

Impaired TNF/TNFR2 signaling enhances Th2 and Th17 polarization and aggravates allergic airway inflammation

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ABSTRACT

CD4⁺T cell differentiation plays an important role in allergic airway diseases. Tumor necrosis factor receptor 2 (TNFR2) has been shown to regulate CD4⁺T lymphocyte differentiation, but its role in allergic airway inflammation is not clear. Here we investigated the role of TNFR2 in allergic airway inflammation. Mouse model was generated by immunization with OVA and intranasal administration of TNFR2 antibody. Airway inflammation and CD4⁺T cell differentiation were measured in vivo and in vitro. Inhibited TNFR2 signaling aggravated airway inflammation and increased the expression of inflammatory cytokines (IL-4, IL-5, IL-17, and TNF- α) in serum and bronchoalveolar lavage fluid (BALF). Impaired TNFR2 signaling promoted Th2 and Th17 polarization but inhibited Th1 and CD4⁺CD25⁺ T cells differentiation in vivo. Furthermore, TNFR2 signaling inhibition promoted Th2 and Th17 polarization in vitro, which may through

the activation of TNF receptor-associated factor 2 (TRAF2) and nuclear factor (NF)- κ B signaling. Therefore, our findings indicate that impaired TNF/TNFR2 signaling enhances Th2 and Th17 polarization and aggravates allergic airway inflammation.

Key words

Allergic airway inflammation, Th2, Th17, TNF, TNFR2

Abbreviations: FoxP3, forkhead box P3; GATA3, GATA binding protein 3; NF- κ B, nuclear factor of kappa B; OVA, ovalbumin; PDTC, pyrrolidinedithiocarbamic acid; ROR γ , RAR-related orphan receptor gamma; T-bet, T-cell-specific T-box transcription factor; TNF- α , tumor necrosis factor- α ; TNFR2, tumor necrosis receptor 2; TRAF2, TNF receptor-associated factor 2; Tregs, regulatory T cells.

Introduction

Asthma is characterized by allergic airway inflammation, bronchial hyperresponsiveness, and airway remodeling, which affects approximately 300 million people worldwide. (5,36,40) Imbalanced CD4⁺T lymphocyte differentiation is considered an important mechanism of the airway inflammation. (21,22) IL-4 and IL-5 secreted by Th2 promote eosinophil maturation and recruitment to the lung. (14) IL-17 secreted by Th17 is elevated in the lung, sputum, and bronchoalveolar fluid (BALF) of asthma patients. (4,23,37) As a pro-inflammatory cytokine, IL-17 induces neutrophil recruitment (26) and is associated with severe asthma. (24) Tregs play an important role in immune homeostasis as well as Th1 cells, which can inhibit Th2 differentiation. However, the frequency and suppressing function of Tregs and Th1 cells have been proved to be decreased in asthma. (16)

Tumor necrosis factor α (TNF- α), a pleiotropic cytokine, is functioned by binding to the receptors designated as TNFR1 and TNFR2. Recent studies have demonstrated that the key role of TNFRs in lung inflammation. Ahn *et al* reported that TNFR1 signaling participated in the secretion of IL-16 furthermore contributed to the lung inflammation of Methicillin resistant *Staphylococcus aureus* (MASA) pneumonia. (1) In a pneumocystis carinii induced pneumonia model, the TNFR signaling resulted in PCP-related immunopathology by recruitment of damaged immune cells. (31, 38) Moreover, TNFR1 knockout attenuated both pulmonary fibrotic response to silica in pulmonary fibrosis and pulmonary emphysema-like changes in COPD. (17,32) Although there have been many studies on the effect of TNFRs knocking down in lung diseases, the role of TNFR2 in allergic airway inflammation is not clear. Our previous study demonstrated that the mRNA and protein expression of TNFR2 decreased significantly in lymphocytes from the spleens and lungs of an OVA-immunized asthma mouse model. However, the mechanism remains unknown. According to Chen and colleagues, CD4⁺T lymphocytes were the primary cell type that expressed TNFR2 in both human and mouse peripheral lymphoid tissues. (7,8) They further demonstrated that TNFR2 was critical for stabilizing CD4⁺FoxP3⁺ Treg cells.(11) In addition, TNFR2-expressing Tregs had been proved to be the most potently suppressive subset of Treg cells. (18,19,20,29) Other CD4⁺ T lymphocyte subsets were also influenced by TNFR2 signaling. A recent study demonstrated that impaired TNF/TNFR2 signaling promotes Th17 differentiation both *in vivo* and *in vitro*. (27)

Since imbalanced CD4⁺T cell differentiation plays an important role in asthma and TNFR2 is crucial for CD4⁺ T cells differentiation, the role of TNFR2 in allergic airway inflammation is not well understood.

We hypothesize that aberrant TNFR2 signaling influences CD4⁺T cell differentiation and exacerbates airway inflammation. In this study, we established an ovalbumin (OVA)-induced allergic airway inflammation mouse model and inhibited TNFR2 signaling with a TNFR2 antibody. We found that inhibited TNFR2 signaling exacerbated airway inflammation by promoting Th2 and Th17 cell polarization and that Th2 and Th17 excessive polarization may occur via activation of nuclear factor (NF)- κ B. Our results indicate an important role of TNFR2 signaling in allergic airway inflammation.

Materials and methods

Mice

Six-week-old female BALB/c mice were purchased from Shanghai SLAC Co.(Shanghai, China).All animal care and handling protocols were approved by Ethic Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. All of the experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Allergic airway inflammation mouse model and TNFR2 antibody administration

Animals were immunized according to previously published protocols. (30) Briefly, mice were sensitized intraperitoneally (i.p.) twice on days 0 and 14 with 100 μ g of OVA (Sigma-Aldrich, St. Louis, MO, USA) and 1mg of Al(OH)₃ (Thermo Scientific, Waltham, MA, USA). On days 25, 26, and 27, mice were challenged with 100 μ g of OVA intranasally (i.n.). For TNF- α receptor 2 inhibition, mice were treated with 10 μ g of TNFR2 antibody (BioLegend, San Diego, CA, USA) i.n.1 day before each sensitization and challenge according to the protocol reported by Choi as shown in Fig. 1. (13)

77 **Collection of serum and BALF**

78 Mice were sacrificed by an overdose of 100mg/kg of pentobarbital intraperitoneally 24 h after the final
79 challenge. Blood was collected by cardiac puncture. After centrifugation at 500g for 15min at 4°C, serum
80 was collected and stored at -80°C for cytokine analysis. We used 0.8 mL cold phosphate-buffered saline for
81 bronchoalveolar lavage. BALF was collected and centrifuged at 400g for 5min at 4°C. The supernatant was
82 collected and stored at -80°C for cytokines analysis, while the precipitate was analyzed for cellular
83 composition using the Wright-Giemsa staining.

84 **Lung histology**

85 The right lungs were removed and fixed in 4% buffered paraformaldehyde for 24h at room temperature.
86 To evaluate airway inflammation and mucus production, the paraffinized lung sections were stained with
87 hematoxylin and eosin (H&E) or periodic acid-Schiff(PAS) and viewed under a light-microscope at $\times 200$
88 magnification.

