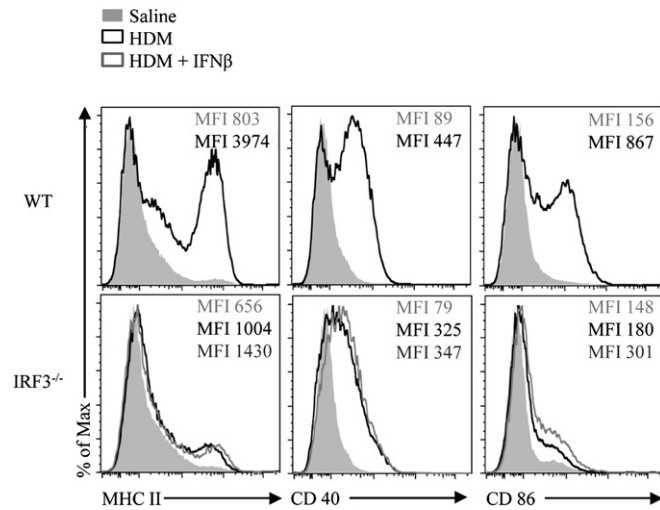


FIG E9. Representative histograms of the expression of MHC class II, CD40, and CD86 on WT and IRF3^{-/-} BMDCs treated with saline, HDM, or HDM and 250 pg/mL recombinant IFN- β 18 hours after treatment.

