

TRAF1 regulates T_H2 differentiation, allergic inflammation and nuclear localization of the T_H2 transcription factor, NIP45

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Abstract

We have previously reported that tumor necrosis factor receptor-associated factor 1 (TRAF1), an intracellular protein, which binds to a range of molecules, including tumor necrosis factor (TNF) receptor family members, regulates TNF-induced NF- κ B and AP-1 signaling as well as TCR-triggered proliferative responses in T cells. In order to define the role of TRAF1 in T_H cell differentiation, we analyzed the responses of TRAF1^{-/-} T cells following TCR activation. Stimulation of TRAF1^{-/-} T cells by antigen resulted in significantly increased expression of the T_H2 cytokines (IL-4, IL-5 and IL-13) compared with wild-type (WT) controls. The T_H2 bias of TRAF1^{-/-} T cells is T lymphocyte intrinsic, since naive CD4⁺CD62L⁺ TRAF1^{-/-} T cells activated with CD3/CD28 produced elevated levels of T_H2 cytokines. Consistent with these observations in cultured T cells, TRAF1^{-/-} T cells induced enhanced T_H2 responses *in vivo*. Transfer of ovalbumin (OVA)-immune TRAF1^{-/-} T cells into naive WT recipients conferred significantly more intense pulmonary inflammation and higher airway hyperresponsiveness following inhaled OVA challenge than did transfer of OVA-immune WT T cells. Biochemical analysis of TRAF1^{-/-} T cells revealed that they have elevated nuclear expression of NFAT-interacting protein (NIP45), a T_H2 cell-associated transcription factor known to potentiate NFATp-driven IL-4 expression. In further experiments, we demonstrated that TRAF1 associates with a fraction of NIP45 in the cytoplasm and prevents its translocation to the nucleus. Taken together these results suggest that TRAF1 may limit the induction of T_H2 responses by decreasing NIP45 concentration to the nucleus and thereby down-regulating the expression of NIP45-dependent IL-4 gene transcription.

Introduction

Tumor necrosis factor (TNF) and its homologues are important in a wide array of biological processes, including the acute-phase response, cell growth and apoptosis, inflammation and lymphocyte activation (1). Tumor necrosis factor receptor-associated factor (TRAF) proteins play a significant role in signaling by both TNF and IL-1R/Toll-like family receptors (2). TRAF1 and TRAF2 were originally identified as TNFR2-associated proteins more than 10 years ago (3). Since that time, TRAFs have been shown to directly associate with the intracellular domains of a number of cell-surface receptors as well as a variety of cytoplasmic proteins (2). Although, the mechanism of action of the TRAF proteins in signaling has not been fully elucidated, it is evident that TRAF family members,

particularly TRAF2 and TRAF6, exert downstream effects on a number of intracellular signaling systems, including the JNK, nuclear factor- κ B (NF- κ B), MAP kinase and E3 ubiquitin ligase pathways (4–7). Typically, TRAF molecules contain a single N-terminal RING finger, several zinc fingers and a C-terminal TRAF domain.

TRAF1 is unique among TRAFs, since it lacks the RING finger and only contains a single zinc finger domain and a TRAF domain. TRAF1 was first identified as a molecule interacting with TNFR2, indirectly via heterodimerization with TRAF2 (3). TRAF1 can also bind to other members of the TNFR2 family, including CD27 (8), CD30 (9), 4-1BB (10), OX40 (11) and HVEM (12). Although, the precise biochemical function of TRAF1 in

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signaling by TNFR family members remains controversial, several *in vitro* studies suggest that TRAF1 acts as an inhibitor of NF- κ B activation (13–16). TRAF1 may modulate the function of TRAF2 following CD40-mediated NF- κ B activation by displacing TRAF2 from lipid rafts into cytosol and preventing TRAF2 degradation and JNK and NF- κ B activation (17). Paradoxically, high levels of TRAF1 appear to inhibit TRAF2-mediated signaling by trapping TRAF2 in soluble complexes outside of lipid rafts and/or by competition with TRAF2 for receptor-binding sites (17).

In addition to TRAF2 and members of the TNFR2 family, TRAF1 has been found to interact with a number of other molecules, including EBV latent membrane protein 1 (LMP1) (18), bcl10 (19), NFAT-interacting protein (NIP45) (20), IKK (16) and TRADD (21). It has been shown that TRAF1 positively regulates LMP1-induced TRAF2-dependent activation of the JNK/AP-1 axis in epithelial cell lines and primary nasopharyngeal carcinomas (22). Bcl10 is a critical positive regulator of antigen receptor- and phorbol 12-myristate-13-acetate (PMA)-mediated NF- κ B activation in B and T lymphocytes (23). TRAF1 has been reported to inhibit bcl10-mediated NF- κ B activation (24). TRAF1 binding to NIP45, a nuclear protein that augments NFAT-driven IL-4 transcription (25) and participates in IL-4 and IFN- γ expression *in vivo*, has been shown to completely block transactivation of the IL-4 promoter (20).

Since NF- κ B and NIP45 are both involved in the regulation of T_H2 cell development, we hypothesized that TRAF1 might modulate the polarity of antigen-driven T_H responses. We tested this hypothesis *in vivo* using TRAF1-deficient ($^{-/-}$) mice previously generated by gene targeting (26). We have shown that these mice have intact T and B lymphocyte development as well as normal antibody responses to T-dependent and both type-1 and type-2 T-independent antigens. TRAF1 $^{-/-}$ T cells exhibit enhanced proliferation following anti-CD3 stimulation and respond to TNF by proliferation and activation of the NF- κ B and AP-1 signaling pathways. We have now undertaken analysis of the effects of TRAF1 on T_H differentiation and have observed that TRAF1 $^{-/-}$ T cells produce higher amounts of the T_H2 cytokines IL-4, IL-5 and IL-13. Adoptive transfer of antigen-stimulated CD4 $^{+}$ TRAF1 $^{-/-}$ T cells to naive recipients followed by inhaled antigen challenge leads to substantially greater increases in airway hyperresponsiveness (AHR), airway inflammation and mucus production than does transfer of wild-type (WT) T cells in an ovalbumin (OVA)-driven murine asthma model. We provide evidence that TRAF1 may negatively regulate T_H2 differentiation by sequestering NIP45 in the cytosol and inhibiting its translocation to the nucleus, thereby down-regulating the expression of NIP45-dependent T_H2 cytokines.

Methods

Mice and cell culture

Generation and initial characterization of TRAF1 $^{-/-}$ mice were described in (26). BALB/c WT mice were purchased from Charles River Laboratories. TRAF1 $^{-/-}$ mice were backcrossed onto a BALB/c background for eight generations. DO11.10 mice (27) were purchased from Jackson Laboratories and bred with BALB/c TRAF1 $^{-/-}$ mice. TRAF1 $^{-/-}$ DO11.10 and WT DO11.10 mice used for the experiments were TCR

transgene hemizygous. Mice were maintained in a specific pathogen-free environment, according to animal protocols approved by Children's Hospital ACUC.

Spleen single-cell suspensions were isolated by density gradient using lympholyte-M (Accurate) and incubated at 10^6 ml $^{-1}$ with graded concentrations of OVA_{323–339} peptide (ISQAVHAAHAETNEAGR) in complete DMEM medium (GIBCO-BRL) supplemented by 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U ml $^{-1}$ penicillin and 100 μ g ml $^{-1}$ streptomycin. Proliferation was assessed by the incorporation of [^{3} H]thymidine, 1 μ Ci per well added during the last 6 h of 72 h cultures.