89 **Measurement of methacholine airway hyper responsiveness (AHR)**

90 24 hours after the last challenge, airway hyper responsiveness was measured by using a Buxco's
91 modular and invasive system (Buxco Electronics Inc., NY, USA). Changes of the airway resistance(RL)
92 and lung dynamic compliance (Cdyn) were measured as our previously reported.(25) Briefly, mice were
93 anesthetized and tracheostomized and intubated with an appropriate cannula and then laid supine inside the
94 body plethysmograph chamber connected to the ventilator. After a stable (<5% variation over 2.5 min)
95 baseline airway pressure was achieved, the mice were treated with aerosolized PBS or various

concentrations of methacholine (3.125, 6.25, 12.5, 25 mg/ml) via a jet nebulizer into the head chamber. The values of RL and C_{dyn} were calculated in response to increasing concentrations of inhaled methacholine. The values were expressed as a percent change from the baseline value as our previously reported.

Cytokine analysis

Lymphocytes derived from the spleen were cultured *in vitro* and stimulated with OVA (10 µg/ml) for 3 days. The culture supernatant was collected and stored at -80°C until cytokines analysis. IL-4, IL-5, IL-10, interferon (IFN)-γ, IL-17A, and TNF-α levels of serum, BALF, and culture supernatant were estimated by enzyme-linked immunosorbent assay kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

T cell isolation and *in vitro* culture

Naive CD4⁺ T cells were isolated from the spleen with an EasySep mouse naive CD4⁺ T cell isolation kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. The naive CD4⁺ T cells were cultured in complete medium (RPMI1640 containing with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) and induced to undergo Th0, Th2, or Th17 polarization, as previously reported. (34) For Th0 differentiation the naive CD4⁺ T cells were stimulated with 5 µg/ml of anti-CD3 mAb, 1 µg/ml of anti-CD28 mAb and 20 ng/ml of IL-2. For Th2 polarization the naive CD4⁺ T cells were stimulated with 5 µg/ml of anti-CD3 mAb, 1 µg/ml of anti-CD28 mAb, 20 ng/ml of IL-2 as well as 100 ng/ml of IL-4, 10 µg/ml of anti-IFN-γ, 10 µg/ml of anti-IL-12. While for Th17 differentiation the naive CD4⁺ T cells were stimulated with 5 µg/ml of anti-CD3 mAb, 1 µg/ml

of anti-CD28 mAb and 1ng/ml of TGF- β , 100ng/ml of IL-6, 10ug/ml of anti-IL4 and 10ug/ml of anti-IFN γ .
In some experiments, 10 μ g/mL of TNFR2 Ab was added to block TNFR2 signaling. While in the other
experiments 10ug/ml of hamster IgG was added for TNFR2 Ab control.

Flow cytometry

For *in vivo* or *in vitro* CD4⁺T cell polarization analysis, cells from the spleen and lung-draining
mediastinal lymph nodes, or *in vitro* culture, were separated and stimulated with leukocyte activation
cocktail (BD Biosciences, Franklin Lakes, NJ, USA) 2 μ l/mL for 5 h. CD4-FITC (0.25 μ g per test) and
CD25-PE (0.125 μ g per test) were used to label CD4⁺CD25⁺ T cells. For intracellular cytokine staining,
lymphocytes were permeabilized and fixed with Cytotfix/Cytopermia Kit (eBioscience, San Diego, CA,
USA). IFN- γ -APC (1 μ g per test), IL-4-PE (0.125 μ g per test), and IL-17-PerCP-Cy5.5 (0.03 μ g per test)
purchased from eBioscience were used to label Th1, Th2, and Th17 cells, respectively. For NF- κ B signaling
analysis, cells were firstly stained with 1 μ l/test of rabbit anti-mouse P65, P-P65, (Cell Signaling Technology,
Boston, MA) tumor necrosis receptor-associated factor 2 (TRAF2; Abcam, Cambridge, UK) for 30 minutes
and then stained with 0.5 μ l/mL of Alexa Fluor 488 goat anti-rabbit IgG (H+L) in darkness for 30 minutes.
All the samples were analyzed by BD FACSCalibur (BD Biosciences).

Real time PCR

Total RNA of lymphocytes from lungs and spleens was extracted using TRIzol reagent. (Takara
Biotechnology, Dalian, China) and cDNA was synthesized by using a PrimeScript TM RT reagent
Kit. (Takara Biotechnology, Dalian, China) The downstream genes expression of TNFRs, Ask1 for TNFR1

and Bmx for TNFR2, were examined with an ABI 7500 real time PCR system using a SYBR Premix Ex TaqTM II kit. The following primer pairs were used: Ask1 forward: 5'-CAAATCAGACAGTCCGACGG-3'; reverse: 5'-GTGCATTCTGGGAGTCATGG-3'. Bmx forward: 5'-GGAAAGTTCCTGTGTTGCCA-3' ; reverse: 5'-TGGGAGCTCTGTGTTTCTCA-3'. Actin forward: 5'-GTGCTATGTTGCTCTAGACTTCG-3'; reverse: 5'-ATGCCACAGGATTCCATACC-3'.

Western blotting

Lymphocytes from spleens and lungs were isolated by lymphocytes separation medium (DKEWE, Beijing, China.) and were restimulated with leukocyte activation cocktail for 5 hours (BD Bioscience). And then the cells were lysed by radio-immunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein samples (50 µg) were run on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After incubation with a blocking buffer [5% non-fat milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (w/v) Tween-20] for 2 h, the membrane was incubated with primary Abs for TNFR2 (1:500; BioLegend, San Diego, CA, USA); for T-bet, GATA3, FoxP3, RORγ (all 1:300, Boster, Wuhan, China), or actin (1:2000, Beyotime, Shanghai, China) and then visualized with a horseradish peroxidase-conjugated secondary Ab(1:2000, Beyotime, Shanghai) and enhanced chemiluminescent reagents.(39)The relative quantities of proteins were estimated by scanning densitometry (ChemiDoc XRS Systems, Bio-Rad Laboratories,Inc) and analyzed by Image Lab 5.0 software(Bio-Rad Laboratories, Inc.)

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Data was expressed by mean±sem, Independent T-test was used for the comparisons between two groups. For multiple comparisons, one-way ANOVA was used first, if significant differences were detected, a LSD post hoc test was performed between pairs of groups. P value<0.05 was considered statistically significant.

Results

TNFR2 signaling is inhibited effectively by TNFR2 antibody

In order to evaluate the blocking efficacy of TNFR2 antibody, we first check the expression of TNFR2 in lymphocytes from lung by western blot. As shown in Fig 2(A-B), the expression of TNFR2 was dramatically decreased in the lung lymphocytes from TNFR2 antibody-treatment group compared with the control group. TNF- α can bind to its receptors TNFR1 and TNFR2, which enhances the expression of the target gene Ask1 and Bmx respectively. (34) To identify whether TNFR2 signaling was inhibited by TNFR2 antibody, we next check the Ask1 and Bmx gene expression. As shown in Fig2 (C-D), the expression of Bmx gene was dramatically decreased in the spleens and lung lymphocytes from TNFR2 antibody-treatment mice compared with that from non-treatment mice. Meanwhile, the expression of Ask1 gene was no significant difference between them. These results indicated that the TNFR2 signaling was inhibited effectively by TNFR2 antibody.

Inhibited TNFR2 signaling aggravates airway inflammation and airway hyper responsiveness.

Lung histology was assessed by HE and PAS staining. As shown in Fig 3A and 3B, the inflammatory cells and mucus production were no significant difference in the lungs between the control and TNFR2 Ab used

alone groups. While the inflammatory response was dramatically exacerbated in lungs from the TNFR2Ab-administrated OVA sensitized mice compared with the non-treatment mice. Furthermore, inflammatory cells in the BALF were typed and quantified by Wright-Giemsa staining. As shown in Fig 3C, TNFR2 Ab administration did not influence the cell amounts in non-sensitized mice but significantly increased the count of total infiltrated cells, eosinophils, neutrophils, and macrophages in OVA sensitized mice. Next, when challenged with various concentrations of methacholine, as shown in Fig 3D, the RL was significantly increased, whereas the Cdyn was significantly decreased in the TNFR2Ab-administrated OVA sensitized mice compared with the non-treatment mice. However, there was no significant difference between non-sensitized mice. These results indicated that inhibited TNFR2 signaling aggravated the OVA induced airway inflammation and airway hyper responsiveness but had no effect on the airway inflammation and airway hyper responsiveness of non-sensitized mice.

