Immunity
Article

The TNF-Family Receptor DR3 is Essential for Diverse T Cell-Mediated Inflammatory Diseases

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DOI 10.1016/j.immuni.2008.04.021

SUMMARY

DR3 (TRAMP, LARD, WSL-1, TNFRSF25) is a death-domain-containing tumor necrosis factor (TNF)-family receptor primarily expressed on T cells. TL1A, the TNF-family ligand for DR3, can costimulate T cells, but the physiological function of TL1A-DR3 interactions in immune responses is not known. Using DR3-deficient mice, we identified DR3 as the receptor responsible for TL1A-induced T cell costimulation and dendritic cells as the likely source for TL1A during T cell activation. Despite its role in costimulation, DR3 was not required for in vivo T cell priming, for polarization into T helper 1 (Th1), Th2, or Th17 effector cell subtypes, or for effective control of infection with *Toxoplasma gondii.* Instead, DR3 expression was required on T cells for immunopathology, local T cell accumulation, and cytokine production in Experimental Autoimmune Encephalomyelitis (EAE) and allergic lung inflammation, disease models that depend on distinct effector T cell subsets. DR3 could be an attractive therapeutic target for T cell-mediated autoimmune and allergic diseases.

INTRODUCTION

Interactions between tumor necrosis factor (TNF)-family ligands and receptors play an important role in shaping specific features of T cell responses. A subfamily of TNF receptors, including CD30, TNFR2, OX40, CD27, GITR, HVEM, and 4-1BB, is expressed on T cells. These receptors mediate distinct aspects of costimulation in specific T cell subsets (Croft, 2003; Watts, 2005). Death Receptor 3 (DR3), also known as TNFRSF25, TRAMP, LARD, or WSL-1, is a death-domain-containing TNF-family receptor that, like its closest paralog TNFR1, binds the adaptor molecule TRADD through its cytoplasmic death domain. TRADD recruitment endows DR3 with dual-signaling capability to activate NF−κB and MAP-kinase signaling or alternatively trigger caspase activation and programmed cell death (Chinnaiyan et al., 1996; Screaton et al., 1997; Wen et al., 2003). However, unlike TNFR1, which is widely expressed, DR3 has been reported to be expressed primarily by T lymphocytes (Screaton et al., 1997; Su et al., 2004). The ligand for DR3 was identified in 2002 as the TNF-family member TL1A (Migone et al., 2002). When added to certain tumor cell lines, TL1A can induce apoptosis after addition of cycloheximide. However, in primary T cells, TL1A has been reported to enhance proliferation and production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) induced by T cell receptor (TCR) crosslinking (Migone et al., 2002; Papadakis et al., 2004). TL1A was originally reported to be expressed exclusively in endothelial cells (Migone et al., 2002), but more recently TL1A has been found to be highly expressed in dendritic cells (DCs) after in vitro activation and in Crohn's disease, rheumatoid arthritis, and mouse models of inflammatory bowel disease, (Bamias et al., 2003; Bamias et al., 2006; Cassatella et al., 2007). Genetic variants in TL1A and DR3 have also been associated with Crohn's disease and rheumatoid arthritis, respectively (Osawa et al., 2004; Yamazaki et al., 2005).

Although exogenous TL1A is a T cell costimulator, the role of DR3 in peripheral T cell responses is not known. DR3-deficient mice exhibit a mild defect in thymic negative selection, but peripheral T cell numbers and subsets are normal (Wang et al., 2001). Spleens from DR3-deficient mice also have normal numbers of myeloid DCs ($CD11b^{+}CD11c^{hi}B220^{-}PDCA^{-}$), plasmacytoid DCs ($CD11b^{-}CD11c^{int}B220^{+}PDCA^{+}$), macrophages, monocytes, and granulocytes (F.M., E.C.Y.W., and R.M.S, unpublished observations). Understanding the physiological and pathophysiological roles of DR3 in immune responses is important for predicting the therapeutic and possibly deleterious consequences of blocking TL1A-DR3 interactions. Here, we have analyzed peripheral T cell responses in mice rendered deficient in DR3 through gene targeting. Exogenous TL1A functioned as a costimulator of T cell proliferation and cytokine production in wild-type (WT) T cells but not in DR3-deficient T cells, confirming the role of DR3 as an authentic and nonredundant costimulatory receptor for TL1A. However, DR3-deficient T cells only displayed defects in proliferation and cytokine production when activated in the presence of DCs, showing that DCs, rather than T cells,

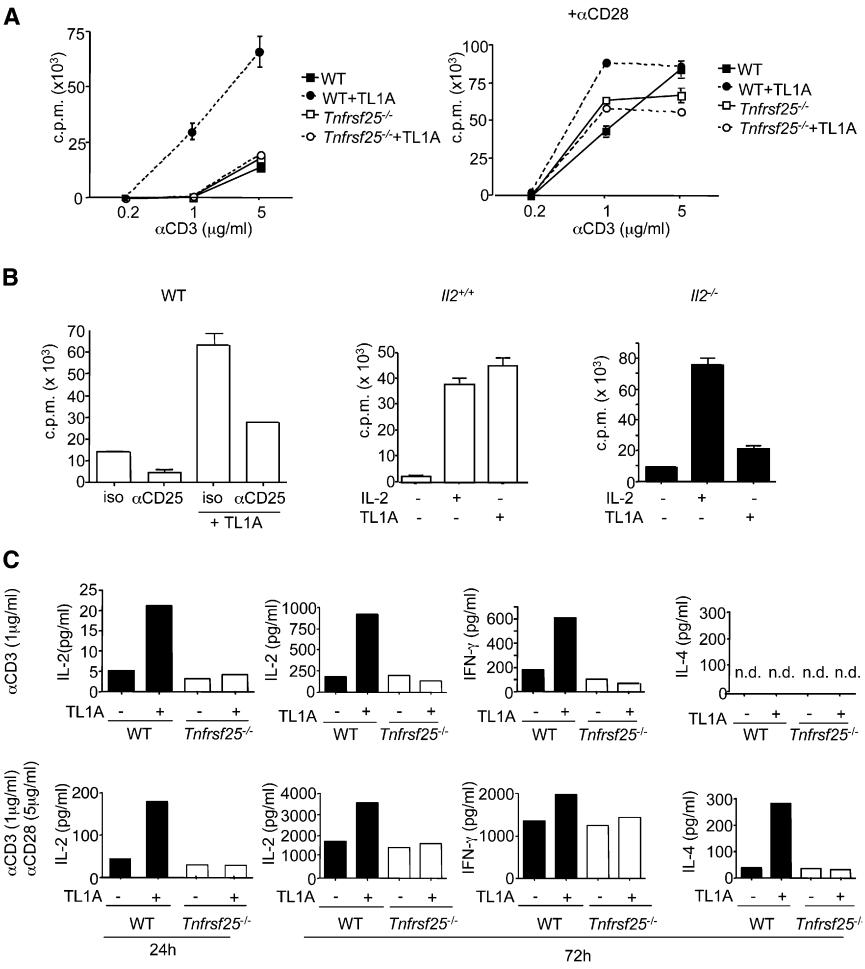

Figure 1. TL1A Costimulates Proliferation and Cytokine Production in CD4⁺ T Cells through DR3

(A) Purified CD4⁺ T cells from WT C57BL/6 or DR3-deficient (*Tnfrsf25*$^{-/-}$) mice were activated with anti-CD3 or anti-CD3 and anti-CD28 in the presence or absence of 10 ng/ml of mouse rTL1A for 3 days. ^{3}H-thymidine was added to the culture, incubated overnight, and analyzed for thymidine incorporation. Error bars represent SEM of triplicate samples.

(B) Purified T cells from C57BL/6 were cultured as above but also in the presence of 10 μg/ml of IL-2Rα (CD25) antibody or isotype control for 3 days (left panel). Purified T cells from *Il2*$^{-/-}$ or *Il2*$^{+/+}$ mice were cultured as above, in the absence or presence of 10 U/ml of IL-2 for 3 days (middle and right panels). Error bars represent SEM of triplicate samples.

(C) Supernatants from CD4⁺ T cells activated and cultured as in (A) were harvested at the indicated time points, and the indicated cytokines were measured with cytokine bead arrays. n.d. indicates "below limit of detection" (4 pg/ml). A representative of three experiments for each panel is shown.

are the physiologically relevant source of TL1A in T cell costimulation. Despite DR3's role in costimulation, DR3-TL1A interactions were not required for polarization of naive CD4⁺ T cells into T helper 1 (Th1), Th2, or Th17 effector cell subtypes, and T cell priming and systemic production of effector cytokines were normal in response to a number of model antigens and pathogens. Strikingly, however, immunopathology was dramatically reduced in the target organs of two different models of T cell-mediated inflammatory disease. These studies reveal a specific, nonredundant function for DR3 in controlling the function of effector CD4⁺ T cells in the target organs of autoimmune and inflammatory diseases.