For 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, splenocytes were washed twice with PBS and incubated at 10^7 ml $^{-1}$ in a pre-warmed 1 μ M solution of CFDA in PBS for 15 min at 37°C. The reaction was stopped by washing with an equal volume of 1% FCS/PBS and incubating in complete medium for 15 min at 37°C.

For cytokine production analysis, supernatants were collected after 5 days of culture. Cytokines were measured by ELISA (BD-Pharmingen). Student's test was used to assess statistical significance of ELISA results. A *P*-value <0.05 was considered statistically significant. Unless otherwise specified, all antibodies and their conjugates were purchased from BD-Pharmingen. For analysis of intracellular cytokine production, T cells were stained with appropriate antibodies using the intracellular cytokine kit (BD-Pharmingen) and analyzed on a FACSCalibur cytometer.

Total RNA was prepared using Trizol (Invitrogen) and ribonuclease protection assays (RPAs) were done using RiboQuant multiprobe kits from BD-Pharmingen.

Naive T cell differentiation

Naive T cells were purified by negative magnetic bead separation using MACS columns (Miltenyi Biotec). T cells were stained with FITC-conjugated anti-CD4 antibody and PE-conjugated CD62L antibody and sorted on a FACS Vantage SE (Becton Dickinson). The CD4 $^{+}$ CD62L ^{hi} populations were collected as naive T cells. For *in vitro* differentiation assays, naive T cells (10^6 ml $^{-1}$) were stimulated with plate-bound anti-CD3 mAb 145-2C11 (50 ng ml $^{-1}$) and anti-CD28 mAb 37.51 (2 μ g ml $^{-1}$) for intermediate (T_Hi) conditions, with 2 ng ml $^{-1}$ recombinant IL-12 (Peprotec, Inc.) and 10 μ g ml $^{-1}$ anti-IL-4 mAb for T_H1 -skewing conditions or with 10 ng ml $^{-1}$ recombinant IL-4 (Peprotec, Inc.), 10 μ g ml $^{-1}$ anti-IFN- γ mAb and 10 μ g ml $^{-1}$ anti-IL-12 mAb for T_H2 -skewing conditions. On day 3, 10 U ml $^{-1}$ recombinant IL-2 (Peprotec, Inc.) was added to all cultures. Seven days after stimulation, cells were washed, counted and re-stimulated (at 10^6 ml $^{-1}$) with plate-bound anti-CD3 mAb (1 μ g ml $^{-1}$). Culture supernatants were analyzed for cytokine production by ELISA 24 h after secondary stimulation.

Western blotting

Whole-cell lysates were prepared from T cells stimulated with PMA (0.2, 1.0, 5.0 ng ml $^{-1}$) and 1 μ M ionomycin (Io) for 15 min. Cells (2.0×10^6 per point) were washed in ice-cold PBS and lysed in lysis buffer (20 mM HEPES, pH 7.9, 1% NP-40, 5 mM EDTA, 150 mM NaCl, 1 mM Na₃VO₄, 50 mM NaF) with EDTA-free complete protease inhibitor (CPI) cocktail (Roche).

Lysates were separated on 14% SDS-PAGE and transferred to nitrocellulose membrane (Pall). Blots were blocked with 3% BSA (Sigma) and incubated with antibodies to TRAF1 (Stressgen Biotechnologies), TRAF2 C-20 (Santa Cruz), bcl10 H-197 (Santa Cruz) and I κ B α (Cell Signaling). Blots were developed with HRP-conjugated secondary antibodies and ECL SuperSignal West Femto kit (Pierce).

For preparation of nuclear and cytoplasmic extracts, purified spleen T cells (3×10^7) were stimulated with plate-bound anti-CD3 ($1 \mu\text{g ml}^{-1}$) and plate-bound anti-CD28 ($2 \mu\text{g ml}^{-1}$) for 24 h. Cells were washed with ice-cold PBS, re-suspended in 500 μl of ice-cold lysis buffer (10 mM HEPES buffer, pH 7.2, 2 mM MgCl_2 , 2 mM KCl, 1 mM dithiothreitol) with EDTA-free CPI cocktail (Roche). Following a 15-min-long incubation on ice, cells were lysed by passing five times through a 27-gauge needle. Part of the lysate was used for the preparation of the cytoplasmic fraction by removing unbroken cells and nuclei for 2 min at 5000 r.p.m. at 4°C. The rest was used for the preparation of a nuclear fraction by centrifugation through a 2.1 M sucrose solution for 30 min at 31 000 r.p.m. ($90\,000 \times g$) at 4°C. The nuclear pellet was re-suspended in 100 μl of lysis buffer. Western blotting was done with antibodies to NIP45 (Santa Cruz), Lamin A/C (Santa Cruz), LAT (BD-Pharmingen) and TRAF1.

Fusion protein constructs and transfection

A piece of the mouse cDNA corresponding to Δ TRAF1 (aa 152–409) was cloned into pEGFP-C1 plasmid (BD-Biosciences) to generate a construct, expressing EGFP- Δ TRAF1. To generate a construct, expressing dsRed-NIP45 fusion protein, the mouse cDNA fragment encoding the full-size NIP45 protein was cloned into pdsRed-C1 (BD-Biosciences). Plasmids were transfected into COS-7 cells using FuGENE 6 transfection reagent (Roche). Fluorescence microscopy was done according to the recommendations from BD-Biosciences, using a Nikon Eclipse E800 microscope.

The 801-bp fragment (spanning 5' non-coding sequences from –801 to +58 bp) of the mouse IL-4 promoter (20) was sub-cloned into pGL-3 Basic vector (Promega). To generate a construct, expressing c-myc-NIP45 fusion protein, the mouse NIP45 cDNA was cloned into pCMV-tag3C expression plasmid (Stratagene). The mouse T hybridoma 68-41 cells were transfected using DEAE-transfection method. 68-41 cells were transfected with increasing amounts of pCMV-c-myc-NIP45. The decreasing amounts of empty pCMV-tag3C were added to achieve equal concentrations of total plasmid DNA in each transfection. Each transfection contained pGL-3-mIL-4-promoter and pRL-TK (*Renilla* luciferase) as a transfection control. Six hours after transfection, 68-41 cells were left unstimulated or stimulated with PMA + Io. Luciferase assays were performed 24 h after stimulation according to the manufacturer's instructions (Dual Luciferase Reporter Assay System; Promega) and using a Dynex luminometer. In each transfection with a particular set of plasmids, fold induction was calculated as a ratio of the specific activities for stimulated cells to unstimulated cells.