Inhibited TNFR2 signaling influences cytokine expression

Cytokines play a critical role in the inflammatory response, then we asked whether inhibited TNFR2 signaling influenced cytokine expression. Cytokines in serum, BALF and cell culture supernatant were detected by ELISA. As shown in Fig 4, the expression of IL-4 and IL-5 were increased in OVA immunized mice and further significantly increased in OVA immunized mice with TNFR2 inhibition. TNF- α is primarily produced by macrophages and has been implicated in airway inflammatory cell influx. We found that inhibited TNFR2 signaling led to increased TNF- α expression in serum and BALF as well as cell culture supernatant. The important neutrophil chemotaxin IL-17 was also increased in our OVA-induced mice model. In addition, a further significant increase of IL-17 was found in the mice treated with TNFR2 Ab. In

contrast, IFN- γ and IL-10, which inhibit pro-inflammatory cytokine synthesis, were both decreased in TNFR2 Ab treatment groups.

Inhibited TNFR2 signaling influences CD4⁺ T cell differentiation *in vivo*

TNFR2 signaling is essential for the stabilization of CD4⁺FoxP3⁺ Tregs and other CD4⁺ T cell subsets(11,27). Whether aggravated airway inflammation induced by TNFR2Ab administration is related to the abnormal differentiation of CD4⁺ T cell subsets is not clear. Next, we checked the percentages of Th1, Th2, CD4⁺CD25⁺ T cells and Th17 in total CD4⁺ T cells derived from the spleen and lung drainage lymph nodes by flow cytometry. As shown in Fig 5 A-D, Th1 and CD4⁺CD25⁺ T cells differentiation were inhibited by TNFR2 Ab administration. OVA immunization induced Th2 and Th17 differentiation, while the frequency of Th2 and Th17 were significantly increased in TNFR2 Ab administration group (Fig 5E-H). Transcript factors play an essential role in the differentiation of CD4⁺ T cell subsets. Whether TNFR2 inhibition affect the expression of Tbet, GATA3, FoxP3, ROR γ is not clear. As shown in Fig 6, the expression of Tbet and FoxP3 in lymphocytes from spleen were decreased while the expression of GATA3 and ROR γ were increased in mice with TNFR2 Ab administration.

Impaired TNFR2 signaling promotes Th2 and Th17 polarization *in vitro*

To clarify the impact of Impaired TNFR2 signaling on CD4⁺ T cells in the setting of airway inflammation, we cultured naive CD4⁺T cells in a microenvironment designed to mimic airway inflammation *in vitro*. Naive CD4⁺T cells were isolated and polarized to Th0, Th2, or Th17 for 96 hours. We inhibited TNFR2 signaling during polarization. As shown in Fig. 7A and 7B, the frequencies of Th2 and

Th17 cells increased in the TNFR2 Ab treatment group when compared with non-treatment group.

NF-κB activation is associated with excessive Th2 and Th17 polarization

As reported previously, NF-κB may be a downstream target of TNF-α/TNFRs signaling. (28) We therefore assessed the expression of P65 and P-P65 (phospho-P65) in CD4⁺T cells during polarization. With TNFR2 inhibition, the expression of P-P65 but not P65 increased in both Th2 and Th17 polarization (Fig. 8A-B). Furthermore, we assessed the expression of Tumor necrosis factor receptor-associated factor 2 (TRAF2), which is a critical mediator of TNF-α/TNFRs signaling, in different polarization conditions by flow cytometry. With TNFR2 Ab administration, TRAF2 expression increased in both Th2 and Th17 polarization (Fig. 8C). As NF-κB signaling is involved in the excessive polarization of Th2 and Th17 following TNFR2 blocking, we inhibited NF-κB with 10μM PDTC. Consequently the excessive polarization of Th2 and Th17 induced by TNFR2 inhibition were ameliorated. (Fig 8D).

Discussion

Recent study has demonstrated that TNFR2 is critical for stabilizing CD4⁺FoxP3⁺ Treg cells and can promote Th17 differentiation. Therefore, we hypothesized that TNFR2 signaling may be involved in allergic airway inflammation. In this study, we inhibited TNFR2 signaling by intranasally administration of TNFR2 antibody and then examined the airway inflammation and CD4⁺T lymphocyte differentiation. Our results suggest that impaired TNFR2 signaling exacerbates airway inflammation by promoting Th2 and Th17 polarization but inhibiting Th1 and CD4⁺CD25⁺ T cells differentiation.

Airway inflammation and high mucus secretion are the most important characteristics of asthma. In the

current study, inhibited TNFR2 signaling aggravated both outcomes. These results are in accordance with the finding of Candel *et al.* that deficiency of TNFR2 may exacerbate oxidative stress-induced inflammation. (6) Another study indicated that loss of TNFR2 signaling aggravated radiation induced nervous system inflammation. (12) Collectively, these findings implicate an important role of impaired TNFR2 signaling in these inflammatory diseases. Upon antigen challenge, we observed a large number of mucus production and inflammatory cells infiltrated into the lung tissue in OVA immunized mice, including both eosinophils and neutrophils. Furthermore, TNFR2Ab administration aggravated allergen-induced airway inflammation. However, the count of lymphocytes was not increased significantly by TNFR2Ab administration, which indicated TNF- α /TNFR2 signaling may not affect the proliferation of lymphocytes. These data were in agreement with those of Choi *et al.* and also shown that lung infiltrations of neutrophils and eosinophils, but not lymphocytes, were significantly increased in mice sensitized with OVA plus TNF- α . (14) In addition, we found that TNFR2 Ab administration did not influence the cell amounts in non-sensitized mice. Similar observations were made in mice sensitized with TNF- α or OVA plus TNF- α by Choi *et al* that TNF- α alone cannot influence airway inflammation significantly. In contrast, OVA plus TNF- α induced airway inflammation greatly. (14) These differences may be explained, in part, by the fact that the activation of TNF- α /TNFRs signaling is only under the condition of OVA sensitization. Airway hyper responsiveness, which is caused by the hypercontractility of airway smooth muscles, is also a critical component and feature of asthma. Amrani *et al* has shown that TNF- α / TNFR1 signaling can induce MAPK activation, which play an essential role in modulating contractile, synthetic and proliferation in airway smooth muscles(2). In our study, we also found that airway hyperresponsiveness was induced dramatically by treatment with TNFR2Ab. These results collectively suggest that inhibition of TNFR2 signaling aggravates the severity of

asthma.

Previous studies had shown that TNFR2 deficiency triggered the induction of genes encoding pro-inflammatory mediators such as TNF- α , IL-1 β , and cyclooxygenase 2b in keratinocytes(6). In the present study, we detected the protein expression of pro-inflammatory cytokines in serum and BALF by ELISA. IL-4, IL-5, TNF- α , IL-17 levels were significantly increased with TNFR2 inhibited, whereas cytokines that can inhibit the inflammatory response in allergic airway inflammation (e.g., IFN- γ and IL-10) were dramatically decreased. Our observations show that the increased airway inflammation by inhibition of TNFR2 signaling may therefore be related to altered cytokine expression.