RESULTS

TL1A Costimulates CD4⁺ T Cells through DR3

By searching through the SymAtlas gene-expression database, we confirmed that DR3 is primarily expressed on T cells in both mouse and man (Figure S1, available online). Exogenous TL1A can costimulate human and mouse T cells, but whether DR3 is the sole costimulatory receptor for TL1A and what role endogenously produced TL1A plays in T cell activation are not known. To investigate, we purified CD4⁺ T cells from spleens and lymph nodes of WT or age- and sex-matched DR3-deficient (*Tnfrsf25*$^{-/-}$) mice (Wang et al., 2001) on a C57BL/6 background and activated them through the TCR in the presence or absence of recombinant murine TL1A. Costimulation by other TNF-family members has been shown to be maximal when CD28-mediated costimulation is blocked (Croft, 2003). At concentrations of 10 ng/ml or above, TL1A increased T cell proliferation most dramatically in the absence of CD28-mediated costimulation (Figure 1A). When CD28-mediated costimulation was present, TL1A costimulated proliferation only at suboptimal doses of anti-CD3 (Figure 1A). The increased thymidine incorporation was due to increased cell division and not to enhanced survival, given that we observed increased CFSE dilution and no significant changes in cellular viability induced by TL1A (data not shown). Importantly, DR3-deficient cells were unresponsive to TL1A, indicating that DR3 is the major receptor that mediates costimulation by TL1A (Figure 1A). However, stimulation of DR3 through endogenous T cell-derived TL1A was apparently dispensable for T cell proliferation, because there were no deficits in proliferation in cultures of purified *Tnfrsf25*$^{-/-}$ T cells (Figure 1A). TL1A costimulation was largely dependent on increased IL-2 production, given that TL1A-induced proliferation was greatly reduced in IL-2-deficient T cells or those to which antagonistic IL-2Rα (αCD25) antibody had been added (Figure 1B).

To investigate the spectrum of cytokines that can be costimulated by TL1A and the dependence of cytokine production on DR3, we measured IL-2, IFN-γ, and IL-4 production in WT or *Tnfrsf25*$^{-/-}$ T cells activated in the presence or absence of recombinant TL1A. TL1A increased IL-2, IFN-γ, and IL-4 production by WT but not by *Tnfrsf25*$^{-/-}$ T cells, with IL-4 most prominently induced by TL1A in the presence of CD28 costimulation (Figure 1C). DR3-deficient T cells were unresponsive to TL1A

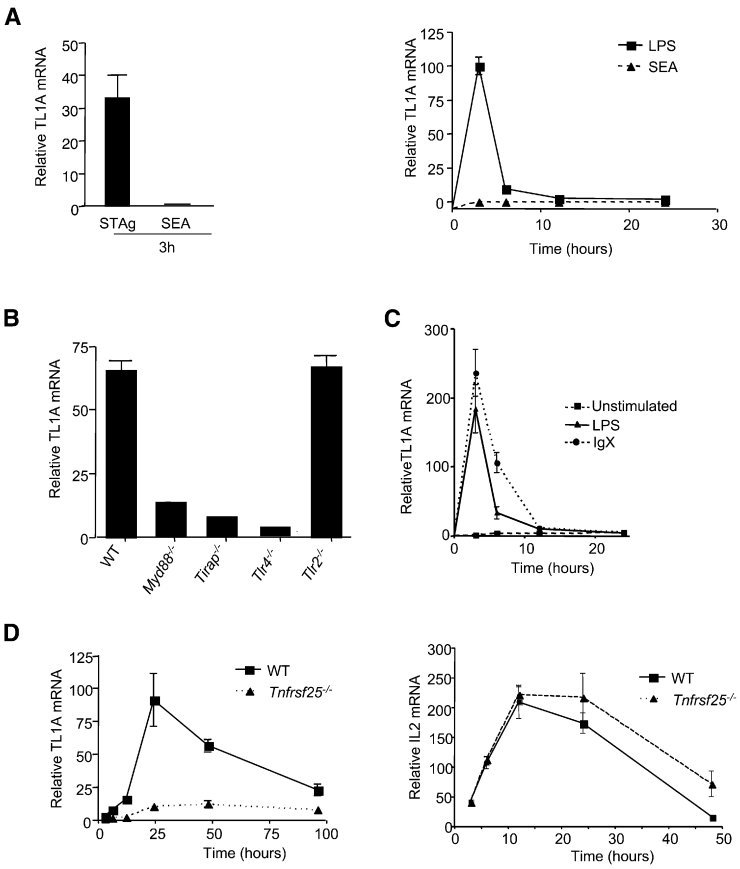

Figure 2. Differential Induction of TL1A Expression in DCs and T Cells

(A) Bone-marrow-derived DCs or CD11c$^+$ DCs from WT C57BL/6 mice were cultured and stimulated for the indicated time with or without 100 ng/ml of LPS, 20 μg/ml of SEA, or 10 μg/ml of STAg. RNA was prepared from each sample and used in reverse-transcriptase quantitative PCR (RT-qPCR).

(B) Bone-marrow-derived DCs from WT C57BL/6 mice or the indicated mutant mice were cultured and stimulated in the presence or absence of 100 ng/ml LPS for 3 hr. RNA was prepared from each sample and used in RT-qPCR.

(C) Bone-marrow-derived DCs from WT C57BL/6 mice were cultured and stimulated for the indicated times with or without 100 ng/ml LPS or Ig cross-linking, and RNA was prepared from each sample and used in RT-qPCR.

(D) Purified T cells from WT C57BL/6 or *Tnfrsf25*$^{-/-}$ mice were cultured and stimulated with 5 μg/ml of anti-CD3 and anti-CD28 for the indicated time. RNA was prepared from each sample and used in RT-qPCR.

Results indicate the amount of TL1A mRNA calculated relative to the untreated cells of each population (A–C) or relative to unstimulated T cells of each genotype (D). TL1A basal mRNA levels in T cells were approximately 50-fold lower than those in DCs. Error bars represent SEM of triplicate samples. A representative of two (A, B and C), one (D right panel), or three (D, left panel) experiments is shown.

but had no defects in cytokine production compared to WT T cells. Thus, as with proliferative responses, DR3 expression is required for TL1A-induced costimulation, but endogenously produced TL1A is not necessary for cytokine production by activated T cells under these conditions.

TL1A has been reported to costimulate memory T cells but not naive T cells (Bamias et al., 2006). To address this issue, we purified CD62L^{hi} CD44^{lo} naive CD4$^+$ T cells from WT and DR3-deficient mice and activated them with or without exogenous TL1A. TL1A mildly enhanced proliferation with or without CD28 costimulation and strongly increased IL-2 (8-fold) and IFN-γ (10-fold) production in a DR3-dependent manner (Figures S2A and S2B), showing that DR3 can function in naive T cells by increasing cytokine production. Percentages of phenotype CD44^{hi} CD4$^+$ memory T cells were also identical in age-matched *Tnfrsf25*$^{-/-}$ and control mice (Figure S2C), indicating that TL1A costimulation of total CD4$^+$ T cells is unlikely to be due to differences in the percentages of memory and naive T cells.

DCs Produce TL1A in Response to TLR and FcγR Stimuli

The lack of proliferation or cytokine-production defects in purified DR3-deficient T cells suggested that other cell types might be the physiological source of TL1A. TL1A has been reported to be produced by human DCs and monocytes after a variety of stimuli, and DCs would be a source of TL1A produced at the appropriate time and place for T cell costimulation. To test this hypothesis, we measured upregulation of TL1A gene expression by Reverse Transcriptase Quantitative PCR (RT-qPCR) in purified splenic CD11c$^+$ DCs and bone-marrow-derived DCs stimulated with a variety of agents. LPS and Soluble Tachyzoite Antigen (STAg) from *Toxoplasma gondii* acts through Toll-like receptors (TLRs) that can induce expression of other TNF-family members. LPS induced TL1A in bone-marrow-derived DCs, and STAg-induced TL1A in splenic DCs that express TLR11 required for STAg responsiveness (Yarovinsky et al., 2005). Expression peaked at up to 100-fold above baseline at 3 hr and rapidly declined after that (Figure 2A). Interestingly, Schistosoma Egg Antigen (SEA) from *Schistosoma mansoni,* which triggers alternative activation of DCs to program T cells for Th2 cell differentiation, did not appreciably induce TL1A mRNA (Figure 2A, left panel). Stimulation of DCs deficient in TLR-signaling components showed that LPS induction of TL1A was mediated by TLR4 in a manner dependent on MyD88 and TIRAP (Figure 2B). Immune complexes acting through low-affinity Fc receptors have recently been shown to be a potent stimulus for TL1A production (Cassatella et al., 2007; Prehn et al., 2007). Stimulation of murine DCs with plate-bound cross-linked mouse Ig (IC) also stimulated TL1A gene expression, comparable to LPS (Figure 2C). Thus, like other TNF-family members, TL1A can be rapidly induced in DCs through TLR and immune complexes.