T cell transfer and allergic airway inflammation

OVA-specific CD4 $^+$ T cells were derived as described in (28). Briefly, WT and TRAF1 $^{-/-}$ mice were immunized intraperito-

neally with 50 μg of OVA/1 mg Alhydrogel 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 $\mu\text{g ml}^{-1}$ of OVA. CD4 $^+$ T cells were purified using MACS columns (Miltenyi Biotec) and transferred intravenously at 2×10^6 cells per 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^{-1}) in 0.9% saline over 30 min. Airflow and 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) as previously described (29). Penh results were analyzed using two-way analysis of variance. A P -value <0.05 was considered statistically significant. Lung inflammation was determined by enumeration of cells in bronchoalveolar lavage (BAL) fluid. Immediately after sacrifice, cells were recovered from the lungs by flushing the trachea with 0.8 ml of BAL fluid (1 mM EDTA, 10% FCS in PBS). Total cell counts were determined for each BAL sample and 100 μl of fluid was cytospun at $400 \times g$ for 4 min onto glass slides using Cytospin 3 centrifuge (Shandon Lipshaw). Differential cell counts were performed after staining with Diff-Quik Stain Set (Baxter). For histological analysis, lungs were inflated with 1.0 ml of 10% formalin instilled through a tracheostomy tube and embedded in paraffin. Multiple 4- μm sections were stained with diastase-periodic acid Schiff and hematoxylin and eosin.

Results

TRAF1 $^{-/-}$ splenocytes have enhanced antigen-dependent proliferation and T_H2 cytokine production

DO11.10 mice, which express a transgenic TCR specific for the OVA_{323–339} peptide presented in the context of I-A ^{d} (27), were used to facilitate the analysis of TRAF1 $^{-/-}$ T cells. Splenocytes from TRAF1 $^{-/-}$ DO11.10 mice exposed to graded concentrations of OVA_{323–339} peptide displayed significantly stronger proliferative responses than splenocytes from WT DO11.10 mice (Fig. 1A). The enhanced proliferation was due to cell division of OVA-specific T cells, identified by the expression of the KJ1-26 $^+$ idiotype (Fig. 1B). During 5-day cultures, CFSE-labeled OVA-specific CD4 $^+$ TRAF1 $^{-/-}$ T cells underwent a higher number of cell divisions than did OVA-stimulated WT T cells (Fig. 1B). The increased proliferation of TRAF1 $^{-/-}$ T cells was not due to impaired activation-induced cell death. The percentage of annexin V-positive CD4 $^+$ T cells was the same in TRAF1 $^{-/-}$ and WT splenocytes after 24 h incubation with antigen (data not shown). These results suggest that CD4 $^+$ TRAF1 $^{-/-}$ T cells are hyperproliferative in response to antigen-bearing antigen-presenting cells (APCs).

Cytokine production by antigen-stimulated spleen TRAF1 $^{-/-}$ T cells was assessed following culture with OVA peptide. Since TRAF1 $^{-/-}$ T cells were hyperproliferative (see Fig. 1A), cells were counted at the conclusion of cultures and cytokine levels were normalized to the cell numbers. OVA-stimulated TRAF1 $^{-/-}$ and WT DO11.10 T cells both produced IFN- γ and the levels were similar (Fig. 1C). However, production of T_H2 cytokines was enhanced in TRAF1 $^{-/-}$ T cells. IL-4 levels were

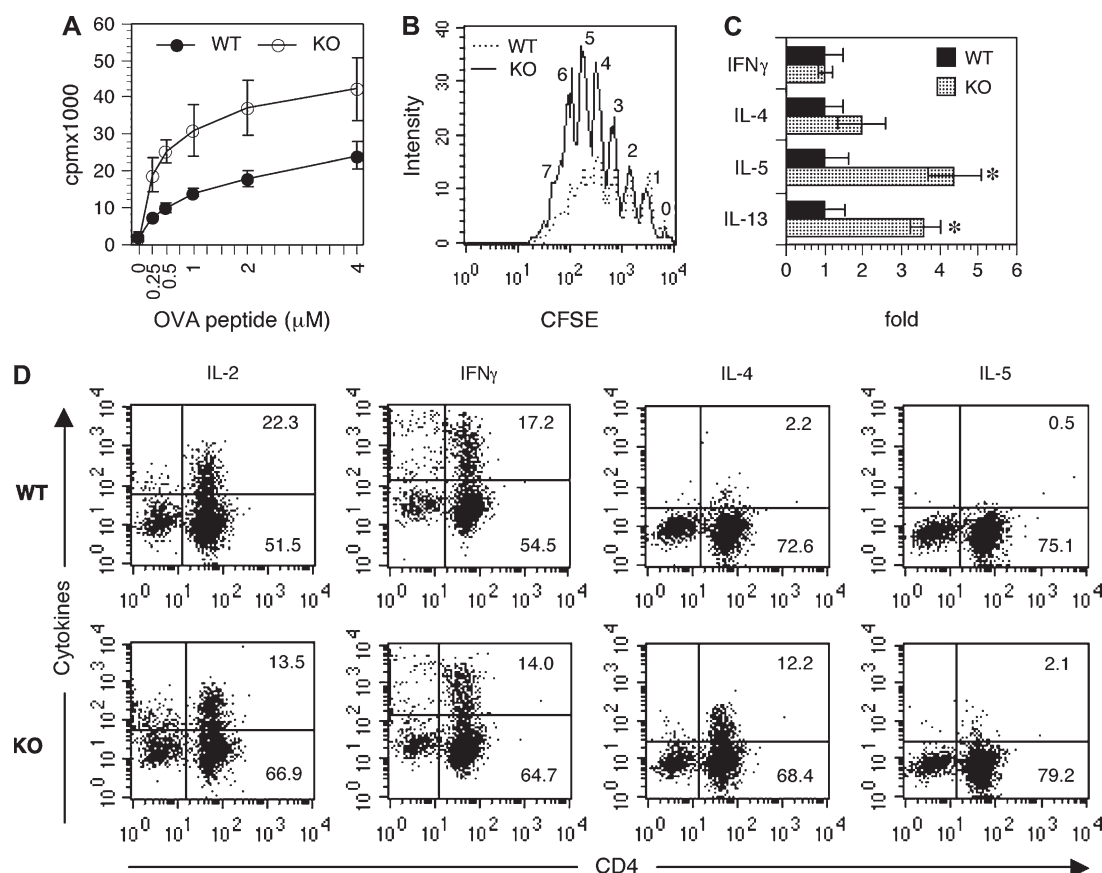


Fig. 1. TRAF1^{-/-} splenocytes have enhanced antigen-dependent proliferation and T_H2 cytokine production. (A) Proliferation of splenocytes from WT (closed circles) and TRAF1^{-/-} (open circles) DO11.10 OVA TCR transgenic mice in response to increasing concentrations of OVA₃₂₃₋₃₃₉ peptide. Each point represents the mean $\pm$ SE within an experiment. These data are representative of three independent experiments. Data represent mean Penh values $\pm$ SEM ($n=3$). * $P < 0.05$. (B) Cell division in the OVA-specific CD4⁺ T cell subset. CFSE-labeled splenocytes from WT (dotted line) and TRAF1^{-/-} (solid line) DO11.10 mice were incubated with OVA₃₂₃₋₃₃₉ peptide (2 μ M) for 5 days. Cell division (CFSE fluorescence) in CD4⁺KJ1-26⁺ T cells was assessed by flow cytometry. The number of divisions is indicated above each peak. The data shown are representative of two independent experiments. (C) Cytokine production by splenocytes from WT (black bar) and TRAF1^{-/-} (dotted bar) DO11.10 mice. Splenocytes were stimulated as in (B). The concentrations of cytokines in supernatants were analyzed by ELISA. Data were normalized to the number of live cells at the conclusion of each culture. The resulting values for TRAF1^{-/-} T cells were expressed as fold induction compared with the values obtained for WT T cells and are shown as the mean between three independent experiments (two mice in each experiment) with error bars representing the SEM. Significant differences ($P < 0.05$) between TRAF1^{-/-} T and WT cells are indicated by asterisks (*). (D) Intracellular cytokine analysis was performed by flow cytometry. Splenocytes were treated as in (B), and re-stimulated on day 5 for 5 h with plate-bound anti-CD3 antibodies (1 μ g ml⁻¹). Data show the results of one representative experiment out of three.