Chronic airway disease is considered to be associated with an imbalance of CD4⁺T cells differentiation. However, the effect of TNFR2 signaling on the CD4⁺T cell differentiation is not well understood. Most studies focused on TNFR2⁺ Treg cells and considered it the most potently suppressive Treg subset. (9,10) Here, we demonstrated the impact of blocking TNFR2 signaling on CD4⁺ T cells differentiation in mice with allergic airway disease. TNFR2 Ab administration decreased the percentages of CD4⁺CD25⁺ T cells and Th1 cells in OVA immunized mice. Th2 cells together with the Th2 cytokines are the initial mediators that trigger eosinophilic inflammation, while IL-17 produced by Th17 is involved in neutrophilic inflammation and severe asthma. In our study, the percentages of Th2 and Th17 cells were both significantly increased with TNFR2 inhibition, both *in vivo* and *in vitro*. These results suggest that lack of TNFR2 signaling may be associated with severe asthma. To clarify the potential mechanism that inhibited TNFR2 signaling regulates CD4⁺ T lymphocyte differentiation, we examined the expression levels of transcript factors of each CD4⁺ T cell subset. Impaired TNFR2 signaling promoted GATA3 and ROR γ expression but

inhibited FoxP3 and T-bet expression. Since the reduced expression of CD4⁺CD25⁺ T cells may promote the differentiation of other subsets in asthma, the unusual differentiation of CD4⁺ T cells induced by TNFR2 inhibition may be directly due to the abnormal expression of transcript factors, or indirectly due to reduced CD4⁺CD25⁺ T cells differentiation.

As NF- κ B may be a downstream target of TNF- α /TNFRs signaling, we checked P-65 and P-P65 expression in CD4⁺T cells during polarization to Th2 and Th17 *in vitro*. We found that the percentage of positive cells and median fluorescence intensities of P-P65 were increased in TNFR2 Ab administration group. These results indicate that NF- κ B signaling is involved in excessive Th2 and Th17 polarization induced by TNFR2 signaling inhibition. Recently, Amrani et al found that agonistic antibodies specific for TNFR1 and TNFR2 both activated NF- κ B-dependent gene transcription in airway smooth muscle cells. However, receptor-blocking anti-TNFR1 antibody but not anti-TNFR2 antibody reduced TNF- α -induced NF- κ B-dependent gene transcription.(2) Therefore, TNF- α regulates NF- κ B activation in airway smooth muscle cells by mainly activating TNFR1, which can partly explain our results that TNFR2 signaling inhibition, in other words TNFR1 signaling dominant enhances NF- κ B signaling under OVA-immunized condition(2) Next, we inhibited NF- κ B signaling by PDTC and found that the excessive polarization of Th2 and Th17 disappeared. Together, these data support a role for NF- κ B signaling in regulating CD4⁺T cell differentiation. TRAF2 is a critical mediator of TNF- α /TNFRs signaling, which is also an activator of NF- κ B. Recent studies have shown that TNFR2 is able to induce JNK activation in a TRAF2-independent fashion, which is responsible for the ability of TNFR2 to induce TRAF2 degradation(5,33) , Furthermore, we found that the percentage of positive cells and median fluorescence intensities of TRAF2 was increased

in TNFR2 Ab administration group. One possible explanation is because blocking TNFR2 may reduce the degradation of TRAF2 and contribute to excessive NF- κ B activation. Together, these data suggest that NF- κ B signaling may affect the expression of transcription factors in CD4⁺T cell subsets and influence their differentiation.

In conclusion, our findings indicate that impaired TNFR2 signaling regulates CD4⁺T cell differentiation via NF- κ B signaling, playing an important role in allergic airway inflammation. Up regulation of TNFR2 may therefore ameliorate airway inflammation.

Figure 1. Timetable for OVA-induced allergic airway inflammation mouse model and TNFR2 Ab administration. (A) Mice were sensitized with 100 μ g of OVA and 1mg of Al(OH)₃ intraperitoneal (i.p.) on days 0, 14 and challenged with 100 μ g of OVA intranasally (i.n.) on days 14, 25, 26, 27. For TNFR2 antibody administration, the mice were treated with 10 μ g of TNFR2 Ab intranasally (i.n.) 1 day before sensitization and challenge. (B) For immunized control 1 mg of Al(OH)₃ and 30 μ l of PBS were used in control and TNFR2 Ab used only groups at the sensitization and challenge times. For TNFR2 Ab treatment control 10 μ g of hamster IgG was used in control and OVA groups at the antibody treatment times.

Figure 2. TNFR2 Ab administration reduced TNFR2 protein and the gene expression of Bmx. (A-B) TNFR2 protein expression in lymphocytes from lungs of the four groups was detected by western blotting. The expression of TNFR2 protein decreased in TNFR2 Ab and OVA groups when compared with control group. TNFR2 protein expression in OVA+TNFR2Ab group was decreased when compared with OVA

group. (C-D)The expression of TNFR2-specific downstream gene Bmx was decreased in TNFR2Ab group when compared with the control and in OVA+TNFR2Ab group when compared with the OVA group.The expression of TNFR1-specific downstream gene Ask1 has no significant difference in the TNFR2Ab administration groups when compared with the control groups. The data were expressed as means $\pm$ SEMs from 6 mice in each group. *P<0.05, **P<0.01. “NS” no significant.

Figure 3. TNFR2 Ab administration aggravated airway inflammation and airway hyperresponsiveness. (A) Lung history analyzed by HE staining. (B) Lung history analyzed by PAS staining. (C) Total cell numbers and cellular composition in BALF were assessed by cytopsin with Wright-Giemsa staining. (D) Airway responsiveness to aerosolized methacholine was evaluated by using a Buxco’s modular and invasive system. Changes in RL and Cdyn were calculated and compared. The data were expressed as means $\pm$ SEMs from 6 mice in each group. *P<0.05, **P<0.01.when compared with the control and TNFR2Ab alone group. #P<0.05 when compared with the OVA group.

Figure 4. TNFR2 inhibition altered cytokines secretion. IL-4, IL-5, TNF- α , IL-17, IFN- γ , and IL-10 in serum, BALF, and cell culture supernatant were assessed by ELISA. The data were expressed as mean $\pm$ SEM (n=6 samples per group) *P<0.05, **P<0.01.

Figure 5. Impaired TNFR2 signaling influenced CD4⁺T cell subset differentiation. Lymphocytes from spleen and lung-draining lymph nodes were restimulated with leukocyte activation cocktail. Differentiation of CD4⁺ T cell subsets in each group were analyzed by flow cytometry. The data shown in right upper quadrant represent the percentage of Th1 (A), Treg (C), Th2 (E) , Th17 (G) in total cells respectively. And then the percentages of each subset ,Th1 (B), Treg (D), Th2 (F), Th17 (H), in total CD4⁺T cells were calculated. The data were expressed as means $\pm$ SEMs (n=6 animals per group)*P<0.05, **P<0.01.

Figure 6. TNFR2Ab administration affected the expression of transcription factors in the lymphocytes from spleen. The protein expression of each transcript factor in lymphocytes derived from spleen of each group was analyzed by Western blotting (A) representative western blots; (B) Densitometry analysis of Tbet, GATA3, FoxP3, ROR γ normalized to actin protein from 3 independent experiments. the data were expressed as mean $\pm$ SEM (n=6 animals per group). *P<0.05.

Figure 7. Blocking TNFR2 promoted Th17 and Th2 differentiation *in vitro*. Naive CD4⁺ T cells were polarized to Th17 and Th2 cells respectively in vitro. The frequency of Th17(A) and Th2(B) in polarization and polarization +TNFR2Ab groups were analyzed by flow cytometry. The experiment was repeated for 3 times and the frequency of Th17(B) and Th2(D) in each group was calculated. The data were expressed as mean $\pm$ SEM. ** P<0.01.

