

Serial OX40 Engagement on CD4⁺ T Cells and Natural Killer T Cells Causes Allergic Airway Inflammation

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Rationale: OX40–OX40 ligand (OX40L) interactions have been proposed to support induction of allergic airway inflammation, which may be attributable to OX40 signaling in CD4⁺ helper T cells for adaptive immune responses. However, a possible involvement of natural killer T (NKT) cells in the pathogenesis suggests that the underlying mechanisms are not yet fully elucidated.

Objectives: We aimed to characterize the OX40-modulated cellular contribution to allergic airway inflammation in a mouse model of house dust mite (HDM) allergen exposure.

Methods: Mice were sensitized to HDM and, 3 weeks later, challenged with HDM on three consecutive days through the airways. Two days after the last exposure, bronchoalveolar lavage fluids and blood samples and lung tissues were evaluated for the airway inflammation.

Measurements and Main Results: The development of HDM-induced eosinophilic airway inflammation was dependent on OX40 of both CD4⁺ T cells and NKT cells; OX40 engagement on CD4⁺ T cells in the sensitization led to pulmonary OX40L augmentation after the allergen challenge, which stimulated pulmonary NKT cells through OX40 to provide the pathogenic cytokine milieu. This was ablated by OX40L blockade by inhalation of the neutralizing antibody during the challenge, suggesting the therapeutic potential of targeting pulmonary OX40–OX40L interactions. Moreover, OX40 expression in CD4⁺ T cells, but not in NKT cells, was reciprocally regulated by the helper T cell type 1–skewing transcription factor Runx3.

Conclusions: OX40 on not only CD4⁺ T cells but also NKT cells is involved in allergic airway inflammation. Notably, pulmonary blockade of OX40 ligation on NKT cells has therapeutic implications.

Keywords: environmental illness; inhalation therapy; neutralizing antibodies

Allergic airway diseases such as bronchial asthma are characterized by the presence of numerous eosinophils in the airway (1–3). The development of the inflammatory process is mediated by helper T cell type 2 (Th2) cytokines, including IL-4, IL-5, and IL-13, which increase the proliferation and differentiation of eosinophils as well as IgE-producing B cells, and recruit these cells to the lungs (2–4). A major source of Th2 cytokines has been attributed to activated CD4⁺ T cells, and thus the costimulatory requirement for effective CD4⁺ T cell responses has been studied

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

OX40 is a T cell–lineage costimulatory receptor believed to be important in allergic lung disease, because of its capacity to provide conventional CD4⁺ T cells with activation signals in promoting adaptive immunity.

What This Study Adds to the Field

We report that OX40 acts on CD4⁺ T cells to achieve allergen-specific sensitization in primary inhalational exposure to an aeroallergen, and also acts on natural killer T cells to develop allergic disease symptoms on secondary exposure. The inflammatory responses were reduced by instillation of a blocking anti-OX40L antibody through the airway in the reexposure phase, suggesting the pulmonary OX40–OX40L interaction as a potential therapeutic target.

with respect to the pathogenesis of allergic lung inflammation (4–13).

Among the costimulatory molecules, particular emphasis has been placed on OX40 (also known as CD134 and TNFRSF4), a member of the tumor necrosis factor receptor superfamily, because of its key activity in stimulating helper activity of CD4⁺ T cells (5, 8, 9, 12–16). OX40 is a 50-kD glycosylated protein that interacts with the extracellular domains of OX40 ligand (OX40L; also known as CD252 and TNFSF4) preferentially expressed on antigen-presenting cells (APCs) (14–16). It is well established that OX40 and its ligand can be induced after activation, and their interaction consequently serves to promote optimal generation of antigen-reactive effector CD4⁺ T cells (14–16). On the basis of this evidence, reduced severity of allergic airway disease in the systemic absence or neutralization of OX40–OX40L interactions has been ascribed to impaired CD4⁺ T cell responses (5, 8, 9, 12, 13).

In addition to CD4⁺ T cells, OX40 costimulation allows the functional activity of other immune cells (14–16). In this context, we and others have demonstrated that OX40 is expressed on invariant natural killer T cells (referred to here as “NKT cells”), and signals transmitted through OX40 can directly increase NKT cell activity (17–19). NKT cells perform innate-like immunomodulatory functions by releasing massive amounts of cytokines, which are thought to underlie the inflammatory cascade of bronchial asthma (20–24). However, the molecular and cellular mechanisms by which NKT cells are involved in allergic airway diseases remain to be clarified, and the clinical implications of NKT cells in the pathogenesis are controversial (25–27).

Here, we show that when mice were sensitized and challenged by inhalation of house dust mite (HDM), an allergen related to human allergic asthma, the development of allergic

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airway inflammation was contingent on OX40 on both CD4⁺ T cells, during the sensitization phase, and NKT cells, during the challenge phase. In the sensitization phase, OX40 engagement on CD4⁺ T cells is required for subsequent pulmonary OX40L induction, which then in the challenge phase provides NKT cells with the OX40 signal to augment the production of Th2 cytokines, thereby enhancing airway eosinophilic responses. The resulting pulmonary OX40–OX40L interactions are underscored as a potential therapeutic target by our finding that the local prevention of the interactions, by an inhaled antagonist of OX40L, ameliorated allergic lung inflammation.

Some of the results of these studies have been previously reported in abstract form at an American Thoracic Society international conference (28).

METHODS

Mice

C57BL/6 mice (*H-2^b*) were purchased from Japan Charles River (Yokohama, Japan). CD4⁺ T cell-deficient mice (B6.129S2-*Cd4^{tm1Mak}*) were obtained from Jackson Laboratory (Bar Harbor, ME) (29). OX40-deficient, NKT cell-deficient, and Runx3-transgenic mice were generated as described previously and have been backcrossed to C57BL/6 mice (30–32). NKT cell-deficient mice lack the α chain of the invariant T cell receptor (TCR), α 18 (30). Runx3-Tg mice express mouse Runx3 under the control of the *lck* promoter, previously shown to be selective for conventional T cells and NKT cells (33). Animals were age (6–8 wk old) and sex (female) matched within each experiment and were housed under specific pathogen-free conditions, in accordance with the guidelines of the institutional animal care and use committee.

Allergen Sensitization and Challenge

Allergen solution (75 μ l) was instilled slowly via the airways into the lungs of anesthetized mice on Day 1 for sensitization and 3 weeks later on three consecutive days (Days 20–22) for challenge. The instilled solution contained 100 μ g of house dust mite extract from *Dermatophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC) or ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS), pH 7. Controls received PBS alone in the sensitization or challenge phase. Two days after the last airway challenge, bilateral bronchoalveolar lavage (BAL) fluids, sera, and lungs were obtained from the mice to assess airway inflammation.

BAL was obtained as described previously (34, 35). Briefly, the lungs were lavaged twice with 0.75 ml of PBS. After centrifugation ($2,300 \times g$, 5 min), the supernatants were stored for cytokine analyses, and cell pellets were resuspended in 1 ml of PBS for total and differential cell counts. Total BAL cells were counted with a hemocytometer. The BAL differential was determined from at least 200 cells in Cytospin preparations stained with Diff-Quik (Sysmex, Japan), and the cells were identified as macrophages, eosinophils, lymphocytes, or neutrophils on the basis of cellular morphology and staining characteristics. Serum was obtained by centrifugation of peripheral blood collected from the cardiac cavity. For histological assessment, lung tissue specimens were fixed with 4% formaldehyde and embedded in paraffin. The sections (5 μ m thick) were stained with hematoxylin and eosin.

ELISA

Serum levels of eotaxin and BAL fluid levels of IL-4, IL-5, and IL-13 were assayed with ELISA kits according to the manufacturer's protocol (BioSource International, Camarillo, CA). For the HDM-specific IgE assay, 96-well immunosorbent plates were coated with HDM extract (100 μ g/ml) in carbonate-bicarbonate buffer (Sigma-Aldrich). After blocking the plates with 1% bovine serum albumin in PBS, sixfold diluted mouse serum was added. The plates were incubated for 2 hours at 37°C. Horseradish peroxidase-conjugated goat anti-mouse IgE antibody (Bethyl Laboratories, Montgomery, TX) was used to detect HDM-bound IgE. The plates were developed with TMB (3,3',5,5'-tetramethylbenzidine) peroxidase substrate (Bethyl Laboratories); the reaction was stopped with H₂SO₄, and the absorbance was determined

at 450 nm. OVA-specific IgE antibodies were measured in serum with a commercial kit (DS Pharma Biomedical, Osaka, Japan).

