

TNF Receptor-Associated Factor 1 Expressed in Resident Lung Cells Is Required for the Development of Allergic Lung Inflammation¹

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TNF is a major therapeutic target in a range of chronic inflammatory disorders, including asthma. TNFR-associated factor (TRAF)1 is an intracellular adaptor molecule important for signaling by TNFR. In this study, we investigated the role of TRAF1 in an adoptive transfer model of allergic lung inflammation. Mice deficient in TRAF1 (TRAF1^{-/-}) and wild-type (WT) control animals were adoptively transferred with WT OVA-immune CD4⁺ T cells, exposed to an aerosol of LPS-free OVA, and analyzed for the development of allergic lung inflammation. In contrast to WT mice, TRAF1^{-/-} recipients failed to display goblet cell hyperplasia, eosinophilic inflammation, and airway hyperresponsiveness in this model of asthma. Neither T cell recruitment nor expression of the proinflammatory cytokines IL-4, IL-5, IL-13, or TNF occurred in the lungs of TRAF1^{-/-} mice. Although purified myeloid TRAF1^{-/-} dendritic cells (DCs) exhibited normal Ag-presenting function and trans migratory capacity in vitro and were able to induce OVA-specific immune responses in the lung draining lymph nodes (LNs) following adoptive transfer in vivo, CD11c⁺CD11b⁺ DCs from airways of TRAF1^{-/-} recipients were not activated, and purified draining LN cells did not proliferate in vitro. Moreover, transfer of WT or TRAF1^{-/-} DCs failed to restore T cell recruitment and DC activation in the airways of TRAF1^{-/-} mice, suggesting that the expression of TRAF1 in resident lung cells is required for the development of asthma. Finally, we demonstrate that T cell-transfused TRAF1^{-/-} recipient mice demonstrated impaired up-regulation of ICAM-1 expression on lung cells in response to OVA exposure. *The Journal of Immunology*, 2008, 180: 1878–1885.

Although TNF is one of the most studied proinflammatory cytokines and has a wide array of immunomodulatory activities (1), its precise role in the pathogenesis of asthma is still unclear. Although increased levels of TNF have been found in the sputum and serum of patients with asthma (2, 3), patients treated with a TNF antagonist had no attenuation of pulmonary eosinophilia and airway hyperresponsiveness (AHR)³ to methacholine (4). In contrast, treatment of patients suffering from severe corticosteroid-controlled asthma with etanercept, a TNFR IgG1-Fc fusion protein, resulted in improvement of symptoms, lung function, and AHR (5). Moreover, etanercept treatment of patients with refractory asthma was associated with improvement in both bronchial hyperreactivity and asthma-related quality-of-life score (6), suggesting that, at the severe end of the disease spectrum, blockade of TNF was highly efficacious in asthma. Only a

few animal studies have been conducted to address the role of TNF in asthma. AHR and inflammation of the bronchial mucosa were induced in rats and mice after exposure to TNF (7, 8). TNF blockade with anti-TNF Ab resulted in significant inhibition of late AHR without affecting early AHR or reducing airway eosinophilia and inflammation (9). Considering our limited knowledge of TNF function in the development of asthma, further studies are required to understand the role of TNF and TNF signaling pathway molecules in Ag-induced T cell-dependent allergic lung inflammation.

There are two distinct receptors for TNF: TNFR1 (CD120a/p55) and TNFR2 (CD120b/p75). These receptors use members of the TNFR-associated factor (TRAF) family for signal transduction. In general, TRAF molecules contain a C-terminal TRAF domain and an N-terminal domain consisting of a RING finger and several zinc fingers (10). Unlike other TRAF family members, TRAF1 lacks a RING finger domain and contains a single zinc finger domain. In addition, expression of TRAF1 is rapidly up-regulated following lymphocyte activation (11). Owing to these features, the precise biological function of TRAF1 remains controversial. Several in vitro studies have suggested that TRAF1 acts as an inhibitor of NF- κ B activation (12–15). Paradoxically, TRAF1 has been identified as a gene target of NF- κ B transcriptional activity that is required to fully suppress TNF-induced apoptosis (16). Moreover, TRAF1 has been demonstrated to promote TRAF2-mediated NF- κ B activation by displacing TRAF2 from lipid rafts to the cytosol and preventing TRAF2 degradation (17). Furthermore, it has recently been shown that, in B cells, TRAF1 cooperates with TRAF2 to enhance CD40-mediated activation signals, including NF- κ B1, NF- κ B2, and JNK activation (18). These results suggest that TRAF1 functions as a TRAF2-interacting partner that enhances TRAF2-mediated signals in CD40-stimulated B cells (18).

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; TRAF, TNFR-associated factor; BAL, bronchoalveolar lavage; DC, dendritic cell; LN, lymph node; WT, wild type.

To more clearly define the biological functions of TRAF1, we have previously generated mice deficient in TRAF1 (TRAF1^{-/-}) (19). In contrast to mice deficient in TRAF2, which die prematurely (20), TRAF1^{-/-} mice develop normally and display normal lymphocyte development (19). TRAF1^{-/-} T lymphocytes are hyperproliferative in response to TCR stimulation and their differentiation is skewed toward a Th2 phenotype (21). In addition to T cells, TRAF1 is also expressed in pulmonary epithelial cells (22). Interestingly, intratracheal administration of TNF to TRAF1^{-/-} mice causes acute liver injury, which is reflected in gross liver abnormalities and a dramatic increase in serum liver enzyme levels in comparison to WT control mice (23). It has also been demonstrated that TRAF1 expressed in resident lung cells is essential for the recruitment of lymphocytes to the pulmonary airspace in a model of endotoxin-induced lung inflammation, suggesting that TRAF1 acts downstream of TNFR1 in facilitating inflammation-induced recruitment of lymphocytes to the lungs (24).