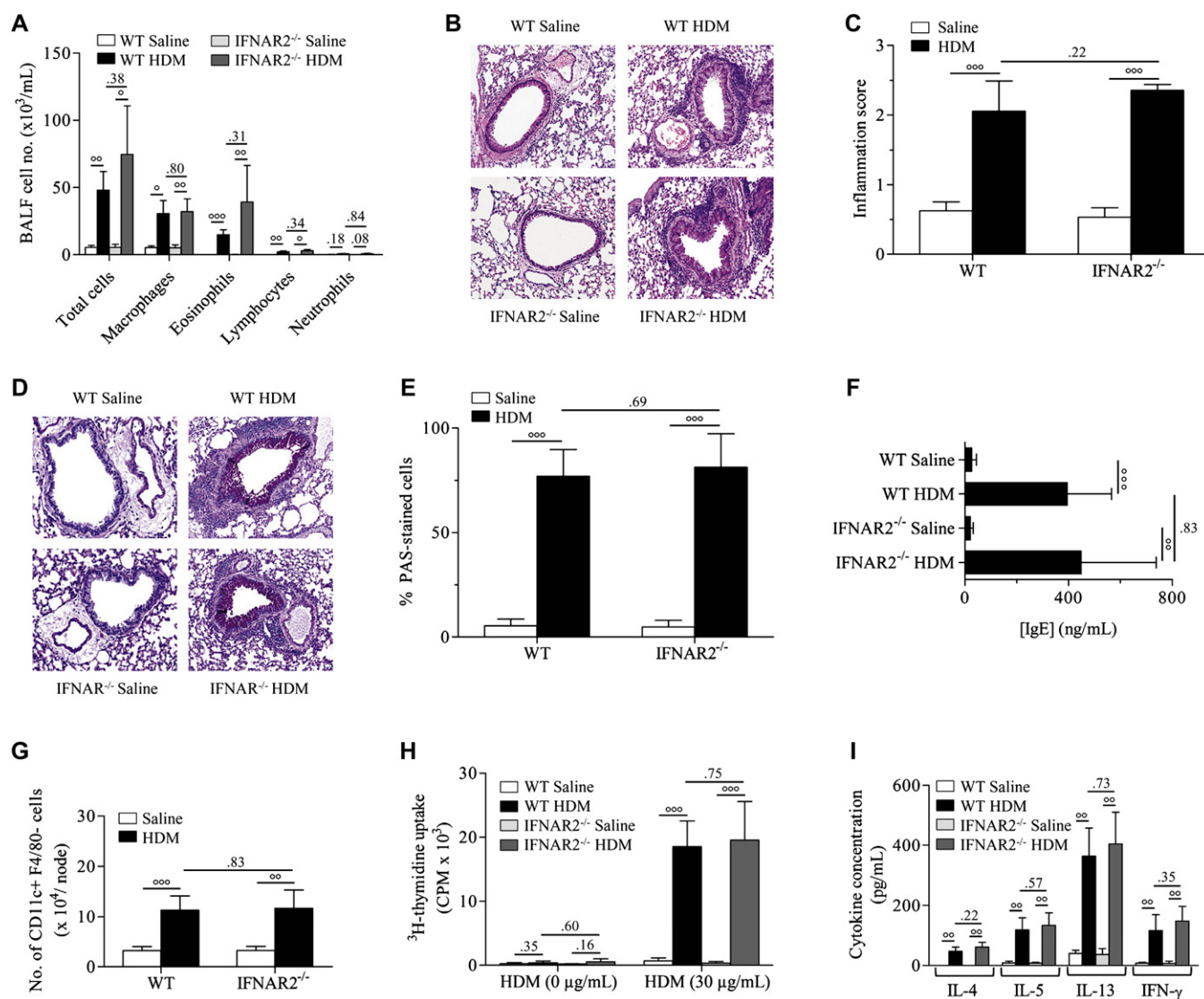


FIG E10. HDM-induced TH2 responses and airway allergy are independent of type I interferon signaling. WT and IFNAR2^{-/-} mice were treated once a week for 3 consecutive weeks with saline or HDM. **A**, Total and differential immune cell counts in the bronchoalveolar lavage fluid (BALF). **B** and **C**, Representative section (original magnification ×100; Fig E10, B) and inflammatory scores (Fig E10, C) of hematoxylin and eosin staining of lung sections. **D** and **E**, Representative staining (original magnification ×100; Fig E10, D) and percentage (Fig E10, E) of periodic acid–Schiff–stained goblet cells per total epithelial cells in randomly selected bronchi. **F**, ELISA measurement of serum IgE levels. **G**, Number of DCs in the BLNs of saline- and HDM-treated WT and IFNAR2^{-/-} mice. **H**, BLN cell proliferation in response to *in vitro* HDM stimulation. **I**, ELISA for IL-4, IL-5, IL-13, and IFN-γ on culture supernatants of HDM-stimulated BLN cells (n = 4). Comparable results were obtained in 2 independent experiments.