Flow Cytometry

Single lung cell suspensions were prepared by collagenase digestion, red blood cell lysis, and discontinuous density gradient purification. For the detection of pulmonary OX40- or OX40L-positive cells, lung cells were stained with biotin-conjugated antibodies against OX40 (clone OX-86; BD Biosciences, San Jose, CA) and OX40L (clone RM134L; eBioscience, San Diego, CA), respectively, and fluorescein isothiocyanate (FITC)-conjugated streptavidin (BD Biosciences). The phenotypes of APCs and T-lineage cells were determined with phycoerythrin (PE)-conjugated antibodies against I-A^b (clone AF6–120.1) and TCR β chain (clone H57–597) (both from BD Biosciences). In some experiments, spleen cells and BAL cells were assessed for surface expression of OX40 and OX40L. The number of NKT cells per lung was examined by double staining with FITC-conjugated antibodies against TCR β and PE-conjugated α -galactosylceramide (α -GalCer)-loaded CD1d tetramers (ProImmune, Bradenton, FL). To determine the phenotype of the pulmonary NKT cell subset, TCR β ⁺ lung cells were isolated by positive selection with magnetically activated cell sorting (MACS; Miltenyi Biotec, Auburn, CA) before cell staining with CD1d tetramers. Flow cytometric measurements of cytokine production in pulmonary NKT cells were done with a Cytofix/Cytoperm Plus kit (BD Biosciences) as per the manufacturer's instructions. Cytoplasmic cytokines were stained with Alexa Fluor 647-conjugated antibodies against IL-13 (clone eBio13A; eBioscience). Flow cytometric analysis was performed with an EPICS XL cytometer, using EXPO32 ADC software (Beckman Coulter, Fullerton, CA).

Transfer of CD4⁺ T Cells and NKT Cells

Splenic CD4⁺ T cells of sensitized mice were isolated by MACS (Miltenyi Biotec) 19 days after allergen exposure. Enriched CD4⁺ T cells (2×10^6) were adoptively transferred by intravenous injection into naive mice 1 day before the 3-day consecutive allergen challenge. For NKT cell reconstitution, splenic NKT cells (10^6) that had been purified from naive mice by the use of PE-conjugated CD1d- α -GalCer tetramers and anti-PE magnetic beads (Miltenyi Biotec) were intravenously transferred into NKT^{−/−} mice that had been sensitized 19 days earlier and would be challenged with HDM on the next three consecutive days. In experiments aimed at blocking pulmonary interactions between OX40 and OX40L, a blocking monoclonal antibody against OX40L (clone MGP34, 150 μ g) was administered intranasally 8 hours before each of the HDM challenges on three consecutive days (36).

Protein Immunoblotting

Spleen cells were isolated from mice that had been sensitized 19 days earlier by intratracheal administration of HDM. The cells at 5×10^6 cells/ml were cultured with HDM (100 μ g/ml) for 3 days. After restimulation, CD4⁺ T cells were purified with MACS beads as described previously, and cell lysates were prepared. For cell lysates of NKT cells, mice were sensitized and challenged with HDM, and 2 days after the last intranasal challenge, pulmonary NKT cells were isolated by MACS, as described previously. Protein lysates from CD4⁺ T cells and NKT cells were resolved by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were immunoblotted with the primary antibodies to OX40 (clone OX-86; BD Biosciences), Runx3 (clone R3-5G4; Medical and Biological Laboratories, Nagoya, Japan), GATA3 (clone HG3-31; Santa Cruz Biotechnology, Santa Cruz, CA), or β -actin (clone AC-15; Sigma-Aldrich) with horseradish peroxidase-conjugated secondary antibodies. The blots were developed with an ECL chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ) and were analyzed for the relative amounts of proteins using Quantity One software (version 4.6.0; Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Multiple group comparisons were made by Tukey-Kramer test. A value of $P < 0.05$ was deemed to indicate statistical significance.

RESULTS

Prevention of Allergic Airway Inflammation by OX40 Deficiency

OX40 was responsible for the development of Th2-associated airway inflammation in a clinically relevant model, using HDM as an inhalant allergen (Figure 1). The experimental protocol consisted of intranasal HDM or PBS challenge on three consecutive days in mice that had been sensitized 3 weeks earlier by intratracheal administration of HDM or PBS (Figure 1A). Using this approach, HDM-sensitized and -challenged wild-type mice revealed typical allergic airway inflammation. The BAL showed increased total cellularity, reflecting increased numbers of eosinophils and lymphocytes, and elevated levels of Th2-type cytokines, including IL-4, IL-5, and IL-13 (HDM-HDM wild-type vs.

HDM-PBS and PBS-HDM, $P < 0.05$ in all measurements; Figures 1B–1E). HDM sensitization and challenge also increased the serum levels of anti-HDM IgE and eotaxin in wild-type mice, compared with PBS controls, including the HDM-PBS and PBS-HDM groups ($P < 0.05$ in both measurements; Figures 1F and 1G). Furthermore, marked peribronchial and perivascular cellular infiltration and increased epithelial thickness were evident in the HDM-HDM wild-type mice, but not in the HDM-PBS or PBS-HDM wild-type mice (Figure 1H). In contrast to wild-type mice, even after sensitization and challenge with HDM, OX40^{-/-} mice displayed morphological features of airway inflammation similar to those in both PBS controls (Figure 1H). All the hallmarks of allergic airway inflammation were significantly reduced in OX40^{-/-} mice, compared with wild-type mice ($P < 0.05$ in all measurements; Figures 1B–1G).