slightly higher and IL-5 and IL-13 significantly higher in supernatants of TRAF1^{-/-} cells (Fig. 1C). This T_H2 bias in cytokine expression by antigen-stimulated TRAF1^{-/-} T cells was confirmed at the single-cell level using intracellular staining of cytokine in WT and TRAF1^{-/-} T cells after re-stimulation with anti-CD3 (Fig. 1D). The percentage of IL-4- and IL-5-positive CD4⁺ TRAF1^{-/-} T cells was higher than that of IL-4- and IL-5-positive CD4⁺ WT T cells, while the percentages of IL-2- and IFN- γ -producing CD4⁺ T cells were actually higher in T cells from WT mice. Taken together, these data suggest that lack of TRAF1 leads to enhanced T_H2 differentiation.

In order to determine the kinetics of T_H2 skewing in TRAF1-deficient T cells, a time course analysis of cytokine transcription was performed using a sensitive and quantitative RPA. On the first day after stimulation, WT and TRAF1^{-/-} splenocytes produced comparable amounts of mRNAs encoding both T_H1

and T_H2 cytokines (Fig. 2A, lanes 2 and 7, respectively). By the second day, IFN- γ transcription by TRAF1^{-/-} splenocytes (lane 3) was actually increased relative to WT splenocytes (lane 8). However, after 5 days, transcription of T_H2 cytokine genes (IL-4, IL-5 and IL-13) in TRAF1^{-/-} splenocytes had markedly increased (lane 10), while expression of T_H1 cytokine transcripts (IL-2 and IFN- γ) was decreased (lane 5). Thus, despite an initial burst of T_H1 cytokine transcription following antigen stimulation, TRAF1^{-/-} T cells eventually differentiated toward a clearly polarized expression of T_H2 cytokines. When T cells subjected to antigen stimulation as above were re-stimulated with anti-CD3, the T_H2 bias of TRAF1^{-/-} cells remained evident (Fig. 2B). Anti-CD3-re-stimulated T cells expressed higher levels of IL-4, IL-5 and IL-13. In fact, WT T cells had no detectable expression of IL-5 mRNA, while TRAF1^{-/-} T cells expressed a very significant amount.

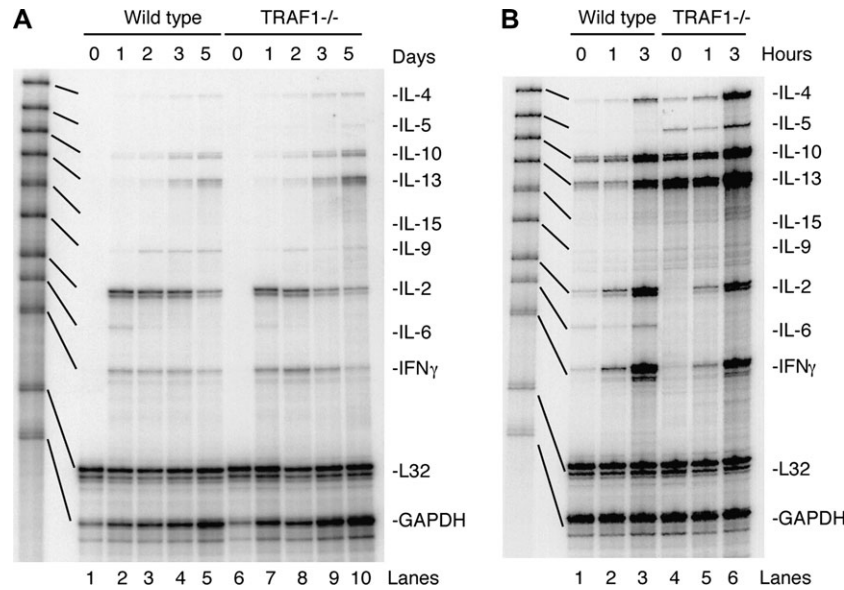


Fig. 2. Time course of induction of T_H2 cytokine transcription and maintenance of polarity upon re-stimulation in TRAF1^{-/-} splenocytes. (A) Splenocytes from WT and TRAF1^{-/-} DO11.10 mice were incubated with OVA₃₂₃₋₃₃₉ peptide (2 μM) for 5 days. (B) Splenocytes were stimulated as in (A) and re-stimulated on day 5 with plate-bound anti-CD3 antibodies (1 μg ml⁻¹). Total RNA was prepared from pooled splenocyte cultures of four mice per lane at indicated time points and subjected to RNase protection assay. L32 and GAPDH are shown as internal controls for RNA processing and loading.

Interestingly, there was no detectable IL-6 mRNA in TRAF1^{-/-} T cells, although it was present on day 1 in TRAF1^{-/-} T cells after stimulation with OVA peptide.

The T_H2 bias of TRAF1^{-/-} T cells exists at the naive stage

The results above indicated that TRAF1^{-/-} DO11.10 T cells are hyperproliferative and develop a strong T_H2 phenotype following stimulation with antigen in the presence of APCs (Fig. 1). In order to assess whether this tendency of antigen-stimulated TRAF1^{-/-} T cells toward T_H2 polarity is T cell autonomous (as opposed to APC driven), the cytokine responses of anti-CD3/CD28-stimulated purified naive T cells were assessed. Highly purified CD4⁺CD62^{hi} WT and TRAF1^{-/-} T cells were cultured with anti-CD3 and anti-CD28 under intermediate (designated T_{H1}), T_H1 or T_H2 differentiating conditions. After 7 days, cells were re-stimulated with anti-CD3 and cytokines were determined in culture supernatants 24 h later. Although there was no difference in cytokine production by WT or TRAF1^{-/-} T cells cultured under either T_H1 or T_H2 conditions (Fig. 3A and B), TRAF1^{-/-} T cells cultured under non-skewing conditions produced considerably higher amounts of IL-4, IL-13 and IL-5 but not IFN- γ when compared with WT T cells (Fig. 3C). These results suggest a T cell intrinsic T_H2 bias in TRAF1^{-/-} T cells.