To test whether T cells could serve as an autocrine source of TL1A, purified T cells were stimulated through the TCR, and TL1A mRNA expression was measured by RT-qPCR. TL1A mRNA was upregulated after TCR stimulation, but with delayed kinetics as compared to that of DCs. Interestingly, TL1A upregulation was specifically dependent on DR3 expression, given that DR3-deficient T cells showed dramatically reduced TL1A induction but normal upregulation of IL-2 mRNA after activation (Figure 2D). Taken together, these data show that T cells can produce TL1A that acts in an autocrine manner to sustain its

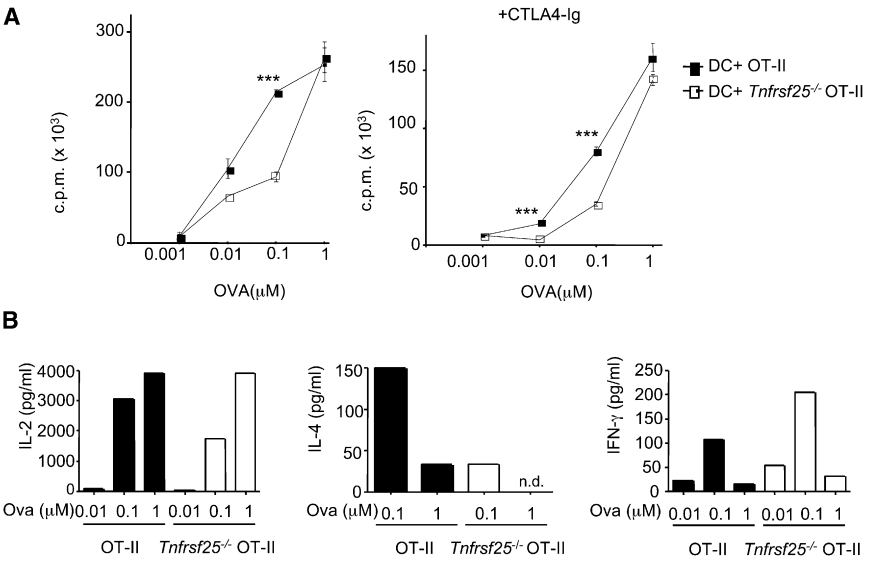

Figure 3. *Tnfrsf25*$^{-/-}$ T Cells Have Reduced Proliferation and Altered Cytokine Production when Cultured in the Presence of DCs

(A) Bone-marrow-derived DCs were cultured with naive OT-II or *Tnfrsf25*$^{-/-}$ OT-II CD4$^+$ T cells in the presence of indicated Ova-peptide concentration and in the absence (left panel) or presence (right panel) of CTLA4-Ig for 3 days. ^{3}H-thymidine was added to the culture, incubated overnight, and analyzed for thymidine incorporation. (B) Supernatants from the above cultures were harvested after 72 hr and tested for cytokine production. n.d. indicates "below limit of detection" (4 pg/ml). A representative of three experiments for each panel is shown.

own expression but that T cell-derived TL1A might not be necessary for proliferation or cytokine production by isolated T cells.

DR3 is Not Required for Th1, Th2, or Th17 Differentiation of Naive T Cells

To study the role of TL1A-DR3 interactions in a more physiological model of T cell activation, we backcrossed DR3-deficient mice to the Ovalbumin (Ova)-specific TCR transgenic line OT-II and cultured naive T cells from *Tnfrsf25*$^{-/-}$ OT-II and OT-II control mice with Ova peptide and WT bone-marrow-derived DCs. Under these conditions, proliferation of *Tnfrsf25*$^{-/-}$ OT-II cells was diminished, especially at low concentrations of Ova (Figure 3A), whether costimulation through B7 was blocked by the addition of CTLA4-Ig or not. The cytokine profile of T cells stimulated with Ova peptide and DCs is characteristically dependent on the dose of antigen, with higher doses favoring IFN-γ production and lower doses favoring IL-4 production (Tao et al., 1997). *Tnfrsf25*$^{-/-}$ OT-II cells produced less IL-2 at lower doses of Ova and less IL-4 at all doses of Ova tested. By contrast, *Tnfrsf25*$^{-/-}$ OT-II cells produced marginally higher amounts of IFN-γ compared with those of controls at all doses tested (Figure 3B). These data suggest that during cognate interactions between antigen-specific T cells and DCs, TL1A-DR3 interactions function to costimulate T cell proliferation and production of IL-2, IL-4, but not IFN-γ.

These alterations in cytokine production and proliferation suggest that DR3 may influence T cell polarization. To test this, we activated naive CD4$^+$ T cells from *Tnfrsf25*$^{-/-}$ or control mice in the presence of DCs, under conditions optimal for differentiation of Th1, Th2, or Th17 effector T cells or under neutral conditions, and we measured cytokine production after restimulation (Figure 4). In the absence of exogenous polarizing stimuli, both WT and *Tnfrsf25*$^{-/-}$ T cells exhibited mild skewing toward a Th1-IFN-γ-secreting profile expected on the C57BL/6 background. In addition, *Tnfrsf25*$^{-/-}$ T cells could be polarized normally toward IL-4-, IFN-γ-, or IL-17-producing cells (Figure 4A). We then set up cultures of *Tnfrsf25*$^{-/-}$ OT-II and control OT-II T cells stimulated with DCs and Ova and polarized them with cytokines or with STAg, which induces TL1A production. These conditions also resulted in normal Th1 cell skewing by antigen-specific *Tnfrsf25*$^{-/-}$ T cells (Figure 4B). Induction of the transcription factors T-bet, GATA-3, or RORγ by appropriate differentiation stimuli was also unaffected in purified *Tnfrsf25*$^{-/-}$ T cells (data not shown). Thus, TL1A-DR3 interactions appear to be dispensable for the in vitro differentiation of naive T cells into Th1, Th2, or Th17 effector cell subtypes.

DR3 is Required for Immunopathology in Experimental Autoimmune Encephalomyelitis

To determine the role of DR3 in T cell differentiation and effector function in the intact immune system, we studied disease models dependent on distinct T cell subsets in *Tnfrsf25*$^{-/-}$ mice. We first studied Experimental Autoimmune Encephalomyelitis (EAE), an autoimmune-disease model dependent on Th17 and Th1 cell subsets. In four separate experiments, *Tnfrsf25*$^{-/-}$ mice exhibited dramatically reduced paralysis, measured by clinical scores (Figure 5A). Despite profound resistance to EAE, T cells from draining lymph nodes of myelin oligodendrocyte glycoprotein (MOG)-primed *Tnfrsf25*$^{-/-}$ mice proliferated normally in response to most doses of MOG (Figure 5B). In accordance with the clinical scores, the total yield and percentage of CD4$^+$ T cells was markedly reduced in spinal-cord homogenates from pooled *Tnfrsf25*$^{-/-}$ mice compared to that of controls (Figure 5C). Within the T cell gate, the percentage of IFN-γ-producing cells was reduced by 50% in T cells from the spinal cords of *Tnfrsf25*$^{-/-}$ mice (Figure 5C). The percentage of IL-17-producing cells was normal in *Tnfrsf25*$^{-/-}$ mice within this gate, but overall, it was reduced due to the decreased percentage of CD4$^+$ T cells in the spinal cord. To examine the absolute amounts of these cytokines in the inflamed spinal cord, mRNA for IL-17 and IFN-γ was measured by RT-qPCR in spinal-cord homogenates. Both cytokines were reduced in spinal-cord preparations from MOG-primed *Tnfrsf25*$^{-/-}$ mice when normalized to the housekeeping gene β2-microglobulin, with IFN-γ the most affected. However, when normalized to the expression of the T cell-specific gene CD3δ, IL-17 and IFN-γ mRNA expression