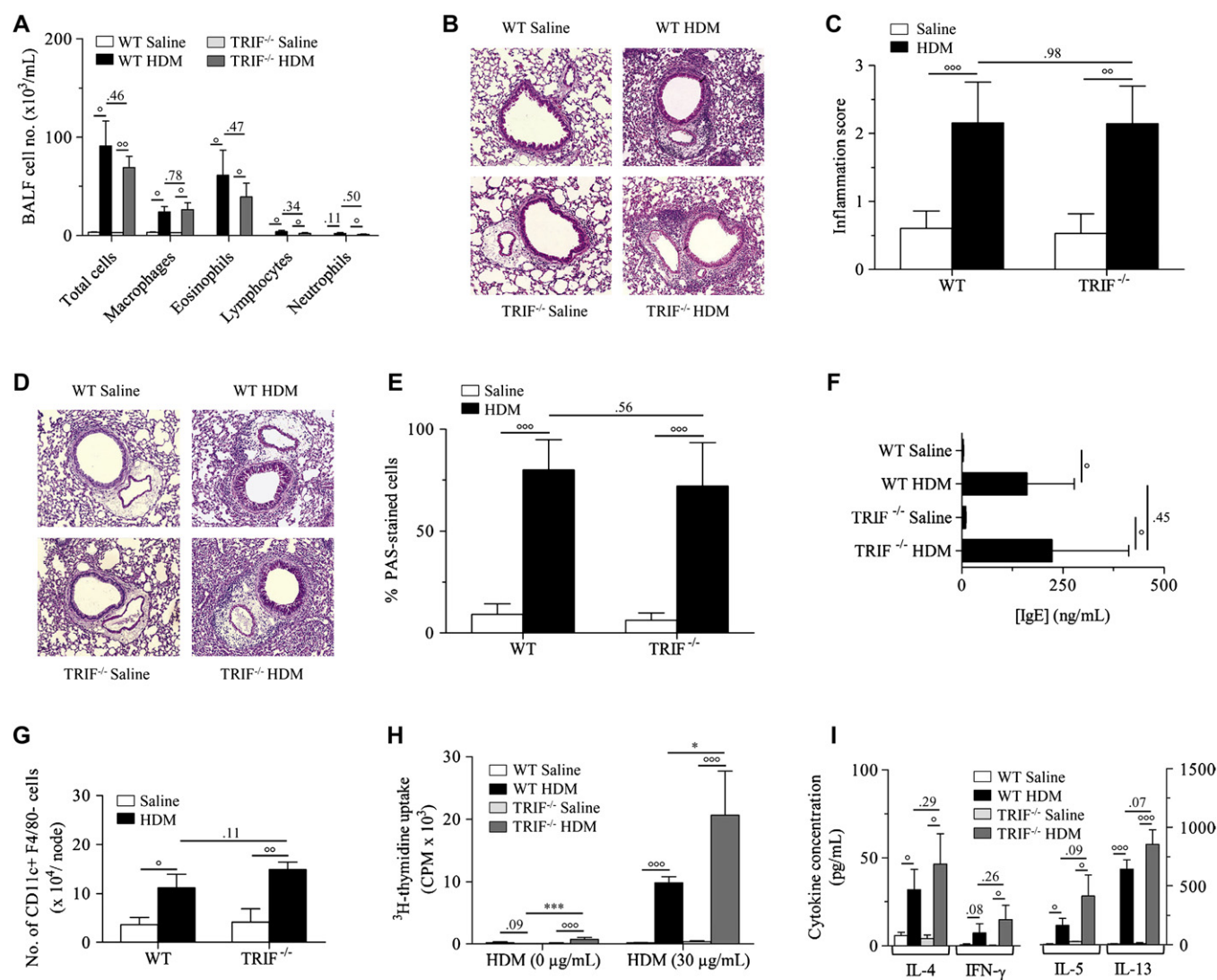


FIG E11. IRF3-dependent T_H2 responses and airway allergy in response to HDM are independent of TRIF signaling. WT and TRIF^{-/-} mice were treated once a week for 3 consecutive weeks with saline or HDM. **A**, Total and differential immune cell counts in the bronchoalveolar lavage fluid (BALF). **B** and **C**, Representative section (original magnification $\times 100$; Fig E11, **B**) and inflammatory scores (Fig E11, **C**) of hematoxylin and eosin staining of lung sections. **D** and **E**, Representative staining (original magnification $\times 100$; Fig E11, **D**) and percentage (Fig E11, **E**) of periodic acid-Schiff-stained goblet cells per total epithelial cells in randomly selected bronchi. **F**, ELISA measurement of serum IgE levels. **G**, Number of DCs in the BLNs of saline- and HDM-treated WT and TRIF^{-/-} mice. **H**, BLN cell proliferation in response to *in vitro* HDM stimulation. **I**, ELISA for IL-4, IL-5, IL-13, and IFN- γ on culture supernatants of HDM-stimulated BLN cells ($n = 4$). Comparable results were obtained in 2 independent experiments.

TABLE E1. List of the antibodies and their respective isotype control used in the study

Anti-mouse antibody (clone)	Host/isotype	Manufacturer
PE–anti-F4/80 (BM8)	Rat/PE-IgG2a	eBioscience
APC–anti-CD11c (N418)	Armenian hamster/APC-IgG	eBioscience
eFluor 450–anti-CD4 (RM4-5)	Rat/eFluor 450-IgG2a	eBioscience
eFluor 450–anti-CD8 (53-6.7)	Rat/eFluor 450-IgG2a	eBioscience
eFluor 450–anti-CD3ε (17A2)	Rat/eFluor 450-IgG2b	eBioscience
Biotin–anti-MHC class 2 (I-Ab; AF6-120.1)	Mouse (BALB/c)/biotin-IgG2a, κ	BD bioscience
APC–anti-IL-4 (11.B.11)	Rat/APC-IgG1, κ	BD bioscience
FITC–anti-CD40 (3/23)	Rat/FITC-IgG2a	Serotec
FITC–anti-CD86 (PO3)	Rat/FITC-IgG2b	Serotec

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.