TRAF1^{-/-} T cells are more efficient in the induction of asthma

To investigate the physiological consequences of the T_H2 bias in TRAF1^{-/-} T cells *in vivo*, their function was investigated using a T_H2 -dependent adoptive transfer model of asthma (28). Effector T cells were prepared by propagating splenocytes from OVA-immune mice in the presence of OVA. Purified

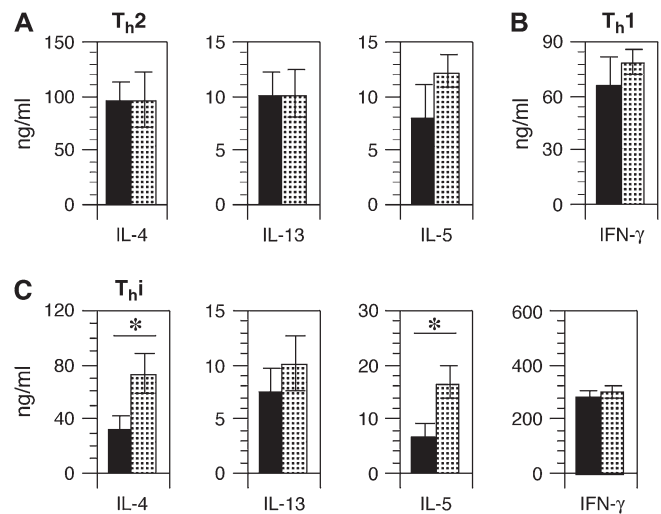


Fig. 3. Naive TRAF1^{-/-} T cells have enhanced production of T_H2 cytokines. Highly purified CD4⁺CD62^{hi} T cells from WT (black bars) or TRAF1^{-/-} (dotted bars) spleens were differentiated *in vitro* under (A) T_H2 -, (B) T_H1 - or (C) non-skewing T_{H1} conditions over 7 days as described in Methods. Live cells were re-stimulated with plate-bound anti-CD3 (1 μg ml⁻¹) for 24 h. Levels of IL-4, IL-5, IL-13 and IFN- γ in supernatants were determined by ELISA. Data represent the mean $\pm$ SEM from three mice and are representative of two independent experiments. *P < 0.05.

CD4⁺ from these cultures were intravenously transferred into WT mice, which were then subjected to OVA aerosol inhalation over 7 days. The recipients were analyzed for the development of AHR, lung inflammation and mucus production by bronchial epithelial cells. As expected, OVA-exposed recipients of WT OVA-specific T cells displayed AHR to methacholine (Fig. 4A).

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However, recipients of TRAF1^{-/-} T cells displayed even greater airway responsiveness. Recipients of either WT or TRAF1^{-/-} T cells displayed intense pulmonary inflammation following OVA exposure but mice that received TRAF1^{-/-} T cells had significantly higher numbers of lymphocytes in their lungs compared with animals infused with WT T cells (Fig. 4B). The secretion of mucus in the airways is T_H2 associated and regulated by IL-13 (30). Mice given WT T cells exhibited increased mucus production by the bronchial epithelium, when compared with mice injected with PBS. Remarkably, mice injected with TRAF1^{-/-} T cells displayed an even higher production of mucus (Fig. 4C). Taken together, these results indicate that allergen-specific TRAF1^{-/-} T cells are more effective than WT T cells in the induction of lung inflammation and AHR *in vivo*.

TRAF1 does not affect TCR-mediated NF- κ B signaling but limits nuclear translocation of NIP45

The T_H2 bias of antigen- or anti-CD3-stimulated TRAF1^{-/-} T cells strongly suggests that TRAF1 interacts with TCR-activated signaling pathways that regulate cytokine transcription. Clues regarding potential sites of TRAF1 engagement with these pathways have been provided by observations that TRAF1 binds to several key regulatory proteins. These include both bcl10, an intracellular protein which is necessary for NF- κ B activation following antigen receptor stimulation in B and T lymphocytes (19, 23), and NIP45, a nuclear protein which augments NFATp- and c-Maf-induced activation of the IL-4 promoter in activated T cells (25). NF- κ B is activated following TCR ligation and enhanced NF- κ B signaling has been linked to a T_H2 bias (31). In order to test the hypothesis that TRAF1 interactions with these proteins modulate their signaling and hence influences the polarity of T cells, their function was evaluated in WT and TRAF1^{-/-} cells.

Bcl10 is a critical intermediate in TCR-mediated NF- κ B activation. Antigen-induced bcl10-dependent activation can be inhibited by a dominant-negative version of TRAF1 (24). It led us to consider the possibility that both T_H2 polarity and enhanced proliferation of TRAF1^{-/-} DO11.10 splenocytes (Fig. 1) were due to stronger NF- κ B activation through bcl10 in the absence of TRAF1-mediated inhibition. In order to evaluate this possibility, bcl10-dependent signaling events were analyzed in splenic TRAF1^{-/-} T cells stimulated with a combination of Io and PMA, which bypasses TCR but results in bcl10-dependent activation of NF- κ B. Bcl10 was normally phosphorylated and I κ B α normally degraded in PMA/Io-stimulated TRAF1^{-/-} T cells (Fig. 5). There was also normal nuclear translocation of NF- κ B as detected by electrophoretic mobility shift assay (data not shown). To confirm that TRAF2 expression was unaffected by the absence of TRAF1 in TRAF1^{-/-} T cells, we analyzed TRAF2 and TRAF1 protein expression in the same samples. WT and TRAF1^{-/-} T cells had similar levels of TRAF2 protein and these levels were unaffected by short-term activation. As expected, TRAF1^{-/-} T cells had no expression of TRAF1 (Fig. 5). Taken together, these results demonstrate that TRAF1^{-/-} T cells have normal bcl10-dependent signaling events, suggesting that TRAF1 does not interfere with TCR-mediated NF- κ B activation.

TRAF1 has also been reported to interact with the nuclear protein NIP45, which can synergize with NFATp and c-Maf to

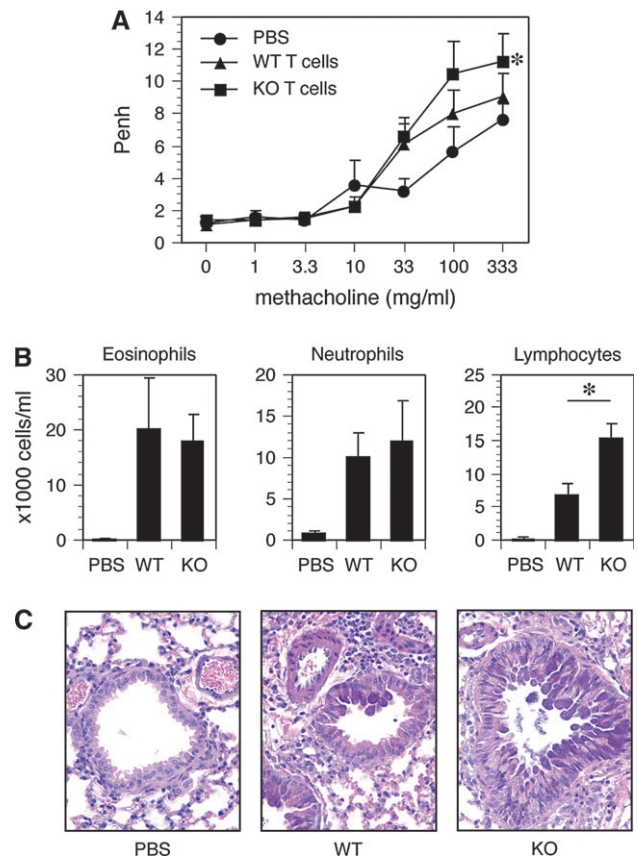


Fig. 4. Adoptively transferred OVA-specific TRAF1^{-/-} T cells confer increased allergic lung inflammation to challenged recipients. WT mice were given intravenous injections of either OVA-specific WT or TRAF1^{-/-} CD4⁺ T cells, which were prepared as described in Methods. Recipient mice were exposed to aerosolized OVA for 30 min daily over 7 days and analyzed 24 h after the last exposure. Control injections were performed using PBS. (A) AHR was measured by whole-body plethysmography on conscious unrestrained mice exposed to increasing doses of nebulized methacholine. Airflow obstruction is expressed as Penh. Data represent mean Penh values $\pm$ SEM ($n = 4$). * $P < 0.05$. (B) The number of eosinophils, neutrophils and lymphocytes in BAL fluid (mean $\pm$ SEM, $n = 4$, * $P < 0.05$ WT versus TRAF1^{-/-}). (C) Representative sections of lungs from recipients of OVA-specific WT or TRAF1^{-/-} T cells or control (PBS) following OVA inhalation. Four-micrometer sections of formalin-fixed paraffin-embedded tissue were stained with periodic acid Schiff reagent for goblet cell hyperplasia.