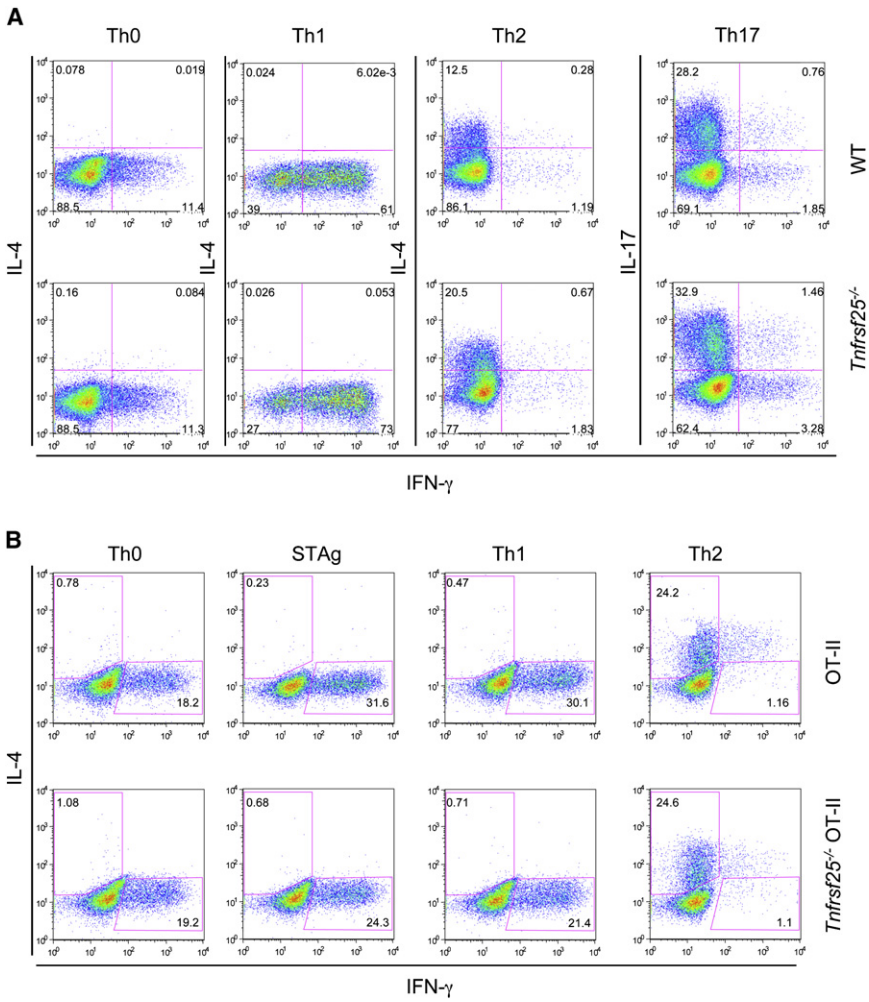

Figure 4. DR3 is Not Required for Th1, Th2, or Th17 Differentiation of Naive T Cells

(A) T-depleted APCs were cultured with C57BL/6 or *Tnfrsf25*$^{-/-}$ purified naive CD4$^+$ T cells in the presence of soluble anti-CD3 (1 μg/ml) and anti-CD28 (3 μg/ml) under Th0, Th1, Th2, or Th17 cell polarization conditions for 4 days. Cells were then restimulated with PMA and Ionomycin for 5–6 hr, stained for intracellular cytokines, and analyzed by flow cytometry.

(B) Sorted CD11c$^+$ DCs were cultured with OT-II or *Tnfrsf25*$^{-/-}$ OT-II purified naive CD4$^+$ T cells in the presence of 1 μM Ova peptide under Th0, Th1, or Th2 cell polarization conditions or in the presence of STAg for 6 days. Cells were then restimulated with anti-CD3 and anti-CD28 for 5–6 hr, stained for intracellular cytokines, and analyzed by flow cytometry.

A representative of two (A) and three (B) experiments is shown.

was not reduced in *Tnfrsf25*$^{-/-}$ spinal cord. (Figure 5D). Thus, DR3 is critical for immunopathology in EAE and appears to act principally by controlling the accumulation of T cells in the spinal cord.

To investigate whether DR3 expression on T cells is responsible for mediating these effects, we performed transfer experiments in which *Tnfrsf25*$^{-/-}$ or WT CD4$^+$ T cells were transferred into TCRα-deficient mice (Figure 5E). The mice were then immunized with MOG. Mice receiving DR3-deficient T cells exhibited dramatically reduced paralysis compared to that of mice receiving WT T cells. As with EAE in *Tnfrsf25*$^{-/-}$ mice, there were fewer CD4$^+$ T cells in the spinal-cord homogenates of mice induced to develop EAE that received DR3-deficient T cells, with the percentages of CD4$^+$ T cells correlating closely with disease score in each mouse (data not shown). Thus, DR3 expression is required on T cells for immunopathology in this mouse model of autoimmune demyelinating disease.

DR3 Is Required for Th2 Cell-Mediated Lung Inflammation but Not for Systemic Th2 Cell Responses

We next investigated a Th2 cell-dependent model of lung inflammation in which mice are primed systemically with Ova and Alum and then locally challenged with Ova (Gavett et al., 1994). In three independent experiments, histological analysis showed that the airways in *Tnfrsf25*$^{-/-}$ mice lungs had less inflammation, including mucin production and peribronchial inflammation (Figure 6A). Standardized histopathology scores and cell counts in bronchoalveolar lavage (BAL) were reduced in Ova-sensitized and -challenged *Tnfrsf25*$^{-/-}$ mice compared with those in WT mice sensitized and challenged in parallel with Ova (Figure 6B). The percentages of CD3$^+$ and CD4$^+$ T cells, invariant Vα14$^+$ natural killer T (NKT) cells, and eosinophils were all significantly reduced in lung cell preparations from Ova-sensitized and -challenged *Tnfrsf25*$^{-/-}$ mice in comparison to controls (Figure 6C). Immunohistochemical localization of CD3$^+$ cells in lung tissue from Ova-sensitized DR3-deficient mice revealed fewer interstitial and peribronchial T cells and increased perivascular localization as compared to controls. Similar increases in perivascular infiltrates were observed for macrophages (Figure S3). Expression of mRNA for IL-5 and IL-13, which are critical for Th2-mediated lung pathology, was markedly reduced in *Tnfrsf25*$^{-/-}$ Ova-sensitized lungs, whereas IL-10 and IFN-γ were produced equally (Figure 6D). By contrast, when *Tnfrsf25*$^{-/-}$-spleen cells from these mice were restimulated with Ova, there was normal production of IL-5 and IL-13, indicating that systemic priming of Ova-specific Th2 T cells was independent of DR3 (Figure 6E). In addition, *Tnfrsf25*$^{-/-}$ splenocytes proliferated normally in response to Ova (data not shown). Systemic Th2 cell function, assessed by the production of Ova-specific IgG1 and Ova-specific IgE after Ova priming, was also normal in *Tnfrsf25*$^{-/-}$ mice (Figure 6F). Thus, in this model of Th2-mediated lung inflammation, DR3 expression is required for accumulation of Th2 effector cells at the site of inflammation but not for systemic differentiation of Th2 cells. Decreased