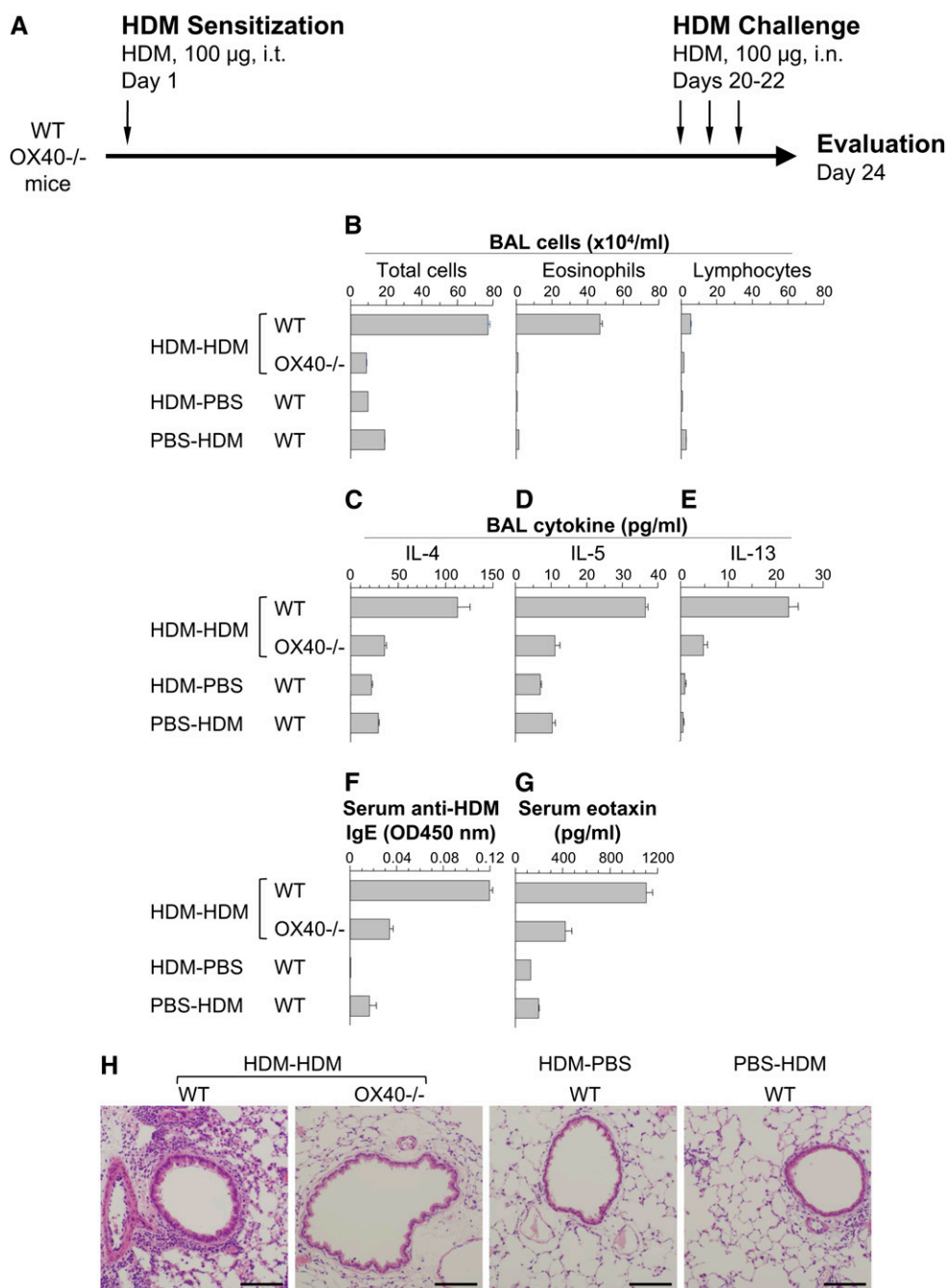


Figure 1. Ameliorated allergic airway inflammation in OX40-deficient mice. (A) Experimental scheme. Wild-type or OX40^{-/-} mice were exposed to house dust mite (HDM) via the airways at the indicated time points in the sensitization phase (Day 1) and the challenge phase (Days 20–22). Alternatively, wild-type mice were exposed to HDM on Day 1 and phosphate-buffered saline (PBS) on Days 20–22 (HDM-PBS), or were exposed to PBS on Day 1 and HDM on Days 20–22 (PBS-HDM). Two days after the last exposure, bronchoalveolar lavage (BAL) and blood samples and lung tissues were taken and evaluated. (B) Total, eosinophil, and lymphocyte airway infiltrates recovered in BAL. (C–E) Cytokine levels in BAL. (F) Anti-HDM IgE levels in serum. (G) Eotaxin levels in serum. For (B–G), the data are presented as means $\pm$ standard error ($n = 3$). (H) Histological lung sections stained with hematoxylin and eosin at an original magnification of $\times 200$. Scale bars, 100 μ m. i.n. = intranasal; i.t. = intratracheal; WT = wild-type.

Role of OX40 on CD4⁺ T Cells in the Sensitization Phase

We next analyzed the relevance of the preferential expression of OX40 on CD4⁺ T cells in the development of allergic airway inflammation (Figure 2). A genetic deficiency of CD4⁺ T cells was linked to significantly ameliorated airway inflammation with reduced airway and systemic allergic responses, compared with responses in HDM-HDM wild-type mice ($P < 0.05$ in all measurements; Figures 2A–2C). To define the role of CD4⁺ T cells in allergen specificity between the sensitization and challenge phases, CD4⁺ T cells from HDM- or OVA-sensitized mice were adoptively transferred into naive mice, followed by allergen challenge (Figure 2D). Adoptive transfer with CD4⁺ T cells from HDM-sensitized mice, but not from OVA-sensitized mice, induced HDM-specific airway inflammation in HDM-challenged recipient mice, demonstrating the HDM-specific effector function of the CD4⁺ T cells ($P < 0.05$ in all measurements except anti-OVA IgE; Figures 2E–2G). This finding was further supported by the converse observation that the transfer of CD4⁺ T cells from OVA-sensitized mice, but not from HDM-sensitized mice, resulted in OVA-specific airway inflammation on challenge with OVA ($P < 0.05$ in all measurements except anti-HDM IgE; Figures 2E–2G). Allergen-specific airway inflammation was not observed when OX40^{−/−} mice were used as donors of sensitized CD4⁺ T cells, compared with the adoptive transfer of wild-type CD4⁺ T cells ($P < 0.05$ in all measurements; Figures 2H–2J). These data indicate that the acquisition of allergen specificity in the sensitization phase is dependent on OX40 expression of CD4⁺ T cells and that these CD4⁺ T cells then mediate allergen-specific airway inflammation in the challenge phase.

Reciprocal Regulation of Runx3 and OX40 in CD4⁺ T Cells

Studies have identified the transcription factor Runx3 as a Th1/Th2 determinant that activates the Th1 pathway while repressing the Th2 pathway (37–39). We next investigated whether Th2-associated HDM airway inflammation could be regulated by Runx3 (Figures 2A–2C). After sensitization to and challenge with HDM, transgenic mice expressing Runx3 (referred to as Runx3-Tg mice) displayed reduced levels of airway leukocytes and allergic mediators, compared with HDM-HDM wild-type mice ($P < 0.05$ in all measurements; Figures 2A–2C). The reduced inflammatory responses in Runx3-Tg mice led us to examine the direct impact of Runx3 expression in CD4⁺ T cells by an adoptive transfer experiment. In naive mice subjected to HDM challenge after HDM-sensitized CD4⁺ T cell transfer, recipients of Runx3-Tg CD4⁺ T cells mirrored the phenotype of those receiving OX40^{−/−} CD4⁺ T cells, that is, significantly less allergic airway inflammation compared with mice receiving wild-type CD4⁺ T cells ($P < 0.05$ in all measurements; Figures 2H–2J).

To further examine the similarly defective function of OX40^{−/−} and Runx3-Tg CD4⁺ T cells, we examined OX40 and Runx3 protein expression in HDM-sensitized CD4⁺ T cells after *in vitro* restimulation (Figure 2K). Immunoblotting revealed the loss of OX40 and an increased protein level of Runx3 in both OX40^{−/−} and Runx3-Tg CD4⁺ T cells (Figure 2K). The expression of the Th2-specific factor GATA3 in both OX40^{−/−} and Runx3-Tg CD4⁺ T cells was about equal and was lower than that in wild-type CD4⁺ T cells. Although it is possible that the lack of OX40 simply reflects either a reduced number of Th2 cells or a reduced number of total allergen-specific CD4⁺ T cells, OX40 deficiency and forced Runx3 expression may be associated with the non-Th2 skewing in reciprocal regulation.

Lung-specific OX40 and OX40L Expression in the Challenge Phase

From a therapeutic perspective, we assessed OX40 and OX40L expression in lung cells in the challenge phase (Figure 3). As determined by flow cytometry, HDM sensitization/challenge increased the frequency of OX40L-expressing cells among lung APCs, but not among spleen APCs, in wild-type mice (OX40L⁺ lung APCs, 30%; OX40L⁺ spleen APCs, 3%; Figure 3A). This increase was not observed among the lung APCs of CD4^{−/−}, OX40^{−/−}, or Runx3-Tg mice (CD4^{−/−}, 2%; OX40^{−/−}, 2%; Runx3-Tg, 1%; Figure 3A). We further examined OX40L expression in lung APCs in recipient mice that had been subjected to HDM challenge after adoptive transfer of HDM-sensitized CD4⁺ T cells (Figure 3B). In mice receiving wild-type HDM-sensitized CD4⁺ T cells, the OX40L⁺ cell frequency among lung APCs was 37%, comparable to that observed in the HDM-sensitized and -challenged wild-type mice (Figure 3B). However, mice receiving OX40^{−/−} or Runx3-Tg CD4⁺ T cells did not show a wild-type response (OX40^{−/−}, 4%; Runx3-Tg, 8%; Figure 3B). Given the defective expression of OX40 in Runx3-Tg CD4⁺ T cells (Figure 2K), these data indicate that pulmonary OX40L induction after HDM reexposure depends on the sensitization of CD4⁺ T cells, in which the expression of OX40 is implicated.