activate the IL-4 promoter (20). Over-expression of TRAF1 has been shown to repress IL-4 promoter transactivation and IL-4 production (20). These observations led us to hypothesize that enhanced T_H2 cytokine transcription in TRAF1^{-/-} T cells might be related to dysregulation of NIP45 expression. To explore potential NIP45 involvement in T_H2 -skewed differentiation of TRAF1^{-/-} T cells, we analyzed the expression of NIP45 protein in WT and TRAF1^{-/-} T cells. For this purpose, we used western blotting to analyze NIP45 expression in purified anti-CD3/CD28-activated splenic T cells. As shown in Fig. 6(A), there was a significantly higher amount of NIP45 protein in the nucleus of TRAF1^{-/-} T cells when compared with WT T cells. The higher amount of NIP45 in the nuclei of TRAF1^{-/-} T cells compared with the nuclei of WT T cells suggests that the

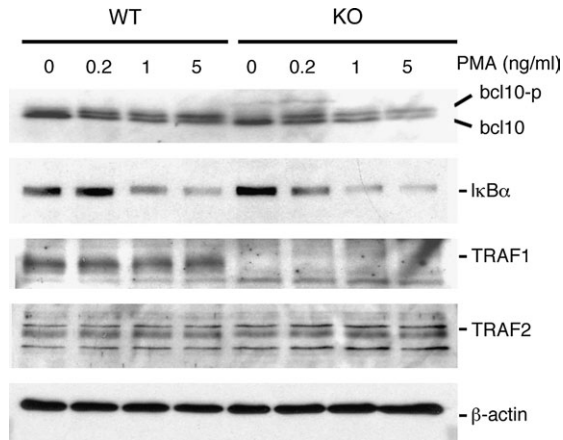


Fig. 5. Normal NF- κ B signaling in TRAF1 $^{-/-}$ T cells. Purified WT or TRAF1 $^{-/-}$ spleen T cells were stimulated with Io (1 μ M) and PMA (at concentrations shown) for 15 min. Whole-cell lysates were prepared and subjected to western blot analysis using antibodies to indicated proteins. β -actin served as a loading control.

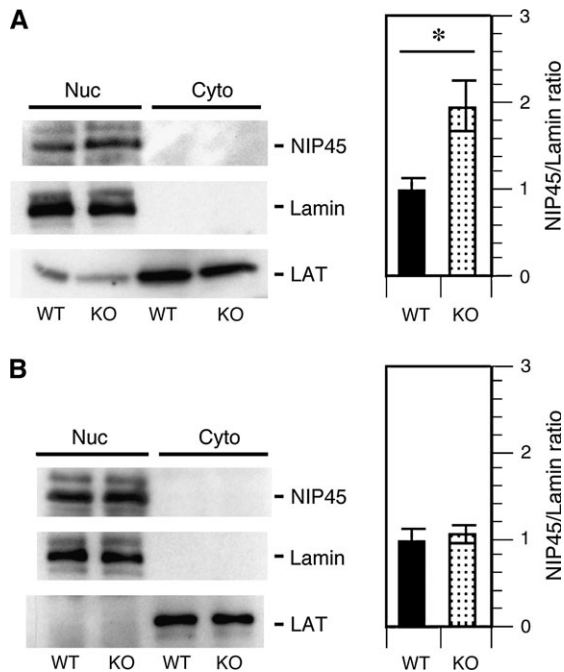


Fig. 6. Increased nuclear levels of NIP45 in TRAF1 $^{-/-}$ T cells. (A) Purified spleen or (B) T_H2 -differentiated WT and TRAF1 $^{-/-}$ T cells were stimulated with plate-bound anti-CD3 (1 μ g ml $^{-1}$) for 18 h. Nuclear and cytoplasmic extracts were prepared and subjected to western blot analysis for NIP45 expression. Lamin A/C and LAT served as internal controls for nuclear and cytoplasmic proteins, respectively. The intensity of NIP45 and Lamin A/C bands in the nuclei was quantified using NIH image software. For quantification of each NIP45 or Lamin A/C band was individually corrected for background intensities by an area of corresponding size in close neighborhood of the respective protein signal. To obtain relative expression values, the ratio between NIP45 and Lamin A/C bands was calculated in WT and TRAF1 $^{-/-}$ samples. Finally, relative NIP45 expression values in TRAF1 $^{-/-}$ T cells were normalized according to the respective values of NIP45 expression in WT T cells. Data show the mean $\pm$ SEM from two independent experiments.

expression of TRAF1 in the cytoplasm of WT T cells down-regulates the amount of NIP45 protein in the nucleus. Furthermore, a diminished NIP45 expression in the nucleus might contribute to enhanced IL-4 transcription and subsequent T_H2 -skewed differentiation of TRAF1 $^{-/-}$ T cells.

It is interesting that TRAF1 $^{-/-}$ and WT T cells differentiated in T_H2 conditions expressed similar amounts of nuclear NIP45 (Fig. 6B). These findings correlate well with the results of the experiments, which analyzed cytokine production by T_H2 -differentiated TRAF1 $^{-/-}$ T cells (Fig. 3A), where no significant difference was detected in the production of IL-4, IL-5 or IL-13 between T_H2 -skewed TRAF1 $^{-/-}$ and WT T cells.

To explore potential mechanisms whereby the cytoplasmic protein TRAF1 might modulate abundance of the nuclear protein NIP45, we expressed EGFP- Δ TRAF1 and dsRed-NIP45 fusion proteins in COS-7 cells. Control transfections with free fluorescent tags showed that both had diffuse cellular distribution with a mild tendency toward nuclear localization for EGFP and cytoplasmic localization for dsRed (Fig. 7A). Most of the EGFP- Δ TRAF1 fusion protein was aggregated in the cytoplasm and displayed no association with free dsRed while dsRed-NIP45 fusion protein restricted to the nucleus and did not show any association with a free EGFP (Fig. 7B and C). Strikingly, when EGFP- Δ TRAF1 and dsRed-NIP45 fusion proteins were co-expressed, a significant amount of dsRed-NIP45 was co-localized with EGFP- Δ TRAF1 in the cytoplasm, while the rest of the dsRed-NIP45 resided in the nucleus (Fig. 7D). These results demonstrate that in COS-7 cells a fraction of NIP45 is associated with cytosolic TRAF1. This result suggests that TRAF1 might limit the nuclear translocation of NIP45 (and consequently activation of T_H2 cytokine transcription) by sequestering a fraction of NIP45 in the cytoplasm.