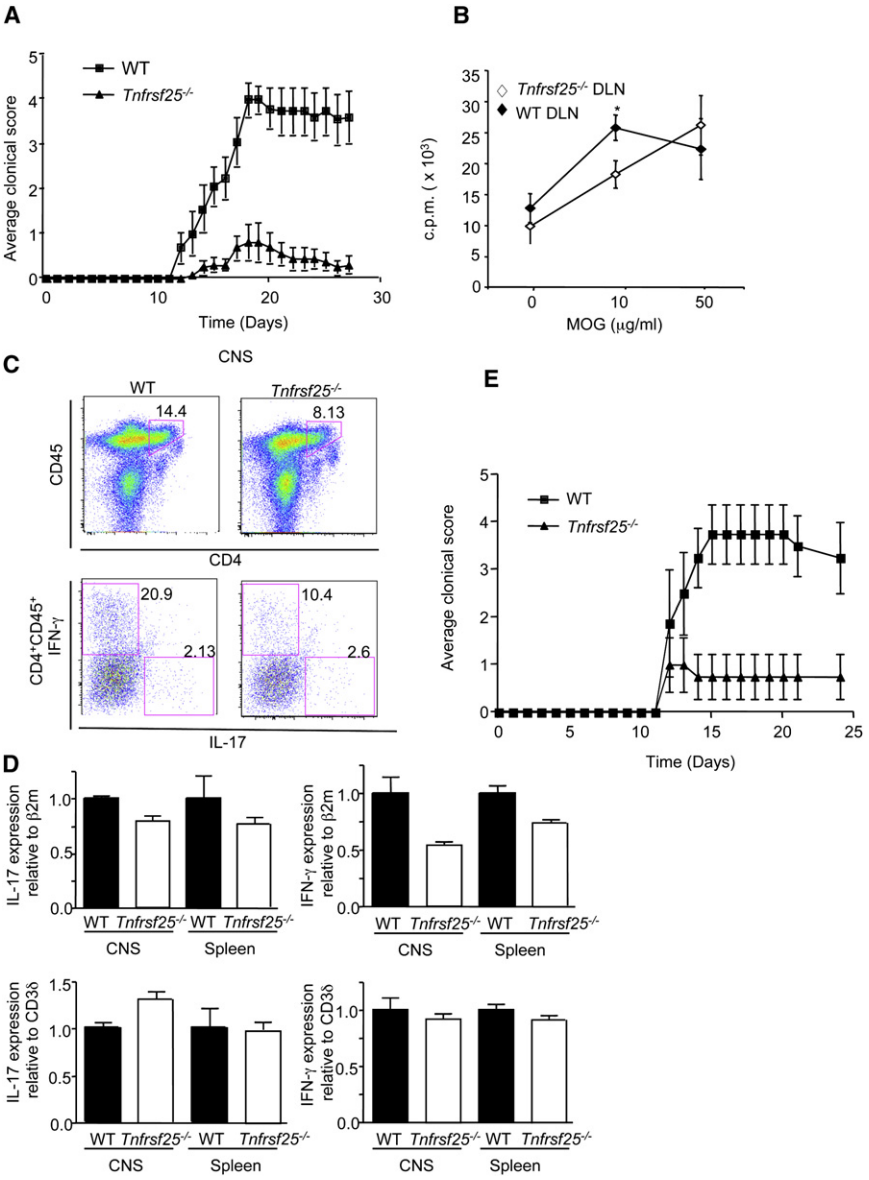

Figure 5. *Tnfrsf25*$^{-/-}$ Mice are Resistant to EAE and Have Reduced Numbers of T Cells in the Spinal Cord

(A) EAE was induced in *Tnfrsf25*$^{-/-}$ mice and C57BL/6 control mice as described in the Experimental Procedures, and clinical scores were measured daily. One of four experimental trials is shown, with a minimum of five mice in each group.

(B) Draining lymph nodes from the site of MOG injection were harvested, and cells were restimulated with the indicated amounts of MOG peptide. T cell proliferation was assessed by ^{3}H-thymidine incorporation after 3 days.

(C) T cell yield and function from spinal-cord preparations. Spinal-cord homogenates were prepared from mice at day 28 of EAE. The yield of CD4^{+} T cells obtained from spinal-cord preparations of WT mice was 2.26 × 10^{5}, versus 1.6 × 10^{5} CD4^{+} T cells from spinal-cord preparations of *Tnfrsf25*$^{-/-}$ mice. The average clinical score of the mice used in this experiment was 2.75 in the WT group and 1 in the *Tnfrsf25*$^{-/-}$ group. Cells harvested from spinal cords were restimulated for 4 hr with anti-CD3 and anti-CD28 and analyzed by flow cytometry for T cell-surface markers, and gated CD45^{+}CD4^{+} cells were analyzed for intracellular cytokine production.

(D) mRNA from spinal cords or spleens of the same groups of mice shown in (C) was analyzed by RT-qPCR for IL-17 and IFN-γ mRNA. Results are normalized to β2 m or CD3δ. Error bars represent SEM of triplicate samples.

(E) EAE was induced in TCRα-deficient mice reconstituted with *Tnfrsf25*$^{-/-}$ or WT CD4^{+} T cells as described in the Experimental Procedures, and clinical scores were measured daily.

Data is representative of four (A), two (B), and four (C) experiments.

T cells in the lung may result in defective recruitment of eosinophils and invariant NKT (iNKT) cells to the site of inflammation, as was observed in the *Tnfrsf25*$^{-/-}$ lung.

To investigate in more detail the role of DR3 in the trafficking and local proliferation of antigen-specific T cells and to determine whether the requirement for DR3 is T cell intrinsic in this disease model, we performed transfer experiments in which *Tnfrsf25*$^{-/-}$ OT-II Ova-specific T cells and OT-II controls were activated in vitro under Th2 cell differentiation conditions and then transferred into naive congenic host mice. Recipients were then given a respiratory challenge with Ova, and responses were analyzed after 1 or 2 days. In this system, when antigen was only delivered locally to the lung, we saw a significant defect in accumulation and proliferation of primed Ova-specific T cells in the lung, with an even more profound defect in the recovery and proliferation of *Tnfrsf25*$^{-/-}$ OT-II T cells in the draining mediastinal lymph nodes (Figures 7A and 7B). Inflammatory cells in the BAL were correspondingly reduced in recipients of *Tnfrsf25*$^{-/-}$ OT-II T cells (Figure 7C). These experiments clearly show that DR3 expression on T cells is necessary for proper local responses of primed T cells to cognate antigen. It is possible that this phenotype could stem from a generalized requirement for DR3 to be expressed on T cells in order for them to acquire the ability to migrate to extralymphatic tissues. However, when naive DR3-deficient T cells were transferred into congenic hosts and Ova was delivered systemically with LPS used as an adjuvant, T cells migrated normally to lung, liver, and intestinal sites as efficiently as WT OT-II T cells (Figure S4). Thus, DR3 expression on T cells appears to be critical for tissue homing and proliferation only for locally restimulated T cells.

The defective autoimmune and inflammatory responses in *Tnfrsf25*$^{-/-}$ mice suggest that cellular responses to infectious organisms might also be dependent on DR3. We have investigated this by studying the role of DR3 in immune responses to *Toxoplasma gondii*, a parasite for which IFN-γ-secreting Th1 cells are critical for host defense and survival of infected mice. After infection with *T. gondii,* all *Tnfrsf25*$^{-/-}$ mice survived acute infection. Spleen cells isolated from infected *Tnfrsf25*$^{-/-}$ mice at

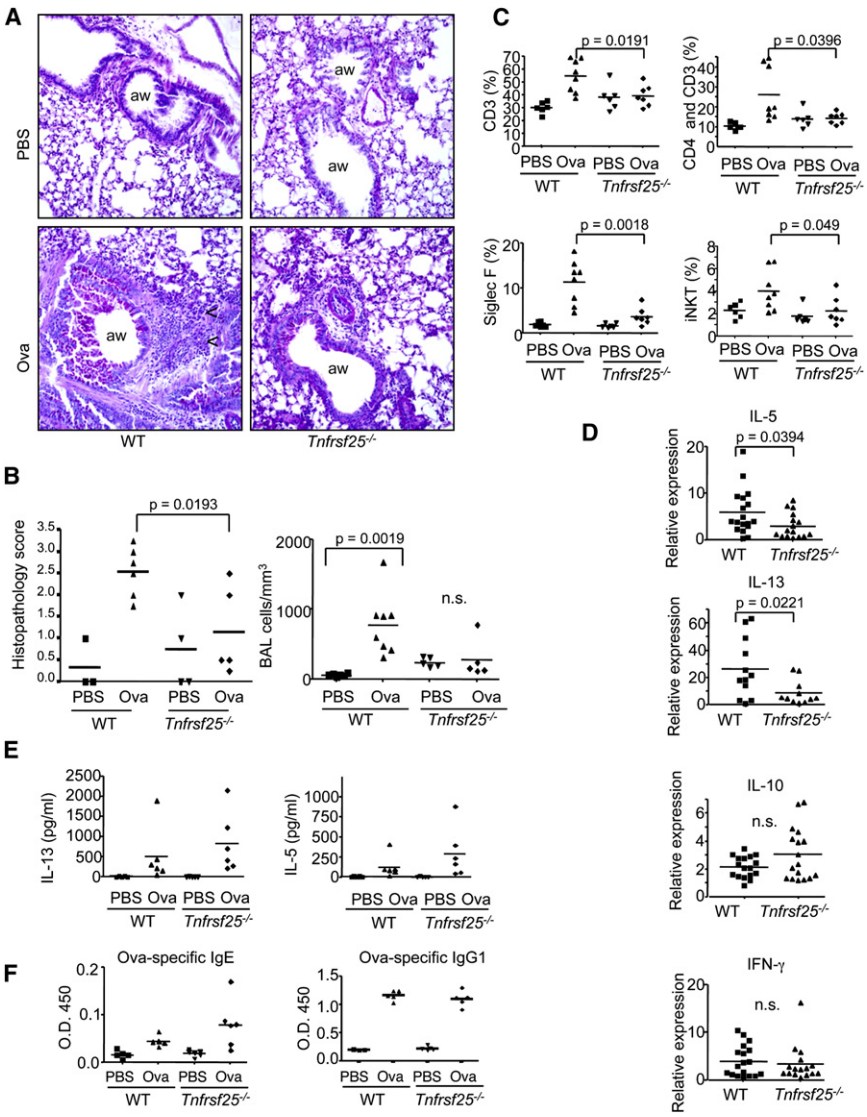

Figure 6. DR3 is Required for Th2 Cell -Mediated Lung Inflammation

Mice were sensitized with Alum + PBS (control) or Alum + Ova. Mice were then challenged with PBS (control) or Ova.