349

350 **Figure 8. NF-κB signaling in different polarization conditions.** (A) P65 and P-P65 expression in Th2
351 polarization and Th2 polarization+TNFR2Ab groups. (i) the calculation of positive cell's frequency of P65
352 and P-P65. (ii) the calculation of mean fluorescence intensity (MFI) of P65 and P-P65. (B) P65 and P-P65
353 expression in Th17 polarization and Th17+TNFR2Ab groups. (i) the calculation of positive cell's frequency
354 of P65 and P-P65. (ii) the calculation of mean fluorescence intensity (MFI) of P65 and P-P65. (C)
355 TRAF2 expression in Th2, Th2 +TNFR2Ab, Th17, and Th17 +TNFR2Ab groups. (i) the calculation of
356 positive cell's frequency of TRAF2. (ii) the calculation of mean fluorescence intensity (MFI) of TRAF2.
357 (D) The Th2 and Th17 polarization with PDTC the NF-κB inhibitor in vitro. All the experiments were
358 repeated for 3 times, The data were expressed as mean ± SEM. *P<0.05,** P<0.01, "NS" no significant.

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363 **Additional information:**

364 **Competing financial interests:** The authors declare no competing financial interests.

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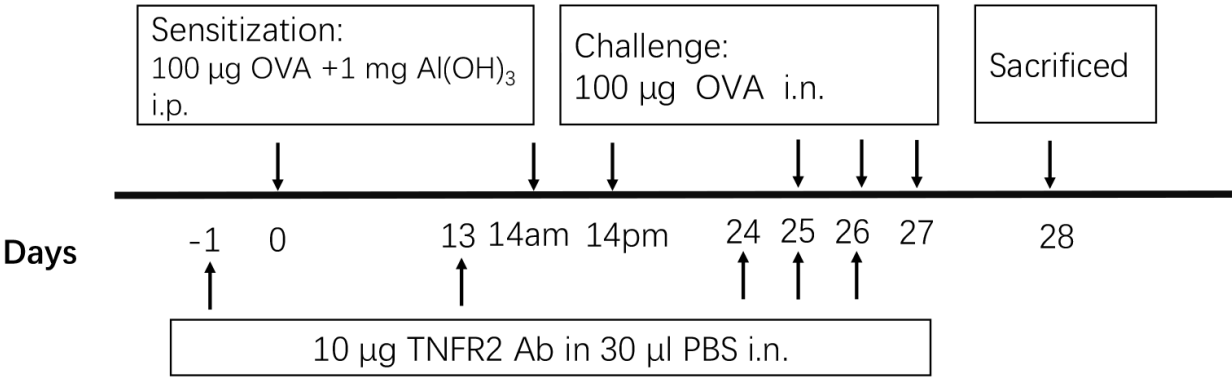
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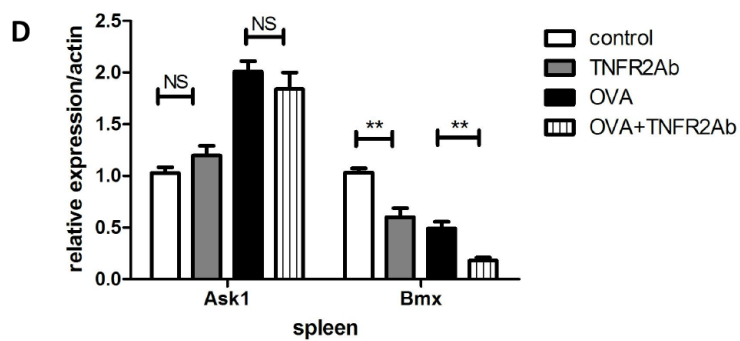
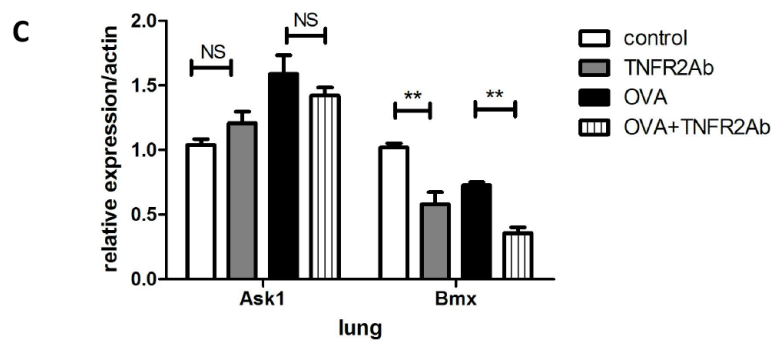
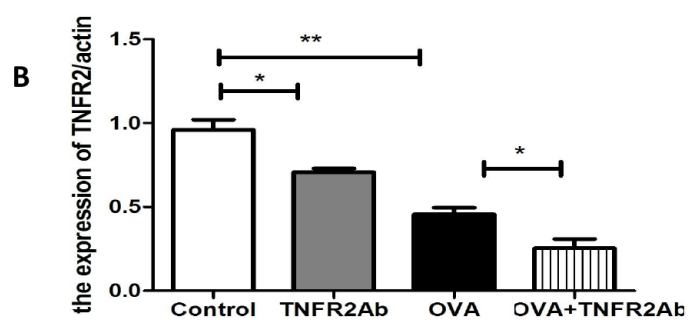
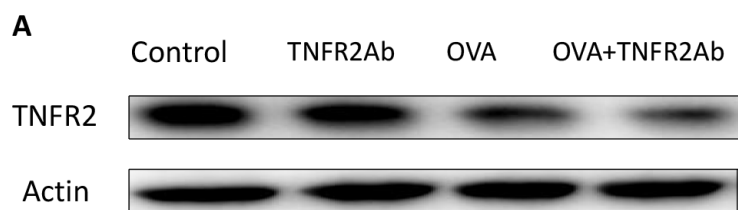
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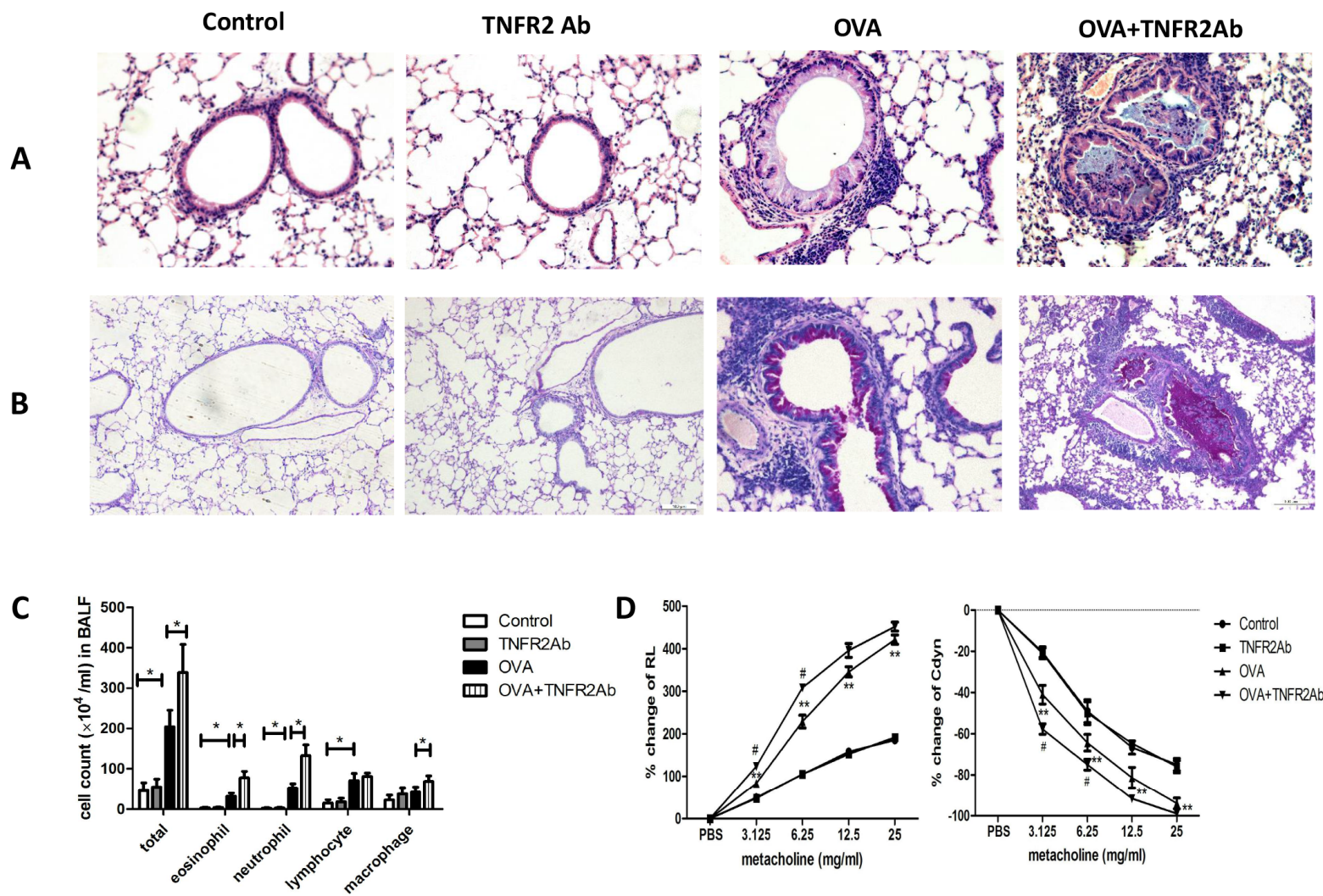
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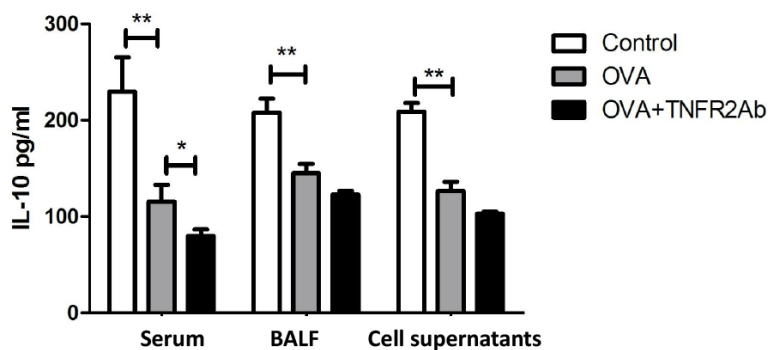
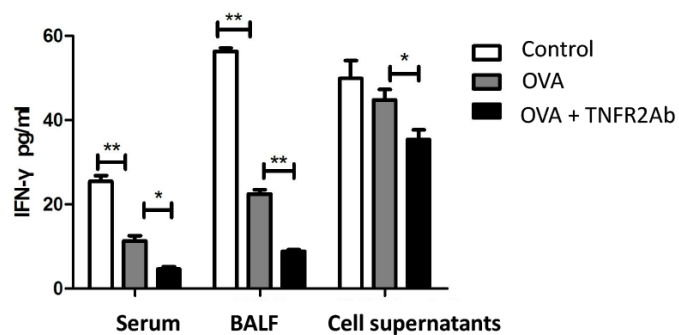
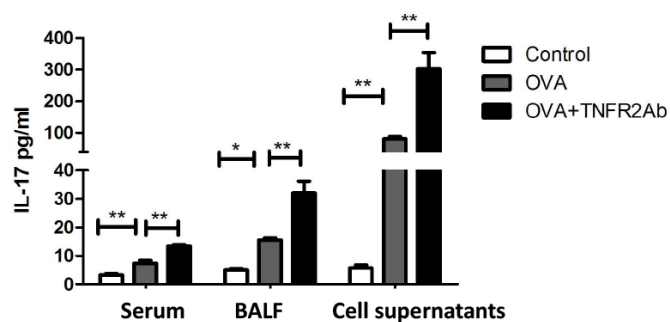
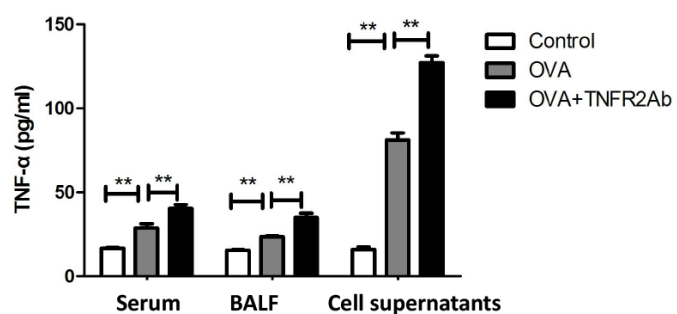
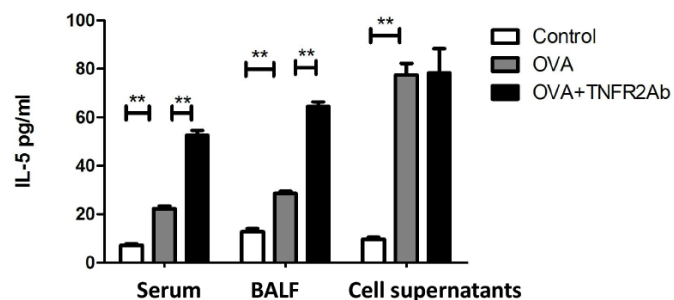
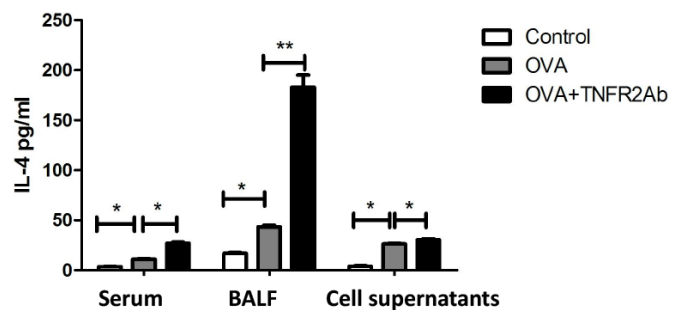