Because endotoxin-induced inflammatory response has been shown to contribute to the development and severity of asthma and other airway diseases, we focus in this study on the physiological consequences of TRAF1 deficiency in resident lung cells in allergic lung inflammation through the use of an adoptive transfer model of asthma. We show that transfer of OVA-immune T cells from WT mice into naive TRAF1^{-/-} recipients failed to result in pulmonary inflammation or increased AHR following OVA inhalation. Moreover, there was no recruitment or activation of WT, allergen-specific T cells in the airways of OVA-challenged TRAF1^{-/-} mice. Airway dendritic cells (DCs) were not activated and did not transport OVA to mediastinal lymph nodes (LNs) in the absence of host TRAF1. When adoptively transferred into TRAF1^{-/-} recipients, purified WT or TRAF1^{-/-} OVA-loaded DCs failed to restore allergic lung inflammation in TRAF1^{-/-} recipients, suggesting that TRAF1, expressed in resident lung cells, is important for the development of asthma.

Materials and Methods

Mice

BALB/c WT mice were purchased from Charles River Breeding Laboratories. DO11.10 mice hemizygous for the TCR transgene were purchased from The Jackson Laboratory (25). TRAF1^{-/-} mice (19) were backcrossed onto a BALB/c background for eight generations. Mice were maintained in a specific pathogen-free environment, according to animal protocols approved by Institutional Animal Care and Use Committee of Children's Hospital and the CBR Institute for Biomedical Research (Boston, MA).

T cell transfer and allergic airway inflammation

OVA-specific CD4⁺ T cells were derived as previously described (26). Briefly, WT mice were immunized i.p. with 50 μ g of OVA/1 mg of alum in 0.9% sterile saline. Seven days after sensitization, splenocytes were purified and cultured in complete HL-1 medium (BioWhittaker) for 4 days in the presence of 200 μ g/ml LPS-free OVA (Worthington Biochemical). CD4⁺ T cells were purified using MACS columns (Miltenyi Biotec) and transferred i.v. at 2×10^6 cells/mouse into naive recipients. Control mice received PBS vehicle. Starting the next day, mice were exposed daily for 7 days to OVA aerosol (10 mg/ml) in 0.9% normal saline solution for 30 min. Obstruction of airflow and lung inflammation were assessed 24 h after the last nebulization. AHR was determined by measuring enhanced pause (Penh) in conscious, unrestrained mice following graded doses of methacholine using whole body plethysmography (Buxco Electronics). Enhanced pause results were analyzed using two-way ANOVA. A value for $p < 0.05$ was considered statistically significant. Collection and evaluation of bronchoalveolar lavage (BAL) fluids and lung homogenates was done as described in (24). Cytokine concentrations were measured by ELISA as described (21). BAL cells were stained with CD4-FITC (RM4-5), CD8-PE (53-6.7), CD11b-PE (M1/70), CD11c-PerCP (N418), and MHC class II (M5/114.15.2) allophycocyanin (BD Biosciences). CFSE labeling of purified CD4⁺ T cells was done as previously described (21). Real-time RT-PCR analysis was performed as described previously (24). The ICAM-1 primers were purchased from Applied Biosystems. The fold induction of

gene expression was calculated as the ratio of gene expression in T cell-transferred mice to PBS-treated mice.

Lung histology and immunohistochemistry

For histological analysis, lungs were inflated with 1.0 ml of 10% formalin instilled through a tracheostomy tube and embedded in paraffin. Multiple 4- μ m sections were stained with diastase periodic acid-Schiff and H&E. For immunohistochemical analysis, 5- μ m sections of lungs were embedded in Tissue-Tek OCT Compound (Sakura Finetek), fixed in acetone, washed in PBS, and incubated with 3% rabbit serum in PBS to prevent nonspecific binding. ICAM-1 expression was detected with the hamster IgG clone 3E2B (Chemicon International) and biotinylated goat anti-hamster IgG (eBioscience). After washing with PBS, slides were incubated with VectaStain ABC reagent (Vector Laboratories), developed using diaminobenzidine substrate kit (BD Biosciences), counterstained with hematoxylin, and cover-slipped with Permount.

BrdU staining

T cell proliferation *in vivo* was measured by BrdU incorporation. BALB/c WT and TRAF1^{-/-} recipient mice were injected i.p. with 1 mg/mouse of BrdU on days 6 and 7 of OVA nebulization. On day 8, BAL cells were harvested and lymphocytes were stained with anti-BrdU, FITC-conjugated mAb (BD Biosciences) to detect proliferating lymphocytes.

Proliferation assay

At 24 h after the last nebulization, mediastinal LNs were prepared using 300 U/ml type I collagenase (Worthington Biochemical) and 100 U/ml DNase I (Sigma-Aldrich) treatment for 60 min in PBS at 37°C. After filtration through a 70- μ m cell strainer, single cell suspensions were isolated by density gradient using Lympholyte-M (Accurate Chemical and Scientific) and incubated with graded concentrations of LPS-free OVA protein (Worthington Biochemical) in complete HL-1 medium (BioWhittaker). Proliferation was assessed by monitoring the incorporation of [³H]thymidine (1 μ Ci/well) added during the last 8 h of 72 h cultures.

DC generation and migration assay

Splenic CD4⁺ DCs were prepared from collagenase D-treated spleens using the CD4⁺ Dendritic Cell Isolation kit (Miltenyi Biotec) according to the protocol provided by the manufacturer. T cells from DO11.10 mice were purified by negative magnetic bead separation using MACS columns (Miltenyi Biotec). Purified T cells were stained with FITC-conjugated anti-CD4 Ab and PE-conjugated anti-KJ1.26 Ab and sorted on a FACS Vantage SE (BD Biosciences). A total of 5×10^3 /well of KJ1.26⁺ CD4⁺ T cells and 1×10^4 /well of splenic CD4⁺ DCs were cultured in the presence of LPS-free OVA for 72 h.

Bone marrow DCs were generated as previously described (27). Briefly, bone marrow cells were isolated and propagated in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 20 ng/ml GM-CSF (PeproTech). On day 7, cells were stimulated with either 1 μ g/ml mouse recombinant TNF (PeproTech) or 1 μ g/ml LPS (Sigma-Aldrich) for 24 h. Chemotaxis assays were performed as previously described (28). Stimulated bone marrow-derived DCs were resuspended at 2.5×10^6 /ml in assay medium (0.1% BSA in RPMI 1640 medium). Assay medium (600 μ l) without or with 200 ng/ml mouse CCL19 (PeproTech) were added per well in 24-well tissue culture plates. A Transwell culture insert (Corning Costar) with 5- μ m pore size was inserted into each well, and 100 μ l (2.5×10^5) of the DC suspension was then added into the top chamber. The plate was incubated at 37°C for 2 h. Cells that migrated to the lower chamber were collected, counted, and analyzed by flow cytometry.