(A) Examples of PAS-stained histology are shown, with airways (aw) and infiltrating cells (arrowheads) shown.

(B) Histopathology of the lungs was scored (left panel), and cells in the BAL were counted (right panel).

(C) Cells were extracted from the lungs and analyzed by flow cytometry for the percentage of the indicated subsets out of the total live cell population. Siglec F was used to measure eosinophils as previously described (Norris et al., 2007). invariant 'type-I' NKT (iNKT) cells were enumerated with CD1d-PBS57 tetramer, which identifies Vα14Jα18$^+$ T cells responsive to alpha-gal-cer sphingolipid.

(D) RNA was prepared from lungs and used in RT-qPCR. Results indicate the amount of cytokine mRNA calculated relative to the lungs of control mice treated with PBS. p values are for unpaired t tests on mRNA levels of the indicated cytokines between *Tnfrsf25*$^{-/-}$ and control mice induced with Ova.

(E) Splenocytes were cultured in the presence of 50 μg/ml of Ova protein or media control for 3 days. Supernatants were analyzed for cytokine production by cytometric bead array.

(F) Serum was tested for Ova-specific IgE and Ova-specific IgG1 concentration by ELISA. p values obtained by comparison of groups with an unpaired two-tailed t test are shown where significant; n.s. indicates "not significant."

A representative of three (A and B), a representative of two (C, E, F), and a pool of two (D) experiments are shown.

seven weeks after infection produced comparable amounts of TNF-α, IFN-γ, and IL-10 in response to STAg as compared to controls (Figure S5A). Immune responses to toxoplasma infection were also intact, as indicated by similar counts of toxoplasma cysts in the brains of WT and *Tnfrsf25*$^{-/-}$ animals (Figure S5B). Infiltrating CD3$^+$ T cells were present in similar amounts in histological sections of the brains of WT and *Tnfrsf25*$^{-/-}$ mice (data not shown). In addition, *Toxoplasma*-infected *Tnfrsf25*$^{-/-}$ mice survived at rates similar to, if not greater than, infected WT mice at later stages of infection (Figure S5C). These data indicate that, at least in the case of *T. gondii*, priming and maintenance of effector T cells responsible for controlling acute and chronic infections are not dependent on DR3.

DISCUSSION

TNF receptors expressed on T cells have been found to play distinct roles in costimulation, survival after activation, and formation of T cell memory (Croft, 2003; Watts, 2005). Here, we have found that TL1A-DR3 interactions can enhance T cell proliferation and cytokine production but are largely dispensable for differentiation into diverse effector T cell subsets in vitro and in vivo. Our findings provide strong evidence that DR3 is required for effective T cell immune responses in the target organs of T cell-mediated autoimmune and inflammatory diseases, whereas DR3 is not required for an effective response to the parasite *T. gondii.*

Our results suggest that the cellular source of TL1A influences the biological outcome of DR3 engagement on T cells. DCs are the most likely source of TL1A for T cells at the time of antigen presentation, and DR3 is required for costimulation by DC-derived TL1A. TL1A is rapidly upregulated in DCs through TLR or FcR and then rapidly returns to baseline expression. T cells are also able to upregulate TL1A after stimulation through the TCR, but mRNA upregulation is slower and is sustained for at least 48 hr after activation. TL1A produced by T cells can bind to DR3 and enhance TL1A production through an autocrine feedback loop. This contrasts with OX40L and FasL, which are hyperexpressed by T cells in the absence of their cognate receptors

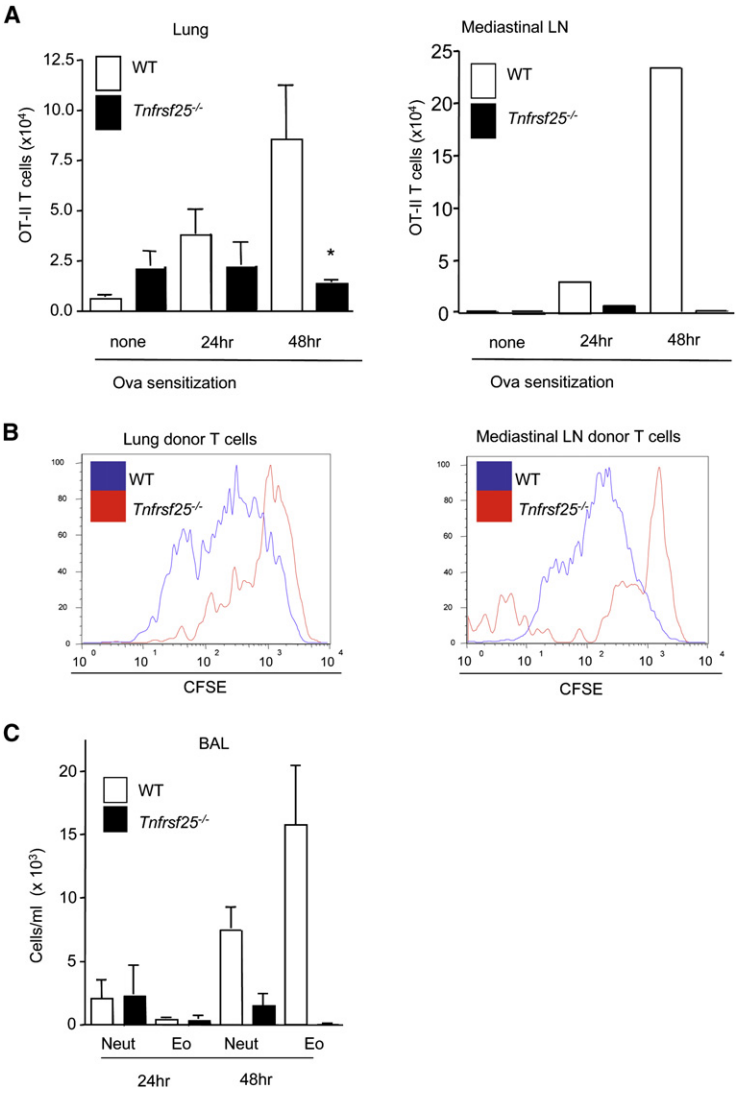

Figure 7. T Cell-Intrinsic Requirement for DR3 in Allergic Lung Hypersensitivity

2×10^6 CFSE-labeled T cells from Th2-polarized *Tnfrsf25*$^{-/-}$ OT-II or OT-II T cells were transferred into CD45.1 congenic recipient mice, and the recipient mice were challenged with i.t.-administered Ova once 24 hr later (24 hr condition) or with i.t.-administered Ova 24 hr after transfer and i.n.-administered Ova 48 hr after transfer (48 hr condition). All mice were killed 72 hr after cell transfer. Four recipients per experimental group were used, and two mice receiving WT and *Tnfrsf25*$^{-/-}$ T cells were kept as unchallenged controls ("none").

(A) Average yield of CD45.2$^+$ T cells, per lung or in pooled mediastinal lymph nodes, from each group.

(B) Representative CFSE profiles of CD45.2 donor cells from recipients of WT and *Tnfrsf25*$^{-/-}$ donor T cells challenged with Ova for 48 hr.

(C) The average ± SEM Eosinophil and Neutrophil cell counts in BAL from mice receiving WT or *Tnfrsf25*$^{-/-}$ T cells challenged with Ova for the indicated period of time. A representative of two experiments is shown.

(Soroosh et al., 2006; Hao et al., 2004). TL1A can be cleaved off the plasma membrane by metalloproteases in endothelial and dendritic cells but not in T cells (Kim and Zhang, 2005; F.M., unpublished observations). TL1A produced by T cells in an autocrine manner may sustain costimulation, although we did not find any functional consequences of DR3 deficiency in isolated T cells in terms of proliferation, cytokine production, or differentiation. Even in the presence of DCs and TL1A-inducing stimuli, DR3 was not required for T cell polarization of Th1, Th2, or Th17 cell subsets in vitro or in vivo. However, these conclusions must be tempered by the fact that we have only examined TL1A expression in mouse T cells at the RNA level and the possibility that there might be very little TL1A produced under these conditions and even less by *Tnfrsf25*$^{-/-}$ T cells.