OX40 expression on T-lineage cells positive for TCR β chain was also analyzed in mice that had been sensitized and challenged with HDM (Figure 3C). An increased frequency of OX40⁺ cells was observed among T-lineage cells in the lungs of all groups, except OX40^{−/−} mice (wild-type, 91%; CD4^{−/−}, 43%; OX40^{−/−}, <1%; Runx3-Tg, 60%; Figure 3C). In parallel experiments with spleen T-lineage cells, we found no difference in the percentage of OX40⁺ cells between HDM-exposed mice and naive mice (8% in both groups; Figure 3C). Notably, OX40 expression on pulmonary T-lineage cells in CD4^{−/−} and Runx3-Tg mice was partially suppressed, compared with that in wild-type mice, suggesting that lung-specific OX40 induction in the challenge phase is, at least in part, attributable to T-lineage cells other than conventional CD4⁺ T cells, in which OX40 expression may not be regulated by Runx3.

NKT Cell Involvement in the Development of Allergic Airway Inflammation

Our previous studies indicated that OX40 acts as a costimulatory receptor on not only CD4⁺ T cells but also NKT cells (19). Thus, NKT cells may represent yet to be determined OX40⁺ T-lineage cells that are engaged in the challenge phase of allergic airway inflammation. To examine this, the binding of CD1d- α -GalCer tetramer (a reliable marker of NKT cells) to OX40⁺ T-lineage cells was examined in the lungs of wild-type mice sensitized and challenged with HDM (Figure 4A). Of the increased number of OX40⁺ lung T-lineage cells on HDM exposure (Figure 3C), about half exhibited CD1d- α -GalCer tetramer binding, characteristic of NKT cells (51%; Figure 4A). OX40 expression on pulmonary NKT cells was also consistent with the flow cytometry data, showing that the percentage of pulmonary OX40⁺ T-lineage cells after HDM sensitization/challenge was considerably lower in NKT^{−/−} mice than in wild-type mice (wild-type, 91%; NKT^{−/−}, 45%; Figure 4B). The abundance of NKT cells in the lung OX40⁺ T-lineage population led us to count the absolute number of NKT cells in the lungs of wild-type mice that had been sensitized and challenged with HDM (Figure 4C). This analysis revealed that sensitization and challenge with HDM induced a significant increase in the absolute number of lung NKT cells, compared with HDM sensitization alone or HDM challenge alone ($P < 0.05$; Figure 4C).

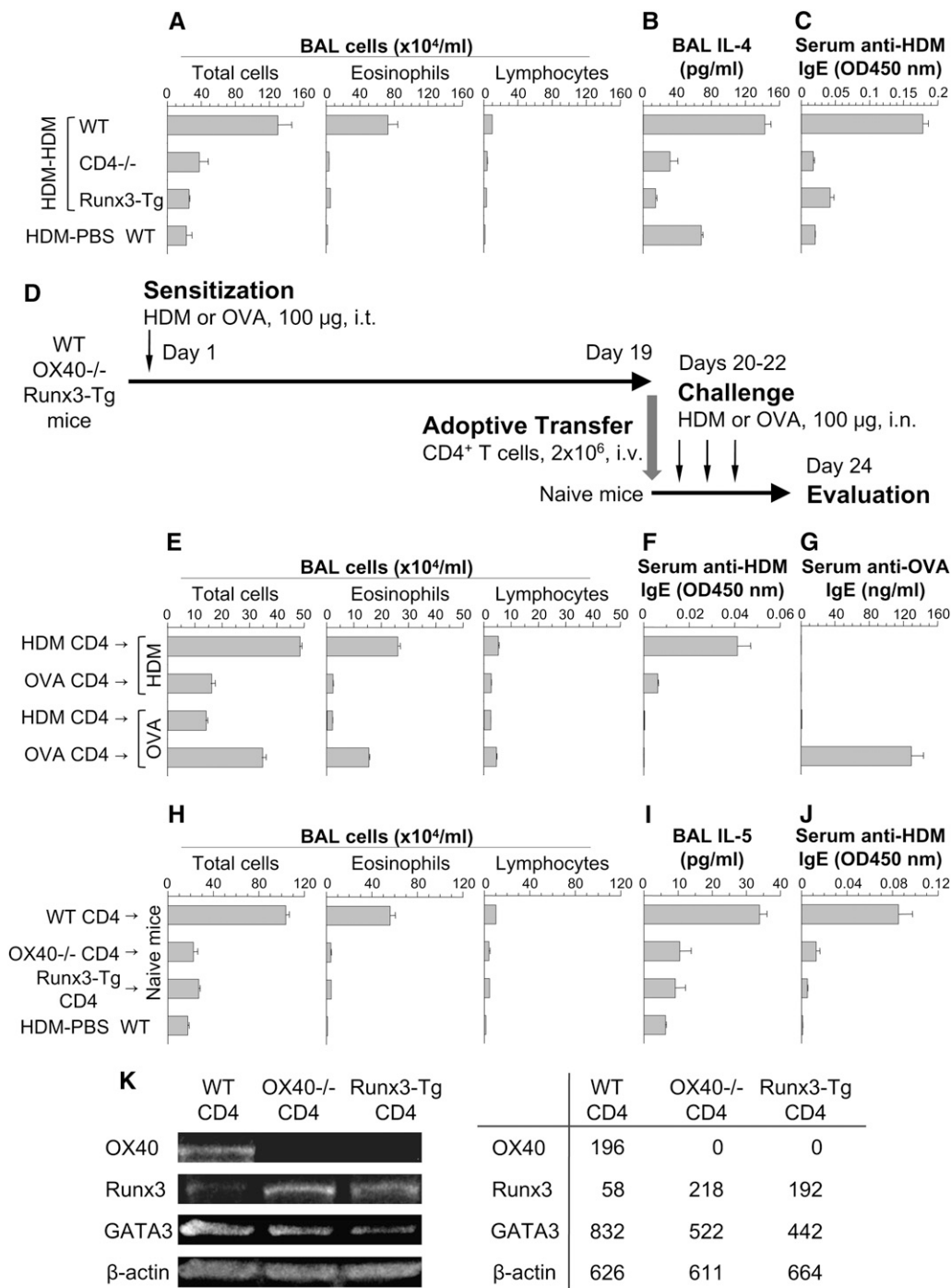


Figure 2. OX40-mediated sensitization of CD4⁺ T cells in developing allergen-specific airway inflammation. (A–C) Wild-type, CD4^{-/-}, and Runx3-transgenic (Tg) mice were exposed to house dust mite (HDM), as described in Figure 1A. Controls included wild-type (WT) mice exposed to phosphate-buffered saline (PBS), instead of HDM, on Days 20–22 (HDM-PBS). (D) Experimental scheme for adoptive transfer of CD4⁺ T cells (E–J). (E–G) CD4⁺ T cells from wild-type mice exposed to HDM or ovalbumin (OVA) on Day 1 were adoptively transferred into naive mice on Day 19, followed by a 3-day consecutive challenge with HDM or OVA on Days 20–22. (H–J) This study was similar to that in (E–G), except that recipient mice with CD4⁺ T cells from HDM-sensitized wild-type, OX40^{-/-}, or Runx3-Tg mice were challenged with HDM. Controls included wild-type mice exposed to PBS, instead of HDM, on Days 20–22 (HDM-PBS). For (A–C) and (E–J), the data are presented as means $\pm$ standard error ($n = 3$); (A, E, and H) Total, eosinophil, and lymphocyte airway infiltrates recovered in bronchoalveolar lavage (BAL); (B and I) cytokine levels in BAL; (C, F, and J) anti-HDM IgE levels in serum; (G) anti-OVA IgE levels in serum. (K) OX40 and Runx3 expression in HDM-stimulated CD4⁺ T cells. Total cell lysates of CD4⁺ T cells from wild-type, OX40^{-/-}, and Runx3-Tg mice exposed to HDM were prepared after *in vitro* restimulation with HDM. Lysate proteins were analyzed by immunoblotting for OX40, Runx3, GATA3, and β -actin. The intensity of each band was quantified and is expressed as arbitrary units, as shown on the right. i.n. = intranasal; i.t. = intratracheal; i.v. = intravenous.