Adoptive DC transfer

For adoptive transfer experiments, *in vitro*-generated bone marrow-derived DCs were stimulated with 1 μ g/ml TNF for 24 h. After extensive washing, 2×10^6 stimulated DCs were intranasally administered to naive BALB/c female mice as previously described (29). Purified OVA-immune WT CD4⁺ T cells (2×10^6 cells/mouse) were i.v. injected into the DC recipients at 24 h after DC transfer. Recipients were exposed daily for 7 days to OVA aerosol and levels of inflammation were assessed 24 h after the last nebulization.

Results

OVA-immune T cells do not confer allergic lung inflammation to TRAF1^{-/-} mice

To investigate whether TRAF1 expressed in cells other than T lymphocytes might play a role in allergic lung inflammation, we

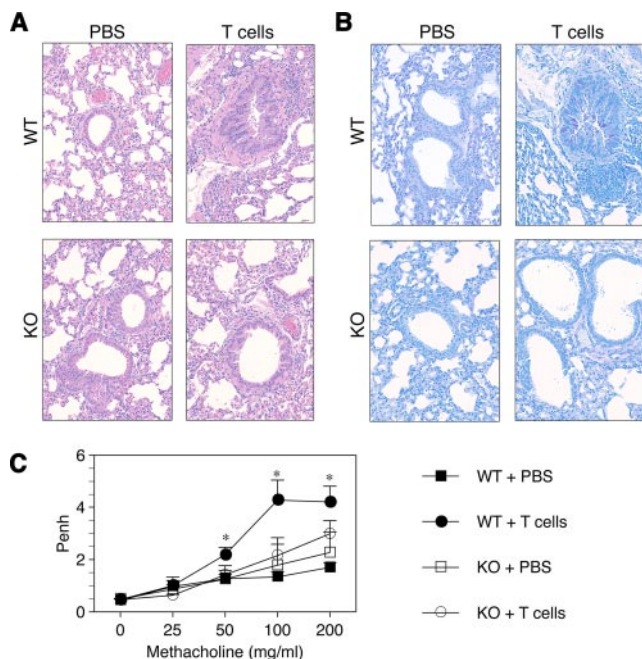


FIGURE 1. Impaired peribronchial inflammation, mucus secretion and increase in AHR in allergen-challenged TRAF1^{-/-} mice. WT and TRAF1^{-/-} (KO) recipients were i.v. injected with PBS or OVA-immune CD4⁺ T cells, then exposed to an aerosol of LPS-free OVA for 30 min daily over 7 days and analyzed 24 h after the last exposure. **A**, Peribronchial infiltrates were analyzed by staining of representative lung sections with H&E. **B**, Goblet cell hyperplasia was analyzed by staining of representative lung sections with periodic acid-Schiff reagent (PAS). **C**, AHR was measured by whole body plethysmography on conscious unrestrained mice exposed to the indicated doses of nebulized methacholine. Mice were adoptively immunized with OVA-immune CD4⁺ T cells (circle symbols) or control PBS (square symbols). Recipients were WT (closed symbols) and TRAF1^{-/-} (open symbols). Airflow obstruction is expressed as the measure of enhanced pause (Penh). Data represent mean measurement of enhanced pause $\pm$ SE ($n = 4$ mice/group). *, $p < 0.05$.

used an adoptive transfer model of asthma (26). OVA-immune WT CD4⁺ T cells were i.v. infused into WT or TRAF1^{-/-} recipients mice. These mice were then exposed daily (for 30 min) to LPS-free OVA aerosol for 7 days and subsequently analyzed for the development of lung inflammation, mucus production, and AHR.

Histologic examination of the lung tissues of OVA-challenged WT recipients, which had previously received OVA-immune T cells, showed an extensive leukocyte infiltration of peribronchial

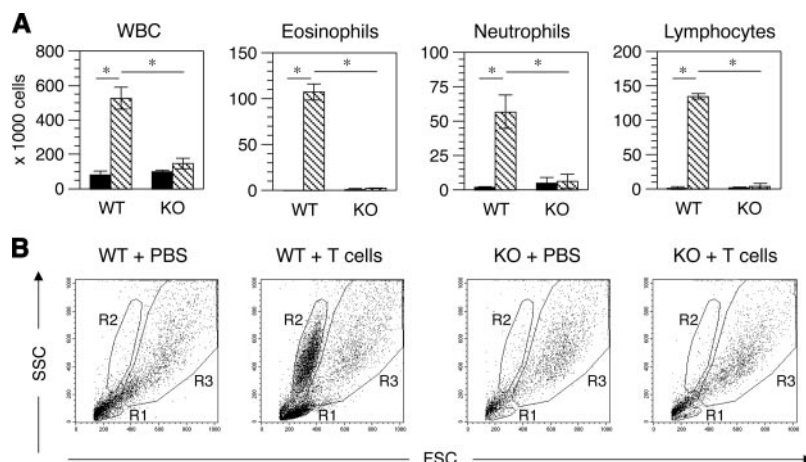
regions compared with WT mice that were injected with PBS (Fig. 1A). In contrast, infiltration of leukocytes into the peribronchial regions was markedly attenuated in TRAF1^{-/-} recipients of OVA-immune T cells (Fig. 1A). Moreover, mucus production in the bronchial epithelium in the lungs of TRAF1^{-/-} mice was impaired compared with production in the lungs of WT animals (Fig. 1B), and the absence of airway inflammation in T cell-injected TRAF1^{-/-} mice was associated with attenuation of airway responsiveness to methacholine (Fig. 1C). Microscopic analysis of BAL fluid from OVA-challenged WT mice, which had previously received OVA-immune T cells, revealed a dramatic increase in the number of airway white blood cells with elevations in eosinophils, neutrophils, and lymphocytes (Fig. 2A). In contrast, TRAF1^{-/-} recipients of OVA-immune T cells did not show any evidence of increased airway recruitment by leukocytes in response to OVA inhalation (Fig. 2B). Although there was a similar number of mononuclear phagocytes in the BAL of all groups of mice (Fig. 2B, R3), only WT mice given OVA-immune T cells displayed significant accumulation of lymphocytes and eosinophils in the pulmonary airspace (Fig. 2B, R1 and R2, respectively). Thus, Ag-immune T cells were not able to confer allergic lung inflammation or AHR to TRAF1^{-/-} recipients, suggesting that the absence of TRAF1 expression in the recipient mice resulted in an impaired ability of transferred allergen-specific T cells to orchestrate an inflammatory reaction in the lungs.