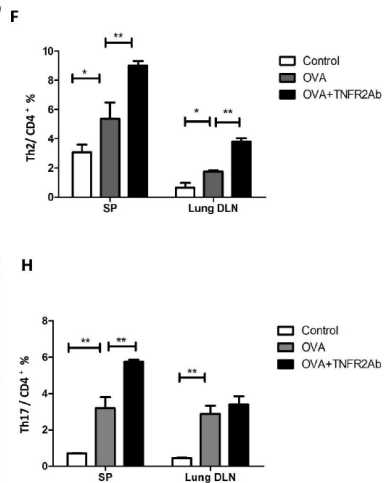
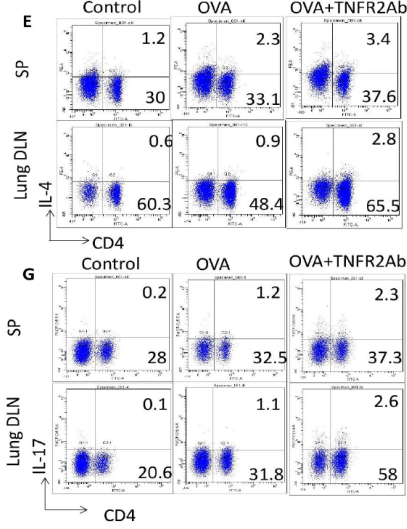
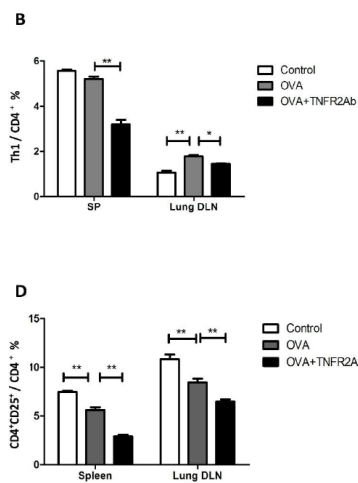
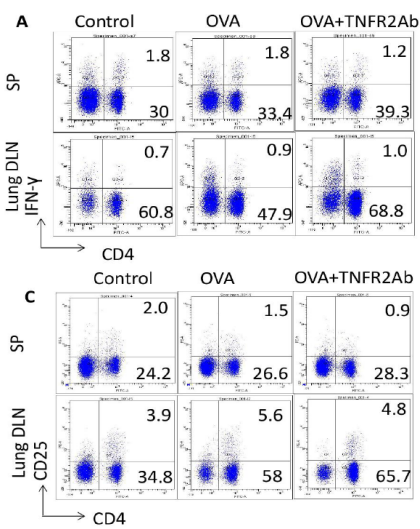
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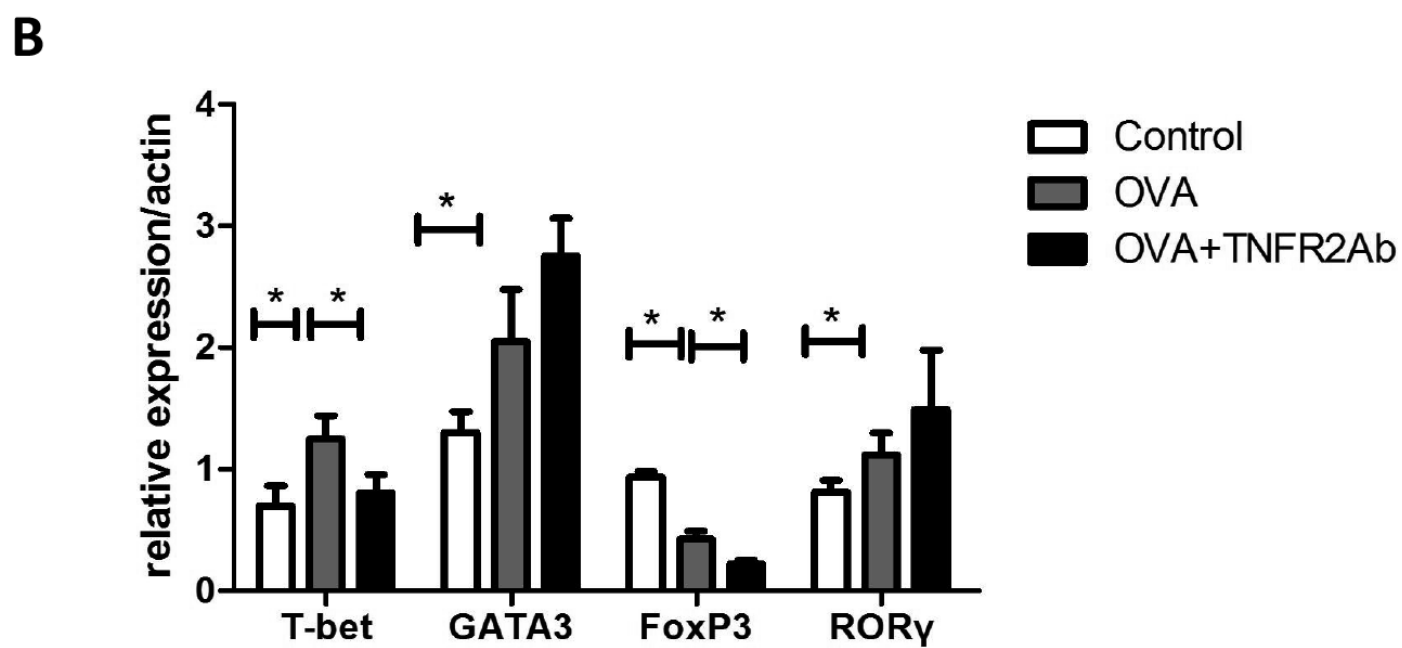
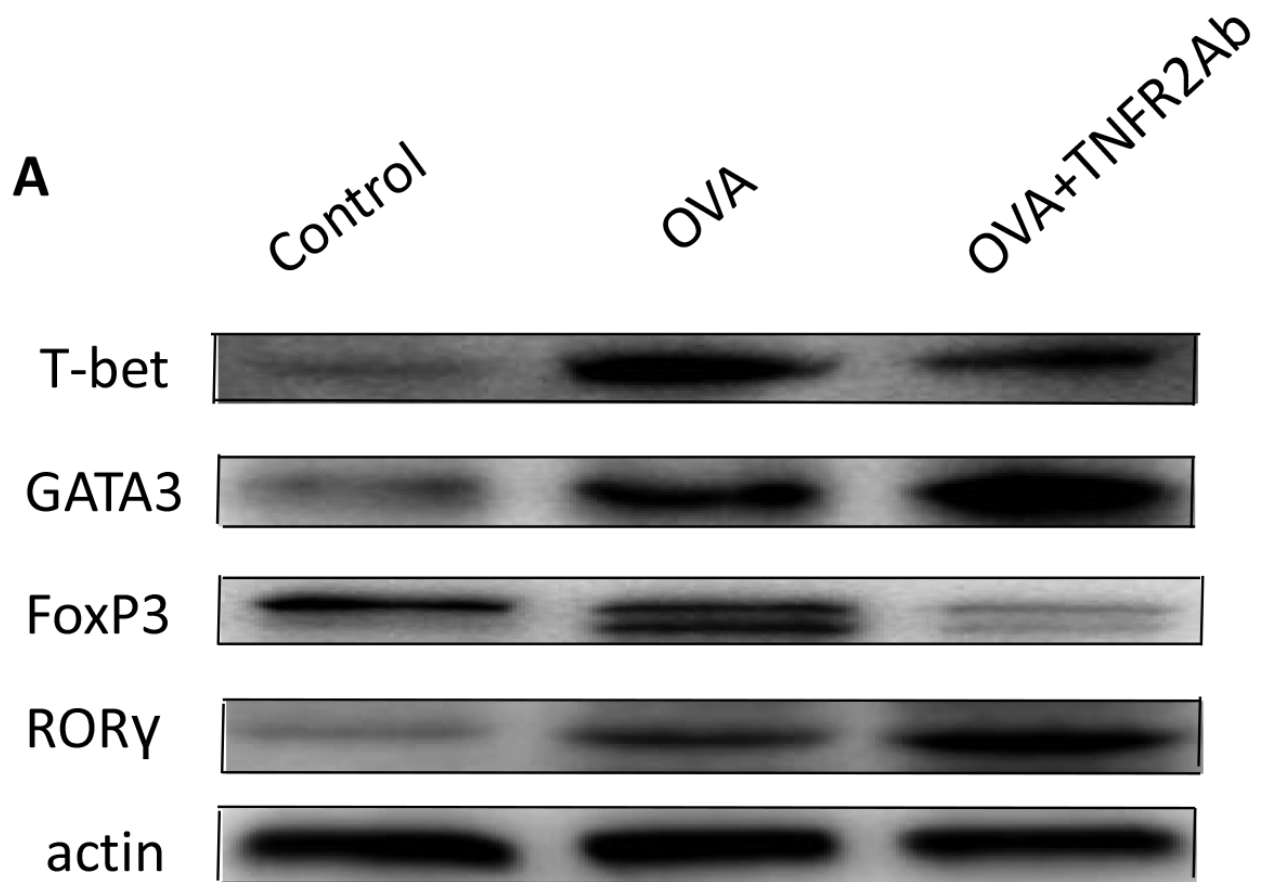
Group	Sensitization: (260 µl i.p.)	Challenge: (30 µl i.n.)	Treatment (30 µl i.n.)
Control	1 mg Al(OH) ₃	PBS	10 µg hamster IgG
TNFR2 Ab	1 mg Al(OH) ₃	PBS	10 µg TNFR2 Ab
OVA	100 µg OVA+1 mg Al(OH) ₃	100 µg OVA	10 µg hamster IgG
OVA+TNFR2 Ab	100 µg OVA+1 mg Al(OH) ₃	100 µg OVA	10 µg TNFR2 Ab