To evaluate the consequences of the increased number of NKT cells on airway allergic inflammation, NKT^{-/-} mice were sensitized and challenged with HDM (Figures 4D–4F). The BAL fluid from the mice exhibited significant cellular and cytokine attenuation, accompanied by a significantly decreased level of serum anti-HDM IgE, compared with that of HDM-sensitized and -challenged wild-type mice (NKT^{-/-} vs. HDM-HDM wild-type, $P < 0.05$ in all measurements; NKT^{-/-} vs. HDM-PBS, $P > 0.05$ in all measurements; Figures 4D–4F). The decrease in HDM-induced pulmonary inflammation in NKT^{-/-} mice was not attributable to impaired OX40L expression on lung APCs as a result of the defect in OX40⁺ NKT cells, because OX40L expression in NKT^{-/-} mice after HDM sensitization/challenge was similar to that in wild-type mice (HDM-HDM wild-type,

42%; NKT^{-/-}, 41%; HDM-PBS, 2%; Figure 4G). These observations suggest that in the challenge phase after sensitization, OX40-expressing NKT cells accumulate in the lung and can be stimulated by pulmonary OX40L, the expression of which was not affected by NKT cells, leading to the development of allergic airway inflammation.

Role of OX40 on NKT Cells in the Challenge Phase

To further investigate the role of OX40⁺ NKT cells in the challenge phase, HDM-sensitized NKT^{-/-} mice were reconstituted with naive NKT cells 1 day before HDM challenge (Figure 5A). Reconstitution of NKT^{-/-} mice with NKT cells from wild-type mice led to increases in total and differential BAL cell counts, BAL fluid IL-5 content, and serum anti-HDM IgE, which

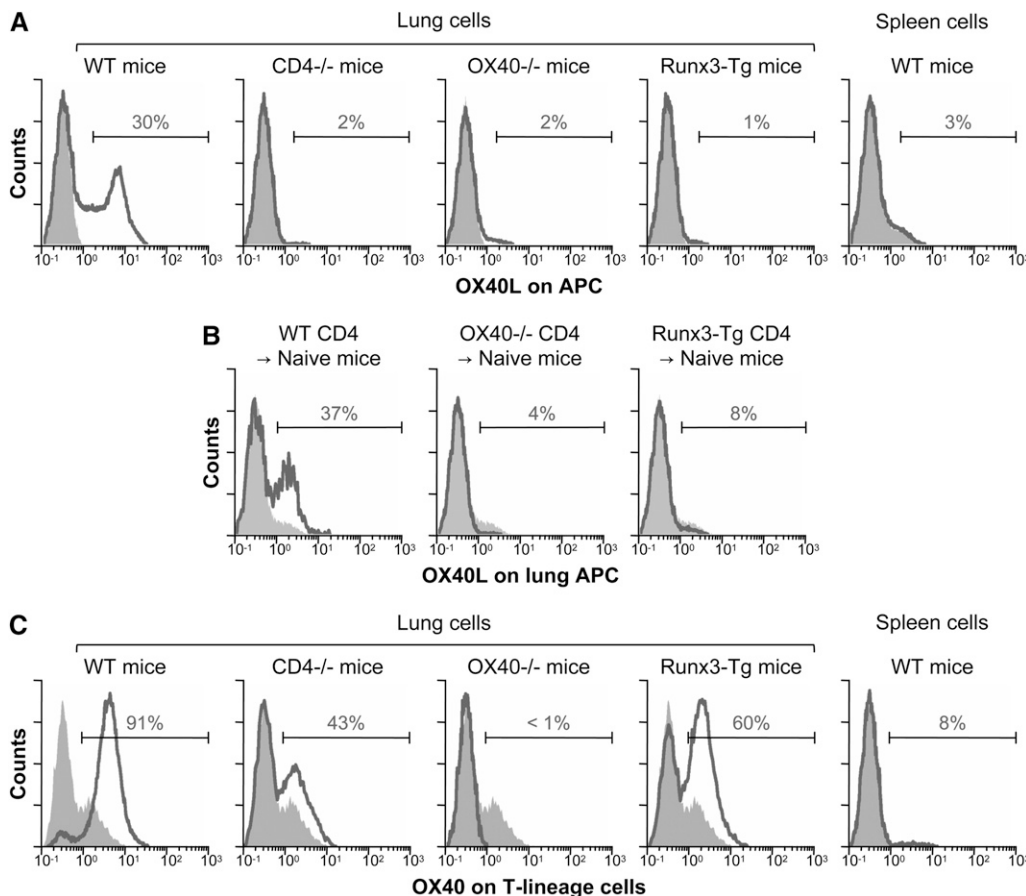


Figure 3. Flow cytometric analysis of OX40 ligand (OX40L) on antigen-presenting cells (APCs) (MHC class II⁺) and OX40 on T-lineage cells (TCR β ⁺) in house dust mite (HDM)-sensitized/challenged mice. (A) OX40L on lung APCs. Lungs of wild-type (WT), CD4^{-/-}, OX40^{-/-}, and Runx3-transgenic (Tg) mice were collected 48 hours after the last exposure of the sensitization and challenge protocol, as described in Figure 1A. Lung mononuclear cells were obtained by enzymatic digestion and were stained with antibodies against OX40L and MHC class II (I-A^b). Histograms were gated on MHC class II⁺ APCs, and the numbers denote the percentages of OX40L⁺ cells. Overlay histogram (gray) depicts lung APCs from naive wild-type mice. Controls included spleen APCs from HDM-exposed (line) and naive (gray) wild-type mice. (B) OX40 on lung APCs after adoptive transfer of CD4⁺ T cells. This study was similar to that in (A), except that lung APCs from the adoptive transferred mice, described in Figures 2H–2J, were analyzed. (C) OX40 on lung T-lineage cells. Similar to (A), lung and spleen cells were prepared, but the cells were analyzed for OX40 expression in TCR β -gated cells.

all reached levels similar to those in wild-type mice that had been sensitized and challenged with HDM ($P > 0.05$ in all measurements; Figures 5B–5D). In contrast, transfer of NKT cells from OX40^{-/-} mice did not restore the capacity of NKT^{-/-} recipient mice to develop allergic airway responses (compared with transfer of wild-type NKT cells, $P < 0.05$ in all measurements; Figures 5B–5D). Consistent with the failure of OX40^{-/-} NKT cells to confer Th2-associated inflammatory responses to HDM-sensitized and-challenged NKT^{-/-} recipients, the frequency of lung NKT cells producing IL-13 was markedly lower than that in recipients of wild-type NKT cells and in wild-type mice (wild-type NKT cell transfer, 23%; OX40^{-/-} NKT cell transfer, 6%; nonreconstituted wild-type, 34%; Figure 5E). These analyses revealed that immune sensitization created by the first HDM inhalation was intact in the absence of NKT cells and that NKT cells transferred into the milieu were stimulated through OX40 to potentiate Th2 cytokine production on the second HDM inhalation.

Runx3-irrelevant OX40 Expression in NKT Cells

To assess whether Runx3 negatively regulated OX40-mediated stimulation in NKT cells, as in CD4⁺ T cells, we repeated the NKT cell reconstitution experiments with NKT cells from Runx3-Tg mice (Figure 5F). After reconstitution into NKT^{-/-} recipients, NKT cells from Runx3-Tg mice and wild-type mice restored the ability to mount HDM-induced airway cell accumulation, similar to that in wild-type mice, and was significantly greater than that in NKT^{-/-} mice (compared with Runx3-Tg NKT cell transfer in all BAL cell counts; wild-type NKT cell transfer, $P > 0.05$; nonreconstituted wild-type, $P > 0.05$; nonreconstituted NKT^{-/-},

$P < 0.05$; Figure 5F). Consistent with the comparable reconstituting function between Runx3-Tg NKT cells and wild-type NKT cells, OX40 expression in Runx3-Tg NKT cells was not less than that in wild-type NKT cells, as we observed the OX40⁺ cell frequency and the OX40 protein abundance in lung NKT cells after HDM sensitization and challenge (Figures 5G and 5H).