TRAF1 expression in cells other than T lymphocytes is required for the development of a pulmonary T cell response to allergen inhalation

Because T lymphocytes are critical for the development of allergic airway inflammation, we analyzed T cell responses in the pulmonary airspace of WT and TRAF1^{-/-} mice. OVA exposure of WT recipients resulted in a remarkably stronger recruitment of CD4⁺ T cells to the pulmonary airspace than did OVA challenge of TRAF1^{-/-} recipients (Fig. 3A). Moreover, WT recipients of immune T cells accumulated a higher number of CD8⁺ T cells in the lungs than did TRAF1^{-/-} mice in response to OVA exposure (Fig. 3A). Furthermore, T cell-injected WT mice had a significantly higher number of BrdU⁺ CD4⁺ T cells in the BAL (Fig. 3B), indicating that a large proportion of CD4⁺ T cells isolated from the lungs of WT recipients with T cells specifically proliferated in response to Ag.

The Th2 cytokines IL-4, IL-5, and IL-13 are important for the development of allergic airway inflammation, mucus secretion, and AHR (30). Analysis of cytokine concentrations in BAL fluid of adoptive T cell transfer recipients revealed elevated levels of

FIGURE 2. Induction of bronchial inflammation in allergen-challenged mice requires TRAF1 expression by non-T cells. **A**, WT and TRAF1^{-/-} (KO) recipients were i.v. injected with PBS (■) or OVA-immune CD4⁺ T cells (▨) and subsequently challenged, then exposed to an aerosol of LPS-free OVA for 30 min daily over 7 days and analyzed 24 h after the last exposure as in Fig. 1. The BAL fluid was removed and analyzed for the number of infiltrating white blood cells, including eosinophils, neutrophils, and lymphocytes, as described in *Materials and Methods*. Data represent the mean of three experiments $\pm$ SE ($n = 3$ mice recipients). *, $p < 0.05$, significant difference between groups of mice. **B**, Flow cytometry analysis of the BAL fluid cells for the presence of lymphocytes (R1), eosinophils (R2), and mononuclear phagocytes (R3) based on forward and side scatter is shown.



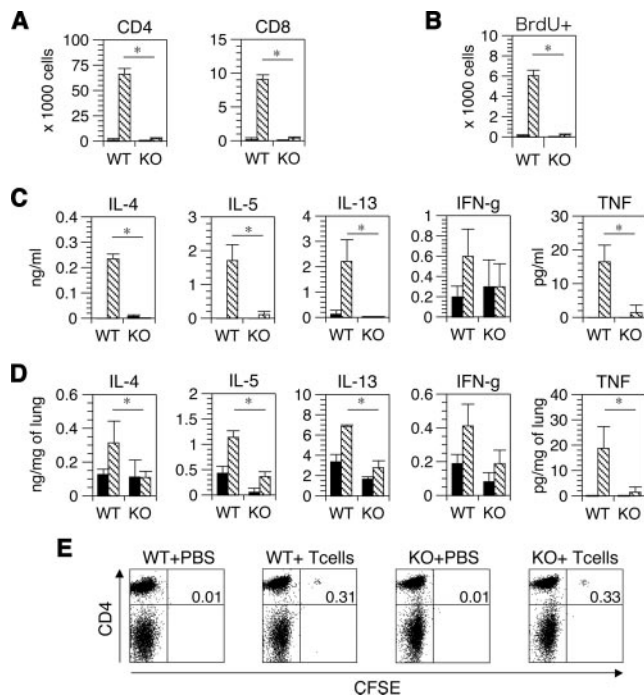


FIGURE 3. A decreased number of T cells and impaired cytokine production in the lungs of TRAF1^{-/-} mice. Groups of WT and TRAF1^{-/-} (KO) recipients injected with PBS (■) or OVA-immune CD4⁺ T cells (▨) were sensitized as described in Fig. 1. *A*, The number of CD4⁺ and CD8⁺ lymphocytes (R1) in the lymphocyte forward and side scatter gate shown in Fig. 2*B* were estimated by multiplying the percentage of each population by the total number of white blood cells in the BAL sample. *B*, The number of BrdU⁺ CD4⁺ T lymphocytes in BAL fluid were assessed by multiplying the percentage of BrdU⁺ cells by total number of CD4⁺ T lymphocytes in the BAL sample. The concentrations of the indicated cytokines in the BAL fluid (*C*) and the lungs (*D*) were determined by ELISA. Results are expressed as the mean of three independent experiments ($n = 4$ mice/group). Error bars represent the SE of the mean. *, $p < 0.05$, significant difference between TRAF1^{-/-} and WT recipients of OVA-immune CD4⁺ T cells are indicated. *E*, Flow cytometry of the inguinal LN lymphocytes from WT and TRAF1^{-/-} recipients. The number in each blot indicates the percentage of CFSE⁺ CD4⁺ T cells. Data are representative of two experiments ($n = 3$ mice/group).

IL-4, IL-5, and IL-13 in T cell-injected WT mice (Fig. 3*C*). In contrast, TRAF1^{-/-} recipients had cytokine levels indistinguishable from control mice injected with PBS. The examination of the

Th1 cytokine IFN- γ levels revealed no significant differences between all four groups of mice (Fig. 3*C*). Importantly, adoptive transfer of OVA-immune T cells conferred a significant production of a major proinflammatory cytokine TNF in the pulmonary air-space of WT mice compared with TRAF1^{-/-} mice (Fig. 3*C*). Cytokine analysis of the whole lung tissue confirmed a robust Th2 cytokine production in response to OVA inhalation occurred only in WT recipients of OVA-immune T cells (Fig. 3*D*). Taken together, these data demonstrate that TRAF1 is necessary for the development of a T cell response in the lungs.