Despite the relatively modest role of DR3 in T cell activation in vitro, our data on T cell-dependent animal-disease models establish for the first time an essential role of DR3 in T cells within target tissues during autoimmune and allergic inflammation. Immunopathology and clinical disease were decreased both in a Th2-driven model of Ova-induced lung inflammation and in EAE, which depends on Th1 and Th17 cells. In both models, the accumulation of T cells in the target tissue and the production of effector cytokines specific for that model were decreased. After submission of this manuscript, two other independent studies using TL1A-deficient mice and DR3-dominant-negative transgenic mice implicated TL1A-DR3 interactions in Th17 cell responses (Pappu et al., 2008) and in allergic lung inflammation (Fang et al., 2008), respectively. Our results show that both disease models require DR3 irrespective of the specific Th cell subtype involved. Remarkably, we found that induction of systemic immune responses to Ova and MOG were, unlike local T cell responses, independent of DR3, as measured by peripheral T cell proliferation, cytokine production, or T cell help for antibody responses. In both lung hypersensitivity and EAE, the requirement for DR3 was T cell intrinsic, as indicated by the transfer experiments in which DR3 was deficient only on T cells.

The requirement for DR3 in T cell-mediated inflammatory disease as seen in these animal models could be due to the induction of TL1A in the target organ or draining lymph node during secondary T cell responses. TL1A produced in the area of inflammation might enhance survival, migration, or proliferation of T cells in target tissues through DR3 signaling. Enhanced survival due to TL1A seems unlikely, given that we have not observed an increase in apoptotic cells in preparations of lung or spinal-cord homogenate from *Tnfrsf25*$^{-/-}$ mice during lung hypersensitivity or EAE (F.M. and R.M.S., unpublished observations). DR3 could also function to enhance effector-cytokine production by T cells at the site of inflammation, although defects in production of effector cytokines on a per-cell basis do not appear to explain the dramatic effects of DR3 deficiency on the severity of EAE. Although fewer cells were detected in the lungs and spinal cords of mice with inflammation in these organs, this does not stem from a requirement for DR3 in extralymphatic trafficking of activated T cells, given the normal ability of T cells to home to the

lung, liver, and gut after systemic priming. Instead, the dramatic reduction in proliferation and expansion of DR3-deficient Ova-specific T cells in the mediastinal lymph nodes and lungs of mice after pulmonary challenge with Ova suggests that a critical function of DR3 stimulation is to costimulate antigen-induced expansion of primed T cells in the target organ of T cell-mediated autoimmune and inflammatory diseases. By contrast, given that this model does not require secondary re-expansion of T cells, DR3 might not be required for the acute Th1 cell response to *T. gondii*. DR3 might not be required for control of brain cysts in the chronic phase of *T. gondii* infection, given that control of brain cysts has been shown to be more dependent on $CD8^+$ than on $CD4^+$ T cells (Parker et al., 1991; Scorza et al., 2003). Thus far, we have not found any defects in primary or secondary $CD8^+$ T cell responses in *Tnfrsf25*$^{-/-}$ mice (F.M. and R.M.S., unpublished observations). DR3's role in T cell immunity to other infectious agents will need to be further tested if we are to know how applicable our findings are to other infections.

Other TNF-family members that costimulate T cells have been reported to contribute to EAE and allergic inflammation, but blockade of most other TNF-family receptors generally affects systemic immune responses as well as target-organ-specific pathology. For example, OX40 has been shown to be important in the generation of a systemic, as well as a local, Th2 cell response in asthma (Arestides et al., 2002; Salek-Ardakani et al., 2003). A blocking antibody against CD70 (ligand for the CD27 costimulatory receptor on T cells) ameliorated EAE but also depressed systemic immune responses (Nakajima et al., 2000). Antibody blockade of OX40L was reported to decrease influx of T cells into the brain in EAE (Nohara et al., 2001). Interestingly, like TL1A, OX40L expression is induced in endothelial cells by pro-inflammatory stimuli.

These results make DR3 a relatively unique TNF-family receptor in terms of its role in promoting T cell accumulation and pathology in autoimmune- and inflammatory-disease models depending on a diverse set of cytokines and Th cell subsets. The role of DR3 in promoting T cell-mediated immunopathology strikingly parallels the role of its paralog, TNFR1, as an amplifier of innate immune responses. The signaling pathways by which DR3 accomplishes this, as well as the molecular mechanisms underlying this specific function of DR3, will be an interesting topic for further study. Because primary T cell responses and host defense are relatively preserved in *Tnfrsf25*$^{-/-}$ mice, blocking the action of TL1A might be less generally immunosuppressive than blocking TNF or non-TNF cytokines. This makes blocking TL1A-DR3 interactions a promising strategy for therapy in a wide variety of T cell-dependent autoimmune diseases.

EXPERIMENTAL PROCEDURES

Reagents and Mice

LPS from E. Coli was obtained from Sigma. STAg was prepared from sonicated *Toxoplasma gondii* tachyzoites, and SEA was prepared from *Schistosoma mansoni* eggs as previously described (Grunvald et al., 1996). C57BL/6 mice were obtained from Jackson Laboratories. DR3-deficient mice, generated as previously described (Wang et al., 2001), were backcrossed to the C57BL/6 background for at least eight generations. DR3-deficient OT-II mice were generated by the crossing of DR3-deficient mice to OT-II TCR transgenic mice (Taconic). *Il-2*$^{-/-}$ mice were a generous gift from Pushpa Pandiyan, NIAID. All antibodies were purchased from BD pharmingen unless indicated otherwise. CD1d/PBS57 tetramers that recognize Vα14 iNKT cells were prepared by the National Institutes of Health (NIH) tetramer core facility. B6.CD45.1 congenic mice were obtained from Jackson Labs or NCI/Frederick. TCRα-deficient mice were obtained from Taconic. Mice were maintained in the NIAMS and NIAID animal facilities under animal-study protocols approved by the appropriate NIH institute animal-care and -use committee.

Cell Preparation and Purification

Splenic DCs were sorted for high expression of $CD11c^+$ on a MoFlo FACS sorter (Dako Carpenteria, CA) from liberase-digested spleens. The purity of $CD11c^+$ DCs was at least 97%. T cells were purified from spleen and lymph-node cell suspensions by magnetic depletion of non-T cells with FITC-conjugated mAb to CD11b, PanNK, B220, NK1.1, CD24, CD16/32, GR-1, and $I\text{-}A^b$ (BD pharmingen), followed by anti-FITC microbeads (Miltenyi). For purification of $CD4^+$ T cells, anti-CD8-FITC was added to the above antibodies. For naive T cells, the $CD62L^+CD44^-$ population of purified $CD4^+$ cells was sorted after staining with PE-Cy5 anti-CD44 and PE anti-CD62L. Bone-marrow-derived DCs were generated by culture with RPMI/10% FCS supplemented with 10 ng/ml of murine GM-CSF (PeproTech, Rocky Hill, NJ). T cell-depleted antigen-presenting cells were obtained by the incubation of spleen cell suspensions with anti-Thy1.2 for 10 min on ice followed by incubation with low-tox-M rabbit complement (Cedarlane laboratories) for 30 min at 37°C. Cells were washed and incubated with 25 μg/ml of mitomycin C (Sigma) for 30 min at 37°C.

T Cell Activation and Polarization

For costimulation studies, $CD4^+$ or naive $CD4^+$ cells were stimulated with plate-bound anti-CD3 mAb (5 μg/ml or at the indicated concentration, 145-2C11; BD PharMingen) in the presence or absence of plate-bound anti-CD28 mAb (5 μg/ml) (37.51; BD PharMingen). Recombinant mouse TL1A (R&D systems) was added at 10 ng/ml. For studies with *Il2*$^{-/-}$ mice, purified T cells were cultured as above but in the absence or presence of 10 U/ml of IL-2. For DC-T cell coculture studies, 10^4 bone-marrow-derived DCs were cultured with 10^5 OT-II or DR3-deficient OT-II naive $CD4^+$T cells per well and the indicated concentration of $Ova_{323–339}$ peptide, with or without 10 μg/ml of mouse CTLA4/Fc (Chimerigen). On day 3, culture supernatants were collected for cytokine measurement, and cells were pulsed with 1 μCi of ^{3}H-thymidine. After an additional 16–20 hr, ^{3}H-thymidine incorporation was measured with a scintillation counter. For polarization studies, 8×10^5 T cell-depleted APCs were cultured with 2×10^5 naive $CD4^+$ T cells from C57BL/6 mice or DR3-deficient mice. Th1 cell polarization was driven with rIL-12 (20 ng/ml) (PeproTech, Rocky Hill, NJ) and anti-IL-4 (10 μg/ml); Th2 cell with rIL-4 (20 ng/ml) (PeproTech, Rocky Hill, NJ), anti-IL-12 (10 μg/ml), and anti IFN-γ (10 μg/ml); Th17 cell with rhTGFβ (5 ng/ml) (eBioscience), IL-6 (20 ng/ml) (eBioscience), anti-IL-12 (10 μg/ml), anti IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml); and Th0 cell with anti-IL-12 (10 μg/ml), anti IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml). After 4 days of culture, intracellular cytokine staining was performed as described below. For polarization studies with STAg, 5×10^4 splenic DCs were cultured with 10^5 OT-II or DR3-deficient OT-II naive $CD4^+$T cells per well with 1 μM of $Ova_{323–339}$ peptide. Th1 cell polarization was driven with rIL-12 (10 ng/ml), Th2 cell with rIL-4 (10 ng/ml), and STAg with 5 μg/ml of STAg. After 72 hr of culture, supernatants were replaced with fresh medium containing 10 U/ml of rIL-2, and after an additional 2–3 days, intracellular cytokine staining was performed as described below.