Pulmonary Blockade of OX40–OX40L Interactions

On the basis of our findings of increased OX40 and OX40L expression limited to allergen-exposed lungs, we finally determined whether OX40 engagement on NKT cells, locally within the lung, may provide a therapeutic target for the inflammatory immune response. To this end, we intranasally administered a blocking antibody specific for OX40L daily for the 3-day challenge phase in the NKT cell reconstitution experiment (Figure 6A). Instillation of blocking anti-OX40L antibody to NKT^{-/-} mice reconstituted with wild-type NKT cells negated the capacity of wild-type NKT cells to restore airway cellular responses against the HDM challenge, as indicated by the decreased BAL cell numbers of total cells, eosinophils, and lymphocytes, with only the decrease in lymphocytes being insignificant (anti-OX40L vs. control IgG and nonreconstituted wild-type, $P < 0.05$ in all BAL cell counts except for lymphocytes; Figure 6B). The attenuated cellular accumulation in the BAL fluid of NKT cell-reconstituted mice treated with anti-OX40L antibody was comparable to that of nonreconstituted NKT^{-/-} mice ($P > 0.05$ in all BAL cell counts; Figure 6B). Consistently, similar results were achieved for all humoral hallmarks of allergic airway inflammation, including Th2-type cytokine levels in BAL

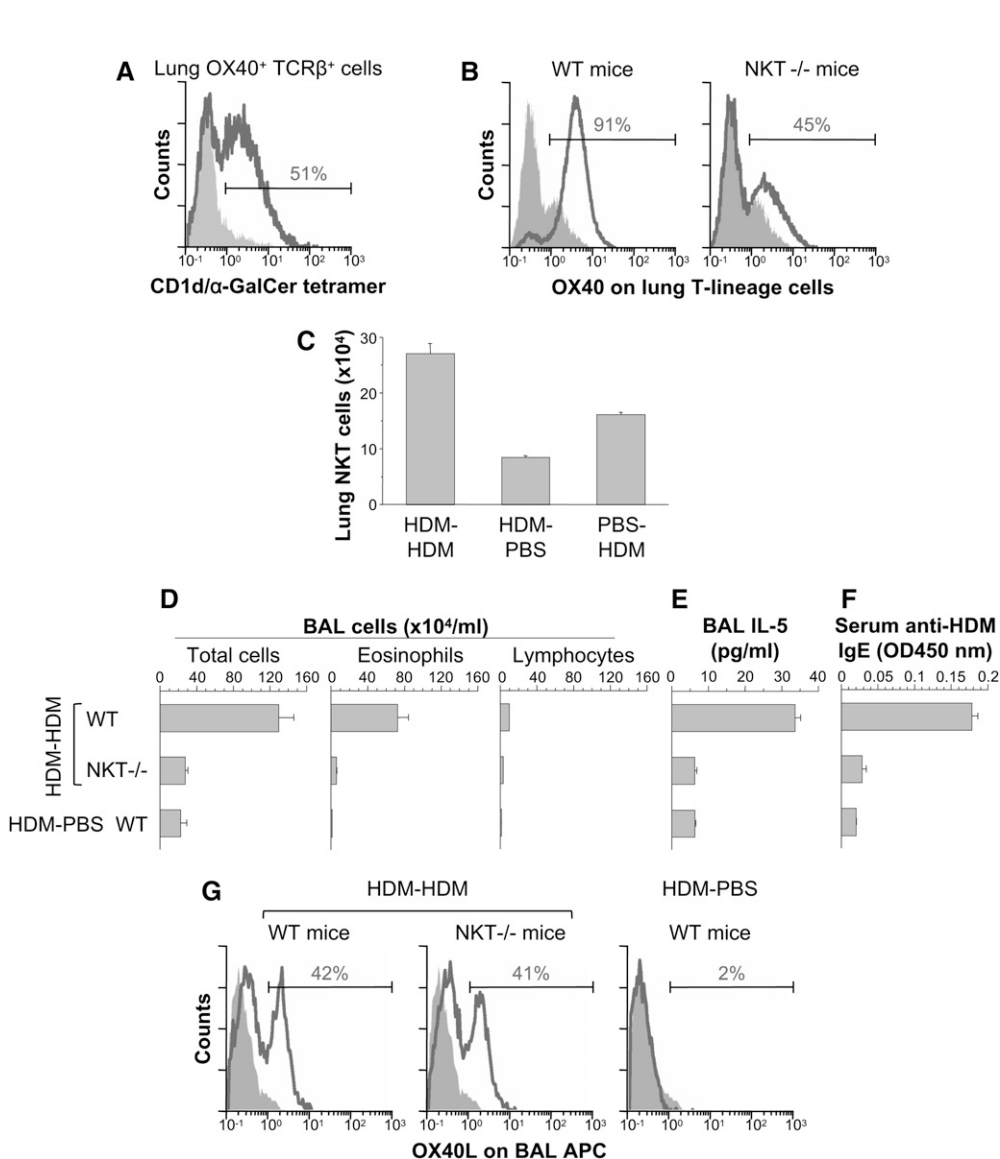


Figure 4. Involvement of lung natural killer T (NKT) cells in allergic airway inflammation. (A) Binding of α -galactosylceramide (α -GalCer)-loaded CD1d tetramer to OX40⁺ T-lineage cells in the lung. TCR β ⁺ lung mononuclear cells were obtained from wild-type (WT) mice that had been sensitized and challenged with house dust mite (HDM), and were stained with anti-OX40 antibody and CD1d- α -GalCer tetramer. The percentages of CD1d- α -GalCer tetramer binding to OX40⁺ T-lineage cells are listed. Overlay histogram (gray) depicts lung OX40⁺ T-lineage cells from naive wild-type mice. (B) OX40 on lung T-lineage cells. After sensitization and challenge with HDM, lung cells from wild-type and NKT^{-/-} mice were analyzed for OX40 expression in TCR β -gated T cells, as described in Figure 3C. (C) Quantification of lung NKT cells. The number of CD1d- α -GalCer tetramer⁺TCR β ⁺ NKT cells in whole lung exposed to HDM or phosphate-buffered saline (PBS), as in Figure 1A, was determined by flow cytometry. The data are presented as means $\pm$ standard error ($n = 3$). (D–F) Wild-type or NKT^{-/-} mice were sensitized and challenged with HDM, as described in Figure 1A. Controls included wild-type mice exposed to PBS in the challenge phase. The data are presented as means $\pm$ standard error ($n = 3$); (D) Total, eosinophil, and lymphocyte airway infiltrates recovered in bronchoalveolar lavage (BAL); (E) IL-5 levels in BAL; (F) anti-HDM IgE levels in serum. (G) OX40 ligand (OX40L) on antigen-presenting cells (APCs) in BAL. The BAL cells were pooled for each group in (D–F) and were analyzed for OX40L expression on APCs, as described in Figure 3A.

fluids and HDM-specific IgE and eotaxin levels in sera (anti-OX40L vs. control IgG and nonreconstituted wild-type, $P < 0.05$ in all measurements; anti-OX40L vs. nonreconstituted NKT^{-/-}, $P > 0.05$ in all measurements; Figures 6C–6G). In accordance with the inhibitory effect of anti-OX40L antibody on the cellular and humoral immune responses, intranasal treatment with anti-OX40L antibody, but not its matched isotype control, improved peribronchial and perivascular cellular inflammation and epithelial thickening, which was detected in lung tissue histology of HDM-exposed wild-type mice, to a level similar to that in NKT^{-/-} mice (Figure 6H).

DISCUSSION

Our data delineate the role of an OX40 signaling pathway that operates in CD4⁺ helper T cells and NKT cells in the sensitization and challenge phases, respectively, in causing allergic airway inflammation. In the sensitization phase, OX40 on CD4⁺ T cells is critical for the generation of allergen-sensitized CD4⁺ T cells, which induce OX40L expression locally within the lung when reexposed to the allergen. In the challenge phase, the resulting

OX40L expression stimulates lung NKT cells through OX40, triggering production of Th2 cytokines for the eosinophilic lung inflammatory response. Pulmonary OX40 triggering in NKT cells in the challenge phase is highlighted as a potential therapeutic target by our finding that intranasal administration of a blocking antibody to OX40L compromised the capacity of NKT cells to mount an allergic airway inflammatory response. Although it is presently unclear why the Th1-associated transcription factor Runx3 differentially affects OX40 expression in CD4⁺ T cells and NKT cells, forced expression of Runx3 reduced its capacity to mediate the inflammatory responses in CD4⁺ T cells, but not in NKT cells.