To eliminate the possibility that transferred OVA-immune WT T cells were rejected by TRAF1^{-/-} recipients, we labeled T cells with CFSE before transfer, exposed mice to aerosol OVA for 7 days, and analyzed the presence of CFSE⁺ CD4⁺ T cells in the LNs. We found a similar number of CFSE⁺ CD4⁺ T cells in the inguinal LNs of WT and TRAF1^{-/-} mice (Fig. 3*E*), indicating that OVA-immune WT T cells were not rejected in TRAF1^{-/-} recipients.

Decreased activation and maturation of CD11b⁺CD11c⁺ DCs in TRAF1^{-/-} airways

CD11b⁺CD11c⁺ BAL DCs have been shown to be the most effective lung APCs for the induction of T cell activation (31). These "myeloid" DCs display a remarkably long-lived Ag-presenting function, contrasting sharply with other types APC in the lungs, which have only a transient ability to present Ag (31). Importantly, these cells appear to be essential for the development of asthma, as their *in vivo* depletion has been shown to abrogate the onset of airway inflammation in Ag-challenged mice (32). We analyzed APC in the BAL of WT and TRAF1^{-/-} mice in terms of their ability to migrate to regional lymphoid tissue and present Ag to T cells.

Although the total number of cells with forward and side scatter properties of mononuclear phagocytes (Fig. 2*B*, R3) was similar among all four groups of mice, WT recipients of OVA-immune T cells possessed a significantly higher number of CD11b⁺CD11c⁺ DCs in the BAL in comparison with control mice injected with PBS (Fig. 4*A*). In contrast, TRAF1^{-/-} recipients had no increase in the number of CD11b⁺CD11c⁺ DCs in comparison with sham WT or TRAF1^{-/-} mice (Fig. 4*A*). As shown in Fig. 4*B*, BAL CD11b⁺CD11c⁺ DCs from T cell-transferred WT but not TRAF1^{-/-} recipients displayed increased expression of MHC class II following exposure to Ag, indicating defective activation and maturation of DCs in the BAL of TRAF1^{-/-} mice.

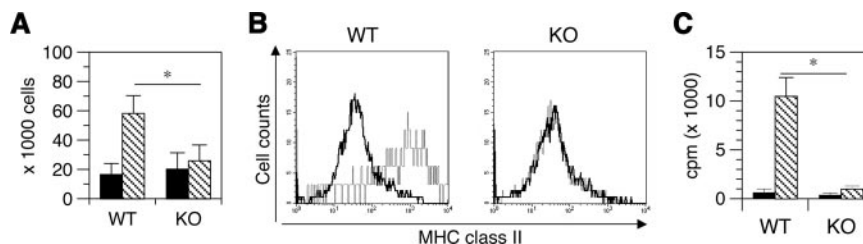


FIGURE 4. Decreased activation of CD11b⁺CD11c⁺ DCs in TRAF1^{-/-} lungs and the absence of Ag in the draining LNs of TRAF1^{-/-} mice. Groups of WT and TRAF1^{-/-} (KO) mice injected with PBS (■) or OVA-immune CD4⁺ T cells (▨) were sensitized as described in Fig. 1. *A*, Flow cytometry analysis of the BAL CD11b⁺CD11c⁺ DCs. BAL-derived myeloid DCs were identified as CD11b⁺CD11c⁺ cells within the mononuclear phagocyte gate (Fig. 2*B*, R3). The number of CD11b⁺CD11c⁺ DCs was estimated by multiplying the percentage of CD11b⁺CD11c⁺ DCs by the total number of white blood cells in the BAL. *B*, Maturation of DCs in the lung mucosa of sensitized mice was determined by evaluating the expression of high levels of MHC class II. Mice injected with PBS (solid histogram); or with T cells (broken histogram) are shown. *C*, Proliferation of cells from the draining LN of sensitized mice. Cells were isolated from mediastinal LN 24 h after the last exposure to OVA and incubated at 5×10^4 /well in 96-well plates. [³H]Thymidine was added during the last 8 h of culture. Results are expressed as the mean of three independent experiments ($n = 3$ mice), and error bars represent the SE of the mean. *, $p < 0.05$, significant difference in *A* and *C* between WT and TRAF1^{-/-} recipients of OVA-immune CD4⁺ T cells is indicated.

Following maturation, Ag-bearing activated DC transport Ag from the airways to the lung draining LNs, where they can present this Ag to specific T cells (33). We assessed Ag-specific responses in the draining LNs of WT and TRAF1^{-/-} mice and observed that mononuclear cells prepared from mediastinal LNs of T cell-injected WT mice displayed significant spontaneous proliferation even in the absence of exogenous Ag (Fig. 4C). These findings are consistent with successful delivery of OVA from the lungs to the draining LNs in WT mice with adoptively transferred T cells and imply an active ongoing immune response in the lung draining LNs of these mice. In contrast, there was no proliferation in cultures from the mediastinal LNs of TRAF1^{-/-} mice injected with OVA-specific T cells (Fig. 4C). These observations suggest that there is no ongoing immune response in the lung draining LNs of TRAF1^{-/-} mice.

TRAF1^{-/-} DCs display normal Ag-presenting function and CCL19-dependent migration in vitro

The absence of proliferation in LN cultures from TRAF1^{-/-} mice could be due to several reasons, including impaired Ag-presenting capability of TRAF1^{-/-} DCs or inability of TRAF1^{-/-} DCs to transport Ag to the draining LNs. To evaluate the Ag-presenting capacity of TRAF1^{-/-} DCs, highly purified CD4⁺ T cells from OVA TCR transgenic DO11.10 mice were cultured alone or with purified splenic CD4⁺ myeloid DCs from WT or TRAF1^{-/-} mice in the presence of increasing concentrations of OVA. We found that TRAF1-negative DCs supported proliferation of DO11.10 T cells to the same extent as WT DCs (Fig. 5A), suggesting that without TRAF1 DCs were able to acquire, process, and present the Ag to cognate T cells. Therefore, TRAF1^{-/-} DCs seem to have normal Ag-presenting capacity.