To directly assess whether increased concentrations of NIP45 contribute to augmented IL-4 promoter transactivation, the 68-41 T cell line was transiently transfected with an IL-4 promoter reporter construct in conjunction with different amounts of c-myc-NIP45-expressing plasmid. Subsequently, cells were stimulated with PMA/Io. Figure 8 demonstrates that transfection of 68-41 cells with increasing amounts of exogenous NIP45 considerably enhances PMA/Io-induced IL-4 promoter-driven transcription in a dose-dependent manner. These transfection experiments suggest that IL-4 gene transcription may be affected by the quantity of NIP45 protein available in the cell.

Discussion

Our understanding of the role of the TRAF family of proteins in intracellular signaling and immune responses is rapidly expanding. In addition to interacting with TNFR family members and modulating their signaling, TRAF members are now known to associate with a variety of other cell-surface receptors and cytosolic proteins and to regulate a number of intracellular signaling processes. Some of these interactions, including TRAF1 association with bcl10 (upstream of TCR-mediated NF- κ B activation) and NIP45 (which potentiates IL-4 transcription in activated T cells), have suggested potential roles for TRAF1 in regulating T_H polarity. We had previously demonstrated that TRAF1 $^{-/-}$ T cells are hyperproliferative in response to anti-CD3 stimulation (26).

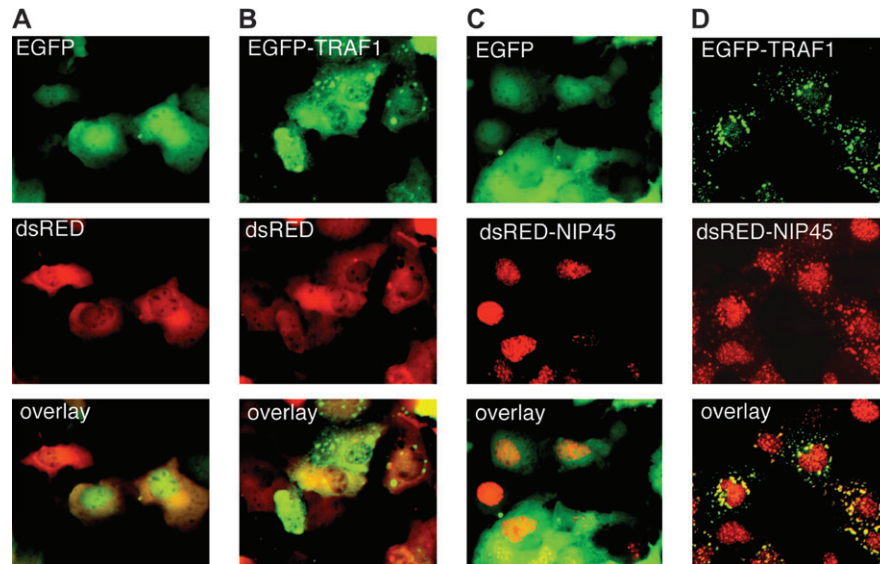


Fig. 7. The nuclear protein NIP45 partially co-localizes with TRAF1 in the cytoplasm of COS-7 cells. Fluorescent NIP45 and TRAF1 fusion proteins were co-expressed by transfecting COS-7 cells with the indicated constructs. (A) EGFP and dsRed. (B) EGFP- Δ TRAF1 and dsRed. (C) EGFP and dsRed-NIP45. (D) EGFP- Δ TRAF1 and dsRed-NIP45. Images were captured using a green filter for EGFP and EGFP- Δ TRAF1 fluorescent proteins (upper images) and a red filter to detect dsRed and dsRed-NIP45 fluorescent proteins (middle images). Green and red images were overlaid using Adobe Photoshop 5.0 software (lower images).

Considering the association of TRAF1 with bcl10 and NIP45, we used T cells from the same TRAF1^{-/-} mice to evaluate the effects of TRAF1 on T_H polarity in both cultured T cells and *in vivo*. Our results demonstrate that antigen/APC-driven TRAF1^{-/-} T cells exhibit enhanced proliferation and increased production of T_H2 cytokines (IL-4, IL-5 and IL-13) (Fig. 1). The T_H2 bias of TCR-stimulated TRAF1^{-/-} T cells develops over several days and is maintained during re-stimulation (Fig. 2). Our observation of increased IL-4, IL-5 and IL-13 production in naive T_H cells stimulated via TCR indicates that the T_H2 bias is T cell autonomous and not driven by TRAF1-regulated alterations in APC co-stimulatory functions (Fig. 3). The regulatory influence of TRAF1 on T_H2 cytokine production by T cells has direct consequences in the setting of an allergic disease model. Using an OVA-driven mouse model of asthma, we have shown that TRAF1 deficiency in T cells is associated with markedly enhanced allergic inflammation and bronchial hyperresponsiveness (Fig. 4).

In order to come back to our original hypothesis that TRAF1 might regulate T cell polarity via interactions with either bcl10-mediated NF- κ B activation or by association with NIP45, we assessed these possibilities directly. TRAF1^{-/-} T cells displayed normal bcl10-mediated signaling, indicating that TCR-driven NF- κ B activation is unaffected by the absence of TRAF1 (Fig. 5). In contrast, nuclear expression of NIP45 protein was enhanced in TRAF1^{-/-} T cells (Fig. 6) and cytosolic sequestration of NIP45 by TRAF1 was observed in co-transfection experiments using fluorescently tagged versions of these proteins (Fig. 7). Taken together, these findings provide strong evidence that TRAF1 deficiency is associated with enhanced T_H2 differentiation as well as augmented allergic inflammatory responses and that this phenotype may result from the absence of a normally inhibitory interaction of TRAF1 with NIP45.

The dominant T_H2 response we have observed in TCR-stimulated TRAF1^{-/-} T cells is similar to the phenotype reported for T cells with defective expression of some other TRAF proteins. For example, TRAF6^{-/-} T cells are hyper-proliferative and secrete predominantly T_H2 cytokines following anti-CD3/CD28 stimulation (32). Irradiation chimeras, in which WT mice are reconstituted with TRAF6^{-/-} fetal liver cells develop a progressive lethal inflammatory disease *in vivo* that is characterized by organ infiltration of T_H2 -polarized CD4⁺TRAF6^{-/-} T cells. TRAF5 deficiency is also associated with T_H2 bias. CD4⁺TRAF5^{-/-} T cells, which mount a normal proliferative response following CD3/CD28 stimulation, have also been reported to secrete elevated levels of T_H2 cytokines (33). This difference in cytokine production between TRAF5^{-/-} and WT T cells is further exaggerated upon anti-OX40 stimulation and is associated with an enhanced T_H2 T cell response and exaggerated allergic lung inflammation in TRAF5^{-/-} mice subjected to inhaled antigen challenge. Tumor necrosis factor receptor-associated factor 2-dominant-negative (TRAF2DN) mice also overproduce T_H2 -specific cytokines (20) and exhibit a decreased proliferative response to TCR stimulation (34). It is possible that this is due to either an inhibitory effect of TRAF2DN and/or dramatically lower expression of endogenous TRAF2 protein (35). Importantly, as we have observed for TRAF1^{-/-} T cells, both TRAF6^{-/-} and TRAF2DN T cells can be readily induced to differentiate toward a T_H1 phenotype (20, 32), suggesting that signaling pathways essential for T_H1 differentiation are intact in T cells with defective expression of these TRAF proteins.