Full T cell activation is generally achieved by TCR engagement with antigen recognition in the presence of additional costimulatory signals provided by APCs (6, 14–16). Among the costimulatory receptors, engagement of CD28 by its ligands, CD80/B7.1 or CD86/B7.2, has been studied in terms of initial T cell priming, whereas several studies have promoted the OX40–OX40L interaction as a key signal in T cell activation subsequent to the initial CD28–B7 interaction (14, 15, 40). From these considerations, studies in immune disease animal models

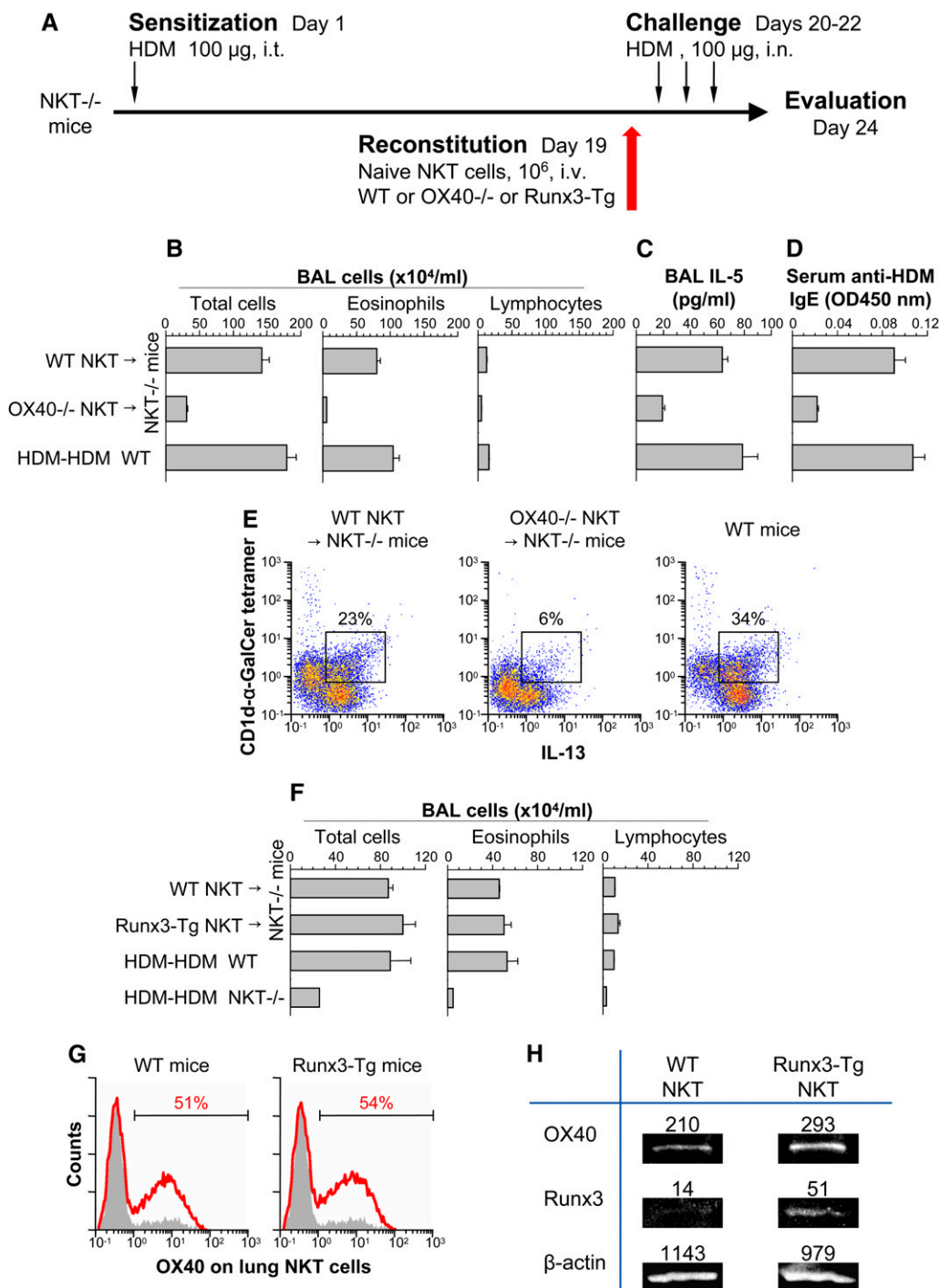


Figure 5. Reconstitution of natural killer T (NKT) cell-deficient mice with naive NKT cells in the challenge phase. (A) Experimental scheme. Similar to Figures 4D–4F, NKT^{-/-} mice were sensitized and challenged with house dust mite (HDM). However, 1 day before the HDM challenge (Day 19), HDM-sensitized NKT^{-/-} mice were reconstituted with NKT cells from naive wild-type (WT), OX40^{-/-}, or Runx3-transgenic (Tg) mice. (B–D) Reconstitution with wild-type or OX40^{-/-} NKT cells. Controls included wild-type mice that were sensitized and challenged with HDM. The data are presented as means $\pm$ standard error ($n = 3$); (B) Total, eosinophil, and lymphocyte airway infiltrates recovered in bronchoalveolar lavage (BAL); (C) IL-5 levels in BAL; (D) anti-HDM IgE levels in serum. (E) IL-13 production in lung NKT cells. TCRβ⁺ lung mononuclear cells were pooled for each group in (B–D) and were analyzed for intracellular IL-13 level and CD1d-α-galactosylceramide (α-GalCer) tetramer binding by flow cytometry. The percentages of IL-13⁺ NKT cells are listed. (F) Reconstitution with wild-type or Runx3-Tg NKT cells; total, eosinophil, and lymphocyte airway infiltrates recovered in BAL. Controls included wild-type and NKT^{-/-} mice that were subjected to HDM sensitization and challenge. The data are presented as means $\pm$ standard error ($n = 3$). (G) OX40 on lung NKT cells. Wild-type or Runx3-Tg mice were exposed to HDM, as described in Figure 1A. Lung mononuclear cells were analyzed for the surface expression of OX40 within the CD1d-α-GalCer tetramer⁺TCRβ⁺ population (NKT cells), using flow cytometry. Overlay histogram (gray) depicts lung NKT cells from naive mice. (H) OX40 and Runx3 expression in pulmonary NKT cells. Total cell lysates of pulmonary NKT cells were prepared from wild-type or Runx3-Tg mice sensitized and challenged with HDM. Lysate proteins were analyzed by immunoblotting for OX40, Runx3, and β-actin. The intensity of each band was quantified and is expressed as arbitrary units. i.n. = intranasal; i.t. = intratracheal.

revealed that systemic blockade of OX40, but not CD28, can prevent ongoing signs of disease when given at the time of the secondary immune response, indicating that OX40 blockade may have potential for the treatment of various immune disorders (12, 15, 36). However, the mechanisms of the therapeutic effects of OX40 ablation are not well understood (14, 15).

We have illustrated two distinct roles of OX40 in primary and secondary immune responses of allergic airway inflammation. Although the immunopathological role of OX40 was shown in pulmonary inflammation, the primary mechanism underlying the OX40 requirement was focused mainly on OX40 engagement

of CD4⁺ T cells to augment helper activity (5, 8, 9, 12, 13). Our study further expands the implications of OX40 signals in developing allergic airway inflammation, with evidence that OX40-dependent CD4⁺ T cell sensitization by allergen inhalation brings about lung-specific augmentation of OX40L, providing pulmonary NKT cells with OX40 signals to produce Th2 cytokines such as IL-13. Because a substantial fraction of NKT cells expresses CD4, the diminished sensitization in CD4-deficient mice may be due in part to NKT cells, but the successful sensitization in NKT^{-/-} mice reconstituted 1 day before the allergen challenge indicates that NKT cells play a minor, if any,