A number of previous studies have demonstrated that maturation of DCs is associated with up-regulated expression of CCR7 and their increased ability to migrate in response to the CCR7 ligands, CCL19, and CCL21 (34). To investigate the transmigration ability of TRAF1^{-/-} DCs in vitro, we generated DCs from bone marrow of WT and TRAF1^{-/-} mice by culturing cells with GM-CSF and inducing their subsequent maturation by stimulation with TNF or LPS. Analysis of the migration of TRAF1^{-/-} DCs revealed that TNF-matured (MHCII^{high}CD11b⁺CD11c⁺) TRAF1^{-/-} DCs have a significantly higher ability to migrate in response to CCL19 than do WT DCs (Fig. 5B). In contrast, there was no difference in migratory ability between WT and TRAF1^{-/-} DCs matured following treatment with LPS (Fig. 5B). Thus, mature TRAF1^{-/-} DCs have an equal, if not higher, migratory capacity when compared with WT DCs in vitro.

Adoptive transfer of DCs is able to restore the immune response in draining LNs, but is not sufficient to induce lung inflammation

To investigate whether TRAF1^{-/-} DCs can also function normally in vivo (e.g., migrate to regional lymphoid tissue and activate T cells), we adoptively transferred bone marrow-derived DCs into TRAF1^{-/-} mice 24 h before adoptive transfer of OVA-immune T cells. Following 7 days of exposure to aerosol OVA, we analyzed proliferation of LN cells in vitro. Fig. 6A shows that, although LN cells from TRAF1^{-/-} mice injected with WT DCs proliferated to the greatest extent, LN cells from TRAF1^{-/-} mice injected with TRAF1^{-/-} DCs proliferated to the same extent as LN cells from mock DC-injected WT mice. These data indicate that bone marrow-derived TRAF1^{-/-} DCs, when transferred into TRAF1^{-/-} mice, can deliver OVA to the draining LNs and induce a T cell immune response to the same extent as WT DCs.

In sharp contrast to WT mice, there was no accumulation of CD4⁺ T cells in the pulmonary airspace of TRAF1^{-/-} mice given

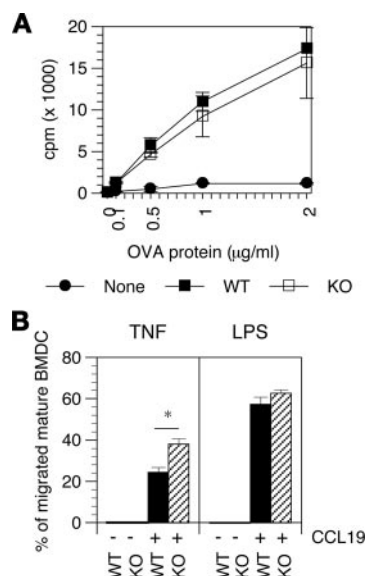


FIGURE 5. Ag presentation and transmigration abilities of TRAF1^{-/-} DCs. *A*, Normal Ag presentation by TRAF1^{-/-} DCs. Splenic CD4⁺ myeloid DCs were isolated from WT or TRAF1^{-/-} (KO) mice and irradiated. A total of 5×10^3 /well of KJ1.26⁺ CD4⁺ T cells from DO11.10 mice and 1×10^4 /well of CD4⁺ DCs were cultured in the presence of OVA protein for 72 h. [³H]Thymidine incorporation over the last 8 h of culture was measured. Results are expressed as the mean of two experiments ($n = 3$ mice), and error bars represent the SE of the mean. *B*, Transmigration of bone marrow-derived myeloid DCs. Bone marrow-derived cells (BMDC) from WT (■) and TRAF1^{-/-} mice (□) were cultured with GM-CSF for 7 days to generate CD11b⁺CD11c⁺ myeloid DCs and were then matured with TNF or LPS for 24 h. For transmigration assay cells were placed in the upper chamber of Transwell inserts and the migration of MHCII^{high}CD11b⁺CD11c⁺ cells toward CCL19 was analyzed using FACS analysis of cells in the bottom chamber. Results of a representative experiment from four independent cell preparations are shown. *, $p < 0.05$, significant difference between WT and TRAF1^{-/-} DCs is indicated.

either WT or TRAF1^{-/-} DCs (Fig. 6B), despite the signs of an ongoing immune response in the draining LNs of DC-injected TRAF1^{-/-} mice (Fig. 6A). Moreover, the expression of MHC class II on BAL CD11b⁺CD11c⁺ DCs from DC-injected TRAF1^{-/-} mice was not significantly different from MHC class II expression on CD11b⁺CD11c⁺ DCs from TRAF1^{-/-} mice without supplementary DCs (Fig. 6, C and D). Taken together these results indicate that the adoptive transfer of preactivated DCs into the lungs of TRAF1^{-/-} mice, although sufficient to restore an immune response in the draining LNs, is not adequate to extend the inflammatory response to the airways.

Decreased expression of ICAM-1 in the airways of sensitized TRAF1^{-/-} mice

We have recently demonstrated that, following inhalation of aerosol LPS, TRAF1^{-/-} mice fail to up-regulate ICAM-1 expression in their lungs and demonstrate impaired recruitment of T lymphocytes to the pulmonary airways (24). To examine the role of ICAM-1 in TRAF1-mediated allergic lung inflammation, we analyzed expression of ICAM-1 in the airways of sensitized TRAF1^{-/-} mice. WT recipients of OVA-immune T cells demonstrated increased transcription of the ICAM-1 gene when compared with WT mice injected with PBS (Fig. 7A). However, there was no significant induction of ICAM-1 gene transcription in the lungs of T cell-injected TRAF1^{-/-} mice (Fig. 7A). Analysis of ICAM-1 protein expression revealed that ICAM-1 was expressed