Given the importance of NF- κ B-mediated responses in allergic diseases (36) and the association of TRAF1 with bcl10, which is essential for NF- κ B activation following TCR stimulation, we anticipated that TRAF1^{-/-} cells would have dysfunctional NF- κ B-associated responses, which underlie the

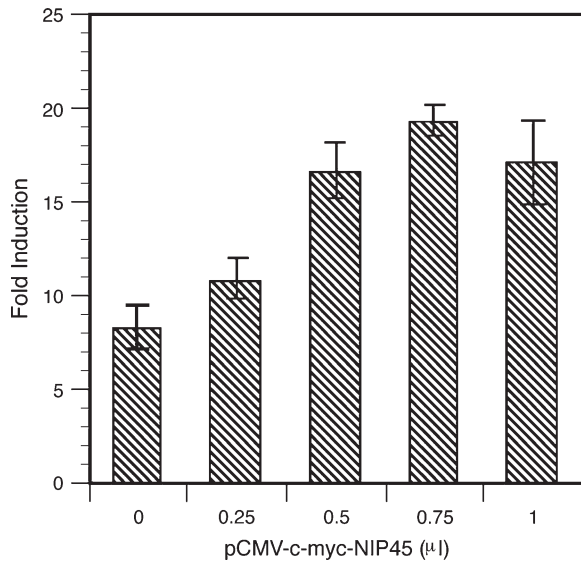


Fig. 8. A vector expressing c-myc-NIP45 increases PMA/Io induction of the mouse IL-4 promoter-luciferase activity. 68-41 T cells were transfected with pGL-3-mIL-4-promoter, pRL-TK and increasing amounts of pCMV-c-myc-NIP45 vector as indicated. The increasing amounts of pCMV-c-myc-NIP45 were balanced by the decreasing amounts of an empty pCMV-c-myc to keep the final amount of transfected DNA even. The cells were stimulated with PMA/Io 6 h after transfection and harvested 24 h later and the dual-luciferase assays were performed. To obtain relative luciferase activity values, the ratio between firefly and *Renilla* luciferases was calculated for each sample. Finally, transcription fold induction values in PMA/Io-activated samples were normalized to the respective values in unstimulated samples. The results shown are representative of two independent experiments. Data show the mean $\pm$ SEM.

T_H2 -skewed differentiation. However, as shown in Fig. 5, this did not turn out to be the case; TCR-mediated activation of bcl10 and the NF- κ B pathway are intact in TRAF1 $^{-/-}$ T cells. These results contrast with previously reported observations that a dominant-negative form of TRAF1 could inhibit bcl10-dependent NF- κ B activation (24). This discrepancy might be explained by anomalous consequences of excessive expression of the dominant-negative TRAF1 in the latter studies.

As TRAF1 had also previously been shown to associate with NIP45 and down-regulate IL-4 promoter activation (20), we hypothesized that the TRAF1-mediated effects on T_H2 cytokines might function through NIP45. Our data reveal that, in COS-7 T cells, TRAF1 binds to a fraction of NIP45 in the cytoplasm (Fig. 7). We propose, given the known interaction between TRAF1 with NIP45 (20), that TRAF1 interaction with NIP45 in the cytosol physically prevents nuclear translocation, thereby negatively regulating the NIP45-mediated activation of IL-4 and other T_H2 -associated cytokines. However, our western blots failed to detect NIP45 in the cytoplasm of WT or TRAF1 $^{-/-}$ T cells (Fig. 6). We suspect that without over-expression of TRAF1, endogenous NIP45 in WT T cells might exist at levels below the limits of detection of the assay. Alternatively, cytoplasmic NIP45 may be sequestered by TRAF1 and degraded by proteolysis since, while NIP45 shares little homology with any known proteins, it does have two consecutive ubiquitin-like domains at its carboxyl-terminus, similar to 2'-5'-oligoadenylate synthetases (OAS), a family of IFN-induced enzymes that

activate RNase L and cause mRNA degradation (37). The precise function of the ubiquitin-like regions in NIP45 is not known, but one of the OAS ubiquitin-like regions has been shown to be important for conformational stability (38). Therefore, the ubiquitin-like domains of NIP45 could participate in the stabilization of NIP45 protein in the cytosol and this possibility will require further examination.

Interestingly, the tendency of TRAF1 $^{-/-}$ T cells to differentiate into T_H2 phenotype and the shift of cytokine balance toward T_H2 in these mice are reminiscent of the T_H2 -skewing phenotype of NFATp $^{-/-}$ mice (39). T_H2 cytokines in both TRAF1 $^{-/-}$ and NFATp $^{-/-}$ mice are affected similarly: IL-5 and IL-13 are severely overproduced while IL-4 is increased to a lesser extent (39). All three genes are located within 120 kb of the T_H2 genomic locus and it has recently been shown that all three promoters are arranged in close proximity to each other forming an initial core chromatin structure providing a base for synchronized or linked transcription (40). Since transcription at the T_H2 locus is NFATp dependent and sequestering NFATp in the cytoplasm by NIP45 would affect all NFATp-dependent genes, the linked ones should be affected proportionally. This is exactly what we observe in the case of TRAF1 $^{-/-}$ mice.

There are several ways in which TRAF molecules might direct T_H2 differentiation of T cells. Analyzing T_H2 -skewed T cell response in TRAF2DN mice, Glimcher and colleagues suggested an intriguing model for TRAF2-regulated T_H2 differentiation of T cells. They proposed that signaling through CD30 (shown to associate with TRAF1 and TRAF5 in addition to TRAF2) might trigger the up-regulation of the IL-4 promoter by disrupting NIP45/TRAF partnering and recruiting TRAF to the cell membrane and thus presumably away from its association with NIP45 (20). The observation that anti-OX40 engagement promoted T_H2 differentiation of TRAF5 $^{-/-}$ T cells suggests a similar scenario for another pair of members of TNFR and TRAF family. It is interesting that TRAF1, TRAF2 and TRAF5 differ in their tissue distribution: while TRAF2 and TRAF5 are ubiquitously expressed, TRAF1 expression is limited to spleen, lung and testis (3). In addition, TRAF1 is up-regulated in T cells following activation (41), revealing a potential mechanism for fine-tuning regulation of T_H2 -skewed differentiation of T cells by a number of TNFR family members, such as TNFR2, CD30, OX40 and CD27, which are expressed in T cells and may associate with TRAF1. Certainly, this possibility warrants future investigation.

In conclusion, our data establish that TRAF1 is a regulator of proliferation and T_H2 differentiation in antigen-specific T cells. The development of a T_H2 bias in TRAF1 $^{-/-}$ naive T cells, stimulated via the TCR, indicates that the regulation of T_H2 polarity by TRAF1 occurs directly in T cells and is not related to APC or other exogenous co-stimulatory influences. We provide evidence that an absence of TRAF1 in T cells leads to increased NIP45 nuclear translocation. As a consequence, higher concentration of NIP45 in the nucleus may lead to an increased expression of T_H2 cytokines.

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Abbreviations

AHR	airway hyperresponsiveness
APC	antigen-presenting cell
BAL	bronchoalveolar lavage
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CPI	complete protease inhibitor
Io	ionomycin
LMP1	latent membrane protein 1
NF- κ B	nuclear factor- κ B
NIP45	NFAT-interacting protein
OAS	oligoadenylate synthetases
OVA	ovalbumin
PMA	phorbol 12-myristate-13-acetate
RPA	ribonuclease protection assay
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
TRAF2DN	tumor necrosis factor receptor-associated factor 2 dominant negative
WT	wild type

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