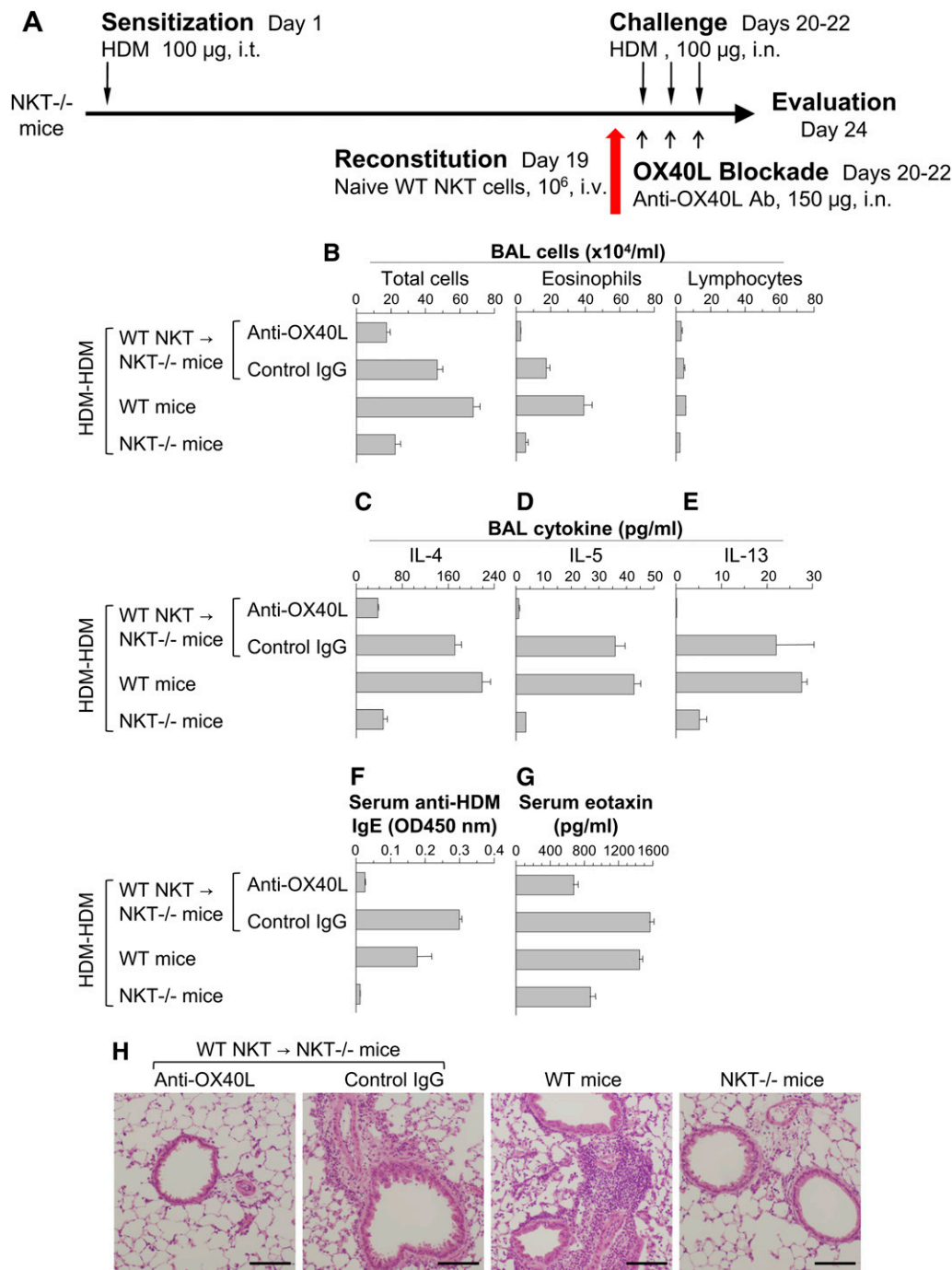


Figure 6. Pulmonary blockade of OX40–OX40 ligand (OX40L) interactions in the challenge phase. (A) Experimental scheme. The study was similar to that of Figure 5A, except that NKT^{-/-} mice reconstituted with naive wild-type (WT) natural killer T (NKT) cells were treated by intranasal (i.n.) administration of neutralizing antibody against OX40L or control IgG on three consecutive days during the challenge phase (Days 20–22). (B) Total, eosinophil, and lymphocyte airway infiltrates recovered in bronchoalveolar lavage (BAL). (C–E) Cytokine levels in BAL. (F) Anti-house dust mite (HDM) IgE levels in serum. (G) Eotaxin levels in serum. For (B–G), the data are presented as means $\pm$ standard error ($n = 3$). (H) Histological lung sections stained with hematoxylin and eosin at an original magnification of $\times 200$. Scale bars, 100 μ m. Controls included HDM-exposed wild-type and NKT^{-/-} mice without NKT cell reconstitution. i.t. = intratracheal.

role during the sensitization (20). The therapeutic importance of pulmonary OX40–OX40L interactions is emphasized by the attenuated allergic airway phenotype after inhalation of OX40L-specific neutralizing antibody during symptomatic reexposure to the allergen. Regarding the OX40L-inducing pathway mediated by OX40, some groups have reported that OX40 binding to OX40L can activate OX40L-expressing cells through bidirectional signaling (41). However, it seems unlikely that the expression level of OX40 simply supports OX40L expression by reverse signaling of OX40–OX40L interactions, as evidenced by defective OX40L expression in the lungs of CD4-deficient and Runx3-Tg mice despite residual OX40 expression. It is probable that allergen-sensitized CD4⁺ T cells are responsible for creating an immunological environment that allows pulmonary APCs to up-regulate OX40L expression in response to allergen reexposure.

Studies have implicated Runx3 as a Th1-skewing transcription factor, which binds to a *cis* regulatory element of the *Il4* locus in cooperation with T-bet and represses IL-4 production in differentiating Th1 cells (37–39). OX40 signals have also been shown to skew naive CD4⁺ T cells toward a Th2 phenotype by enhancing nuclear accumulation of GATA3; this binds to the Th2 cytokine locus for early expression of Th2-type cytokines including IL-4, which in turn provokes further nuclear GATA3 accumulation for autocrine IL-4 production (42, 43). The present study provides mechanistic clues, demonstrating that defective OX40 signaling was associated with higher Runx3 and lower GATA3 transcription factor levels in HDM-stimulated CD4⁺ T cells. Collectively, it is conceivable that triggering of OX40 cooperatively antagonizes Runx3 induction in CD4⁺ T cells to release the IL4 suppression, which may apply an

essential brake on the GATA3/IL-4 positive feedback circuit for Th2 polarization. Consistent with this scenario, it has been reported that forced expression of GATA3 inhibits binding of Runx3 complexes to the *Il4* silencer in CD4⁺ T cells (39).

Contrary to the functional activity of Runx3 in promoting Th1 polarization of CD4⁺ T cells, the forced expression of Runx3 in NKT cells exerted no functional influence on their capacity to reconstitute Th2-type airway eosinophilic inflammation in NKT^{-/-} mice. Although it is possible that the *lck* promoter-driven production of Runx3 was insufficient to alter cellular function in NKT cells, our finding suggests that the molecular mechanism that governs cell differentiation is distinct between CD4⁺ T cells and NKT cells. Although there is little evidence of an NKT cell-intrinsic mechanism by which the Th1/Th2-like differentiation of NKT cells can be modulated, it is becoming clear that external stimuli in NKT cell TCR signaling events can influence the cytokine production profile of NKT cells (22, 24, 44–46). In this regard, studies of NKT cell ligands have shown that compared with the original α -GalCer, some variants with shorter sphingosine have stimulatory properties, biasing NKT cells toward the secretion of Th2-type cytokines in TCR recognition (20, 24, 47–50).

In this context, we and others have found costimulatory activities of Th2-related OX40 signaling in NKT cells (17–19, 51). We have now extended this knowledge by demonstrating that repeated inhalation of HDM leads to NKT cell accumulation in the lung, where NKT cells are activated after OX40 engagement and produce Th2 cytokines essential for the development of HDM-induced allergic airway inflammation. Studies suggest that NKT cells contribute to the pathogenesis of allergic airway diseases such as asthma in mice and humans (20, 23, 25, 27, 52, 53). However, the pathogenic role of NKT cells in allergic airway inflammation could still be argued, as bronchial NKT cell expansion in patients with asthma has been disputed with differences among patient cohorts; moreover, animal studies have used an extremely artificial setting, in which mice are intraperitoneally sensitized with OVA plus the adjuvant aluminum hydroxide and subsequently challenged by OVA inhalation (25, 27, 52, 53). On this point, we designed our experimental protocol to be more like the clinical setting, using the natural allergen HDM inhaled with no adjuvant. This experimental study suggests that OX40 on pulmonary NKT cells plays a major role at least in HDM-induced allergic airway responses and that the diversity of pathogenic aeroallergens may account for the controversial clinical observations. Further study of pulmonary NKT cells in other clinical conditions may enhance our understanding of asthma pathogenesis.

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