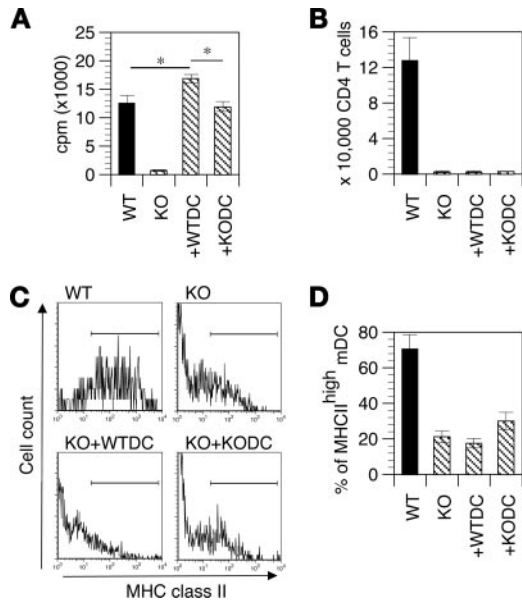


FIGURE 6. Adoptive transfer of exogenous bone marrow-derived DCs into TRAF1^{-/-} mice restores the LN, but not the lung immune response. Bone marrow-derived WT and TRAF1^{-/-} (KO) DCs were intranasally transferred into TRAF1^{-/-} mice 1 day before adoptive transfer of OVA-immune WT T cells and at the beginning of 7 days of OVA aerosol exposure. **A**, Proliferation of the mediastinal LN cells from sensitized WT (■) and TRAF1^{-/-} (▨) mice transferred with or without WT and TRAF1^{-/-} DCs was done by exposure to OVA and incubation at 5×10^4 /well in 96-well plates, as described in Fig. 4C. [³H]Thymidine was added during the last 8 h of culture. *, $p < 0.05$. **B**, The number of CD4⁺ T lymphocytes in the BAL of sensitized WT (■) and TRAF1^{-/-} (▨) mice injected with WT and TRAF1^{-/-} DCs, or with a PBS control. **C**, FACS analysis of MHC class II expression on WT and TRAF1^{-/-} BAL myeloid DCs from representative mice as indicated. MHCII⁺CD11b⁺CD11c⁺ DCs were gated and further analyzed in **D**. **D**, Quantification of MHC class II expression on WT and TRAF1^{-/-} BAL myeloid DCs. The percentage of BAL MHCII⁺CD11b⁺CD11c⁺ DCs in the gated regions of the FACS histograms presented in **C**. Results are expressed as the mean of two independent experiments ($n = 3$ mice), and error bars represent the SE of the mean.

in PBS-treated WT and TRAF1^{-/-} mice (Fig. 7B, left panels). This is consistent with previously published observations that have found significant levels of ICAM-1 in the alveolar structures and vascular endothelium of normal untreated lungs (35). Importantly, WT recipients of OVA-immune T cells displayed higher levels of ICAM-1 expression than did PBS-treated WT mice (Fig. 7B). In contrast, we found no significant increase in ICAM-1 protein expression in the lungs of T cell-injected TRAF1^{-/-} mice compared with PBS-treated controls. Taken together with an essential role of ICAM-1 in allergic lung inflammation (36–38), our results suggest that ICAM-1 expression in the pulmonary airways might be important for the development of TRAF1-mediated allergic lung inflammation.

Discussion

TRAF1 is a unique member of the TRAF family due to an unusual primary structure and limited tissue distribution compared with other TRAF molecules: TRAF1 mRNA has only been detected in the lung, spleen, and testis (39, 40). Our previous studies demonstrated that TRAF1^{-/-} T lymphocytes are hyperproliferative in response to TCR stimulation and preferentially differentiate toward a Th2 phenotype with increased expression of IL-4, IL-5 and IL-13 (19, 21). However, analysis of TRAF1^{-/-} mice in an active immunization model of asthma revealed no enhancement of AHR, recruitment of eosinophils, or expression of proinflammatory cy-

tokines and chemokines in the lungs of TRAF1^{-/-} mice when compared with the lungs of WT mice (data not shown), suggesting that TRAF1 expressed outside of the T cell compartment might play a role in facilitating allergic lung inflammation. In this study, we address the role of TRAF1 expressed in cells other than T lymphocytes by examining TRAF1^{-/-} mice in an adoptive transfer mouse model of asthma. Our results demonstrate that, in contrast to WT mice, TRAF1^{-/-} mice did not develop pulmonary inflammation or AHR following adoptive transfer of OVA-immune T cells and exposure to aerosol OVA (Figs. 1 and 2). Sensitized TRAF1^{-/-} recipients had a lower number of CD4⁺ and CD8⁺ T cells in their BAL and expressed negligible amounts of the Th2-associated cytokines IL-4, IL-5, and IL-13 in comparison to similarly treated WT recipients (Fig. 3). TRAF1^{-/-} mice that were injected with OVA-immune CD4⁺ T cells not only exhibited impaired OVA-dependent immune responses in the lungs but also failed to mount an immune response in the pulmonary draining LNs (Fig. 4C). Taken together these results indicate that the development of allergic inflammation in the lungs requires TRAF1 expression in non-T cell compartments.

Because DCs are known to express TRAF1 (41), we hypothesized that DCs may potentially be responsible for the absence of asthma in TRAF1^{-/-} recipients of OVA-immune T cells. Therefore, we investigated the function of TRAF1^{-/-} DCs in this model. Our findings reveal that there was no a significant increase in the number of DCs in the lungs of T cell-transferred TRAF1^{-/-} mice in comparison to either WT or sham-injected TRAF1^{-/-} mice (Fig. 4A). In addition, residual DCs in T cell-transferred TRAF1^{-/-} mice were not activated as indicated by low expression of MHC class II (Fig. 4B). These data correlate well with previous observations demonstrating that TRAF1^{-/-} DCs fail to up-regulate CD86 expression in response to CD40L stimulation and have a decreased survival rate in response to CD40L or TNF (17). However, TRAF1^{-/-} DCs induced normal Ag-dependent T cell proliferation in vitro, indicating that TRAF1 is not essential for Ag presentation and stimulation of T cells by DCs (Fig. 5A). In addition, like WT DCs, TRAF1^{-/-} DCs, when matured in the presence of LPS, displayed a more trans migratory capacity than did DCs in the presence of TNF (Fig. 5B). This result is in agreement with a previous observation that LPS is more effective than TNF in terms of inducing phenotypic and functional maturation of bone marrow-derived DCs (42). Interestingly, TNF-matured TRAF1^{-/-} DCs migrated even more efficiently than WT DCs (Fig. 5B), indicating that, although it is dispensable for LPS-mediated maturation of bone marrow-derived DCs, TRAF1 plays a negative role in TNF-mediated DC maturation. These findings are consistent with our previous results, which demonstrated that TRAF1 is a negative regulator of TNF signaling (19). In fact, in vivo bone marrow-derived mature TRAF1^{-/-} DCs, as well as WT DCs, were able to deliver sufficient amounts of Ag to the draining LNs to drive the local immune response (Fig. 6A). Taken together, these results suggest that TRAF1^{-/-} DCs, although competent to transport and present Ag, do not receive an activation signal in the lung mucosa of TRAF1^{-/-} mice and therefore fail to migrate to the lung draining LNs.