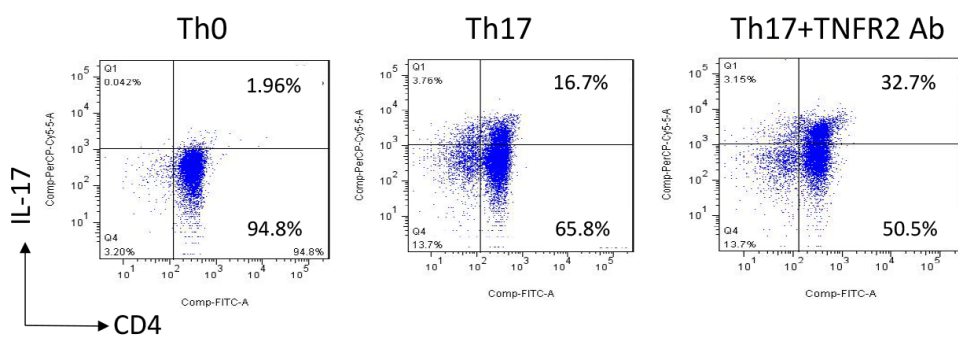




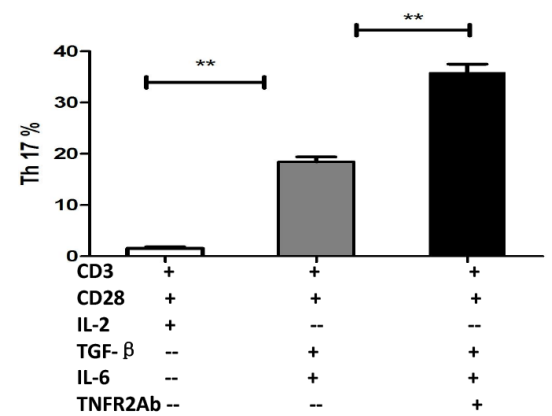




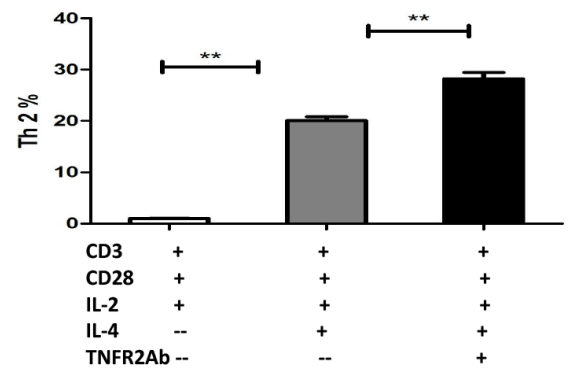
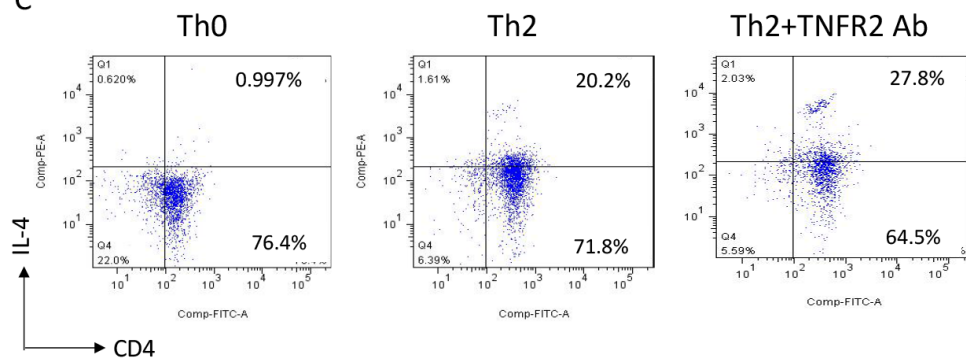
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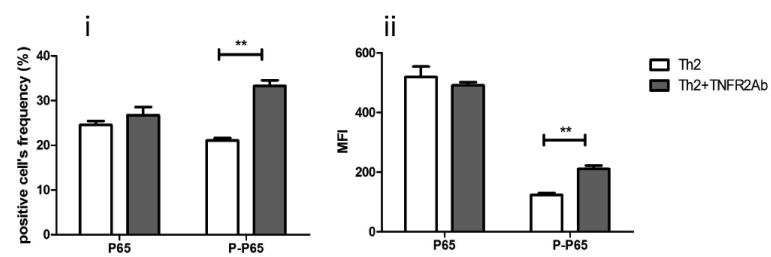
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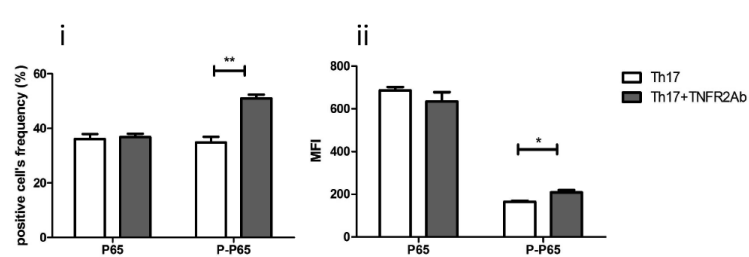
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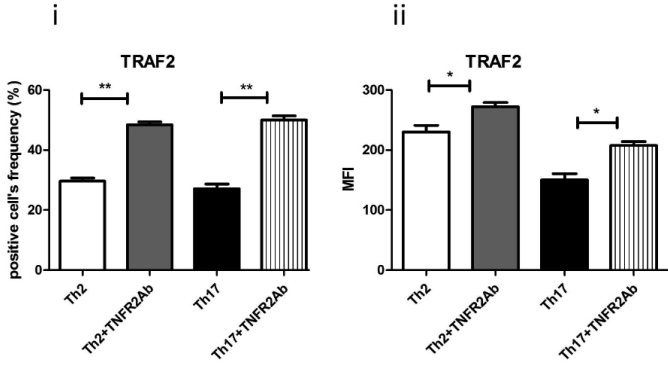
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