Induction of Experimental Autoimmune Encephalomyelitis

Mice were immunized subcutaneously with myelin oligodendrocyte glycoprotein (MOG) 35-55 peptide in CFA, with pertussis toxin administrated intraperitoneally (i.p.) on days 0 and 2 to induce EAE. Five to eight mice were included per group and were scored.

For transfer experiments in EAE, 2×10^7 $CD4^+$ T cells from C57BL/6 or DR3-deficient mice were transferred into TCRα-deficient mice. Mice were immunized with MOG peptide 24 hr after the transfer, and they were scored by clinical assessment. Clinical assessment of EAE was performed daily according to the following criteria: (0), no disease; (1), tail paralysis; (2), hind-leg weakness; (3), full hind-leg paralysis; (4), complete hind-limb paralysis plus front-limb paraparesis; (5), death. Cells from the spinal cord were isolated with the use of the Neural Tissue Dissociation Kit from Miltenyi Biotec according to the

manufacturer's recommended protocol. Lymph-node and spleen cells from MOG-sensitized animals were isolated with CD4 beads. The cells were re-stimulated in the presence of irradiated T cell-depleted splenocytes as APCs and the indicated concentrations of MOG peptide in 96 well plates. On day 3, the cells were pulsed with ^{3}H-thymidine for 6 hr and then harvested and counted on a scintillation counter.

Ova-Induced Lung Hypersensitivity

On days 0 and 7, mice were sensitized systemically via a 200-μl i.p. injection containing either 100 μg Chicken Ova (Sigma) or PBS emulsified in an equal-volume mixture with alum (Pierce Laboratories, Rockford, IL). For assessment of pulmonary inflammation, mice were challenged with 100 μg Ova or PBS/30 μl inoculum, intratracheally (i.t.) on day 14 and intranasally (i.n.) on day 15. Mice were euthanized 48–72 hr after the final challenge for evaluation of cell infiltration, cellular inflammation in the lung, and cytokine levels in the sera and BAL fluid. For transfer experiments, OT-II or DR3-deficient OT-II CD4 T cells were polarized under Th2 cell conditions for 5 days. The cells were CFSE-labeled, and 2 × 10^{6} cells were transferred into CD45.1 mice. Mice were challenged with 100 μg Ova or PBS/30 μl inoculum administered either i.t. after 24 hr and i.n. after 48 hr or i.t. after 48 hr. Mice were euthanized 24 hr after the final challenge for evaluation of cell infiltration, cellular inflammation in the lung, and cytokine levels in the sera and BAL fluid. BAL fluid was obtained by direct cannulation of the lungs with a 20-gauge intravenous catheter and lavage with 500 μl 1% fetal bovine serum (FBS) in PBS (for cytokine analysis) and 750 μl 1% FBS in PBS (for analysis of cellular infiltration). Samples for cytokine analysis were stored at −80°C. Samples for cellular analysis were prepared as a cytospin (Thermo-Shandon, Pittsburgh, PA) for differential cellular analysis after staining with Kwik-diff (Thermo-Shandon), and a portion was used for determination of total cell counts. Lung histology was scored by a reader, with experimental conditions masked as described previously (McConchie et al., 2006). Inflammatory lung score was scored as follows: (0) normal lung, minor inflammation, no goblet cells; (1) minor perivascular inflammation (PVI)/cuffing (PVC); (2) moderate PVI and peribronchial inflammation (PBI)/cuffing (PBC); (3) increased PVI and PBI, increased goblet cells, smaller airways; (4) severe PVI and PBI, goblet cell infiltration of both small and large airways (practically solid lungs).

Toxoplasma Infection

T. gondii cysts from the ME-49 strain were prepared from the brains of infected C57BL/6 mice. For experimental infections, mice were inoculated i.p. with an average of 20 cysts/animal. At 7 weeks after infection, the number of cysts in the brain of individual infected animals was determined. Spleen cells were harvested and cultured, then stimulated with either anti-CD3 and anti-CD28 or 5 μg/ml of STAg. Supernatants were harvested after 72 hr and analyzed for cytokine production.

Trafficking of T Cells

5 × 10^{6} CD4^{+} T cells from DR3-deficient OT-II or OT-II control mice were transferred into CD45.1 congenic recipients, and the next day mice were challenged with 100 μg Ova protein and 5 μg LPS injected i.p. Three days later, lymphocytes were prepared from the indicated organs and analyzed by flow cytometry.

Cytokine and Immunoglobulin Measurement

Detection of IFN-γ-, IL-4-, and IL-17-producing cells was determined by intracellular cytokine staining with anti IFN-γ-APC, anti IL-4-PE, or anti-IL-17-PE (BD Biosciences), respectively. In brief, cells were stimulated for 5 hr with anti-CD3 and anti-CD28 or phorbol myristate acetate and ionomycin, with monensin added after two hours. Cells were fixed in 3% paraformaldehyde, permeabilized in 0.1% saponin, and analyzed on a FACS Calibur flow cytometer (Becton Dickinson). Cytokine production in cell-culture supernatants was analyzed by Cytometric Bead Array (BD Biosciences). Serum immunoglobulins were measured by ELISA per the manufacturer's instructions (Bethyl Labs), and Ova-specific IgG1 and IgE were measured by IgG1- or IgE-specific ELISA with plates coated with 50 μl of Ova (100 μg/ml).

TL1A Induction in DCs and T Cells

Bone-marrow-derived DCs or splenic CD11c^{+} DCs from C57BL/6 mice and the indicated knockout mice were cultured and stimulated for the indicated time with or without 100 ng/ml of LPS, 20 μg/ml of SEA, or 10 μg/ml of STAg. Stimulation with Ig crosslinking was performed by the coating of plates with 0.5mg/ml of mouse IgG (Jackson Immunoresearch) for 1 hr at 37°C, followed by 50 μg/ml of sheep anti-mouse IgG (Jackson Immunoresearch) for 1 hr at 37°C. Purified T cells were stimulated with 5 μg/ml of anti-CD3 and anti-CD28 for the indicated time.

Measurement of RNA by Quantitative RT-PCR

Total RNA was isolated from cells with the use of TriZOL and the pure link Micro-to-Midi Kit (Invitrogen). Quantitative RT-PCR was performed with the use of an ABI PRISM 7700 sequence-detection system with SuperScript One-Step RT-PCR System (Invitrogen). Predesigned primer/probe sets were from Applied Biosystems, with the exception of TL1A, which was detected with primers designed to recognize full-length TL1A (forward: 5′-CCCCGGAA AAGACTGTATGC-3′; reverse: 5′-GGTGAGTAAACTTGCTGTGGTGAA-3′; probe: 5′-TCGGGCCATAACAGAAGAGAGATCTGAGC-3′). Probes specific for β2-microglobulin or CD3δ were used as internal controls. Each measurement of TL1A was normalized to expression of β2-microglobulin or CD3δ (delta Ct), then measurements of stimulated and unstimulated were compared (delta delta Ct). The inverse log of the delta-delta Ct was then calculated to give the fold change. The level of TL1A in each sample was normalized to the TL1A level of the same control cells.

SUPPLEMENTAL DATA

Supplemental data include five figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/1/79/DC1/.

ACKNOWLEDGMENTS

This research was supported in part by the Intramural Research Programs of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) and the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Françoise Meylan was supported in part by the Swiss National Science Fundation FNRS Project no: PBGEA-104622. Michelle Kinder was supported by the Molecular and Cell Biology Training Grant at the University of Pennsylvania. We would like to thank John O'Shea for critical reading of the manuscript; Bill Paul, Pam Schwartzberg, and Terri Laufer for helpful advice; Hillary Norris, Brittany Wetzel, Satish Madala, Shweta Trivedi, and Agnieszka Boesen for technical assistance; Patricia Zerfas for assistance with immunohistochemistry; Dr. Allen Cheever for analysis of brain sections; and Joseph Woo and the NIAMS animal facility for assistance with animal care.

Received: August 16, 2007
Revised: March 23, 2008
Accepted: April 21, 2008
Published online: June 19, 2008

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