After acquisition of Ag, migration of mucosal DCs to the secondary lymphoid organs, particularly regional LNs, requires the presence of so-called “danger signals” (43). Because WT and TRAF1^{-/-} mice were exposed to LPS-free OVA to eliminate any nonspecific signals that might activate innate immune responses through TLRs, transferred OVA-immune T cells were the most likely providers of early activating signals to airway mucosal DCs following cognate interactions (44). Taking into account that mucosal TRAF1^{-/-} DCs were able to transport Ag to the draining

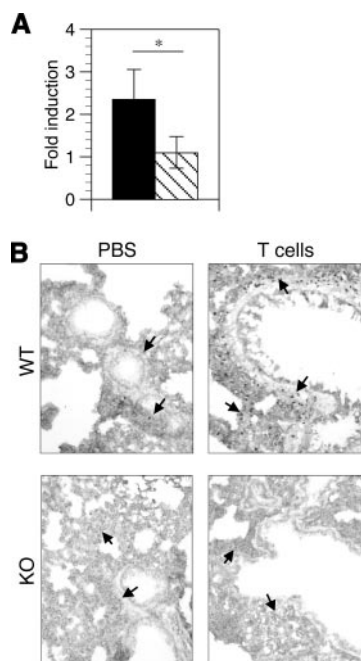


FIGURE 7. Decreased ICAM-1 expression in the lungs of TRAF1^{-/-} mice. Groups of WT and TRAF1^{-/-} (KO) mice were sensitized as described in Fig. 1. **A**, Expression of ICAM-1 was measured by quantitative real-time PCR in the lung tissue of WT (■) and TRAF1^{-/-} recipients (▨) injected with PBS or OVA-immune CD4⁺ T cells. Relative quantification of gene expression was done by the threshold comparative C_T method. The mRNA expression of the indicated molecules was normalized to that of β_2 -microglobulin from the same sample. The fold induction was calculated as the ratio of gene expression for CD4⁺ T cell-transferred mice to PBS-transferred mice. Data are expressed as mean + SEM of two independent experiments, and each experiment included three mice per group. *, $p < 0.05$. **B**, Immunohistochemical detection of ICAM-1. Mouse lungs from sensitized mice were stained with hamster Ab 3E2B to mouse ICAM-1 as the primary Ab. Arrow indicates ICAM-1 antigenicity. Original magnification at $\times 100$. Results are representative of four mice in each group.

LN (Fig. 6A), the absence of danger signals driving DC migration may be explained by the failure of Ag-specific effector CD4⁺ T cells to be recruited to the TRAF1^{-/-} lungs. T cells can supply danger signals to drive DC migration to the draining LNs through the expression of CD40L and TNF (45, 46). Although, it has recently been shown that TRAF1 cooperates with TRAF2 in CD40-mediated activation of B cells (18), it is doubtful that signaling through CD40 plays a role in the mouse model of allergic lung inflammation, as allergen-challenged mice, possessing a targeted deletion of CD40, display normal AHR and eosinophil influx into the bronchial mucosa (47, 48). Therefore, we speculate that in our experimental model TNF, which is produced by recruited T cells, plays a major role in driving the emigration of lung DCs to the regional LNs.

The process of T lymphocyte recruitment into the lung airways can be divided into three major stages including 1) the attachment of circulating lymphocytes to the capillary endothelium, 2) trans-endothelial migration, and 3) crossing of the epithelium into the alveolar airspace. Although the distribution and subsequent activation of adoptively transferred naive CD4⁺ T cells in the whole body has been well documented (49), very little is known about the immediate distribution of adoptively transferred activated CD4⁺ T cells. Dixon et al. (35) have demonstrated that shortly after i.v. administration, the majority of transferred T lymphocytes are retained in the lungs. Interestingly, adherence of these cells in the

lungs is significantly reduced by neutralization of LFA-1 or by ICAM-1 deficiency (35). In support of these observations, a study of the in vivo migration and localization of adoptively transferred CD8⁺ T cells demonstrated that activated CD8⁺ T cells are retained within the pulmonary vasculature and, subsequently, actively transmigrate into the pulmonary interstitium (50). Most importantly, the retention of activated T cells in the lungs is dependent on their expression of LFA-1 (50). In WT mice, the T cells that have migrated to the lungs might be further activated to express TNF and to induce the expression of adhesion molecules, such as ICAM-1, on resident lung cells following the series of aerosolized OVA exposures (Fig. 7). Thus, TRAF1-dependent up-regulation of ICAM-1 expression in the lungs may be part of an essential pathway that facilitates the recruitment of additional OVA-specific T cells into the lungs from the draining LNs.

TNF has been implicated in many pulmonary diseases, including asthma, chronic bronchitis, chronic obstructive pulmonary disease, and other disorders (51). A recent study has demonstrated that both TNFR1 and TNFR2 are important for Ag-induced allergic lung inflammation (52). TRAF1 has been shown to associate with both of these receptors (53, 54). Although lung epithelial and endothelial cells express only trace amounts of TRAF1 in the quiescent state, TRAF1 expression is dramatically increased following stimulation of these cells with TNF (22, 55) or other NF- κ B-inducing signals (56, 57). Taken together, our observations strongly imply that TRAF1 plays an important role in the up-regulation of ICAM-1 on resident lung cells, as well as in the resulting airway recruitment of T cells during allergic lung inflammation. Nevertheless, further studies are required to obtain a detailed understanding of the molecular pathways and types of resident lung cells responsible for the impaired allergic pulmonary inflammatory response demonstrated by TRAF1^{-/-} mice.

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Disclosures

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