The TNF-family ligand TL1A and its receptor DR3 promote T-cell mediated allergic immunopathology by enhancing differentiation and pathogenicity of IL-9 producing T cells


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Abstract

The TNF family cytokine TL1A (Tnfsf15) costimulates T cells and type 2 innate lymphocytes (ILC2) through its receptor DR3 (Tnfrsf25). DR3-deficient mice have reduced T cell accumulation at the site of inflammation, and reduced ILC2-dependent immune responses in a number of models of autoimmune and allergic diseases. In allergic lung disease models, immunopathology and local Th2 and ILC2 accumulation is reduced in DR3 deficient mice despite normal systemic priming of Th2 responses and generation of T cells secreting IL-13 and IL-4, prompting the question of whether TL1A promotes the development of other T cell subsets that secrete cytokines to drive allergic disease. Here we find that TL1A potently promotes generation of murine T cells producing IL-9 (Th9) by signaling through DR3 in a cell-intrinsic manner. TL1A enhances Th9 differentiation through an IL-2 and STAT5-dependent mechanism, unlike the TNF-family member

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OX40, which promotes Th9 through IL-4 and STAT6. Th9 differentiated in the presence of TL1A are more pathogenic, and endogenous TL1A signaling through DR3 on T cells is required for maximal pathology and IL-9 production in allergic lung inflammation. Taken together, these data identify TL1A-DR3 interactions as a novel pathway that promotes Th9 differentiation and pathogenicity. TL1A may be a potential therapeutic target in diseases dependent on IL-9.

Introduction

Cytokines in the TNF superfamily have important roles in host defense and amplification of both innate and adaptive immune responses. The TNF family cytokine TL1A costimulates T cells and innate lymphocytes through its unique receptor DR3 (Tnfrsf25). TL1A is synthesized in response to TLR ligands and other proinflammatory stimuli and optimizes T cell responses to bacterial pathogens such as salmonella and viral infections (1, 2). TL1A may also be involved in autoimmune disease pathogenesis, as TL1A levels are elevated in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (3-5). Genetic variants in the TL1A locus are associated with human inflammatory bowel disease (6, 7), and a duplication in the DR3 locus has been linked to rheumatoid arthritis (8). TL1A/DR3 interactions are required for maximal pathology in diverse autoimmune disease models including collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE), and multiple forms of induced colitis (9-13). In allergic disease models, TL1A plays an important role in T cell-dependent airway hypersensitivity induced by ovalbumin (10, 14) and in ILC2-driven allergic lung disease triggered by papain exposure (15, 16).

While TL1A has been shown to activate ILC2s and promote IL-13 production in allergic disease (15, 16), the mechanism by which TL1A enhances the adaptive arm of allergic responses remains less clear. IL-9 is an important driver of allergic disease (17-21), and although its production was historically attributed to the Th2 subset of CD4+ T cells, recent work has highlighted another T cell subset, named Th9, as a major producer of IL-9 (22). Th9 can be generated from naïve T cell precursors by activation in the presence of TGFβ and IL-4, with IL-4 acting through Signal Transducer and Activator of Transcription 6 (STAT6) (23-25). Alternative cytokines and signaling molecules such as IL-25, IL-1, TSLP, Jagged-2, calcitonin gene-related peptide and the TNF-family member OX40 ligand (OX40L) have been proposed to enhance Th9 differentiation and/or T-cell production of IL-9 (17, 26-30), while cyclooxygenase-2-derived prostaglandins, programmed cell death ligand 2, and 1,25-dihydroxyvitamin D3 inhibit Th9 polarization (31-33). In addition to murine allergic lung disease, T cell production of IL-9 has been shown to promote T-cell mediated inflammatory eye disease, EAE, colitis, and clearance of intestinal nematode infection (23, 24, 34-38). In the lung, IL-9 acts on mast cells, epithelial cells and smooth muscle cells to promote production of cytokines and chemokines such as IL-8, IL-13 and eotaxin, which enhance recruitment and activation of neutrophils, eosinophils and mast cells in allergic inflammation. IL-9 also induces smooth muscle hyperplasia and goblet cell metaplasia, important factors in the airway remodeling seen in asthma and allergic airway disease (19, 39, 40). Importantly, IL-9 has been shown to induce airway remodeling in an IL-13 dependent manner, placing IL-9 ‘upstream’ of IL-13 in these disease models (37, 41).
We investigated the role of TL1A in promoting IL-9 production in allergic and autoimmune disease, and found that during T cell-dependent induced airway hypersensitivity and the T cell phase of allergic lung disease, total IL-9 and the number of IL-9-producing T cells are reduced in concert with decreased allergic pathology in Tnfrsf25−/− mice, leading to the hypothesis that TL1A may drive Th9 differentiation. Indeed, TL1A specifically and potently enhances the differentiation of IL-9 secreting T cells from naïve T cell precursors. Enhancement of IL-9 secretion depends on cell-intrinsic signaling through DR3, as well as TGFβ. TL1A can act independently of STAT6, but IL-2 signaling through STAT5 is absolutely required for its enhancement of Th9 differentiation. TL1A also acts independently of the ETS family transcription factor PU.1, which has previously been found to be important for Th9 generation (18). In addition to promoting Th9 differentiation, TL1A enhances pathology induced by Th9 in models of experimental eye inflammation and allergic airway disease. DR3-deficient Th9 transferred into a wild-type host are defective in inducing allergic airway inflammation, indicating that endogenous TL1A signaling through DR3 is necessary for Th9 to promote allergic pathology. These results define TL1A as a cytokine that can promote the generation and pathogenicity of IL-9 secreting T cells through a pathway distinct from those previously defined for this T helper subset.

Materials and Methods

Mice

Wild-type C57BL/6 and CD45.1+ mice were obtained from Taconic. Tnfrsf25−/− mice were generated as previously described (42), and were back-crossed to the C57BL/6 background for at least ten generations. This line was subsequently crossed to OT-II Rag-deficient mice. Stat5a/bfl/fl CD4Cre OT-II and Stat5a/bfl/fl OT-II mice were from the NIAMS Taconic mouse colony, and Stat6−/− mice were from Jackson Labs. Sfpi1fl/fl LckCre mice were produced as previously described (18). Mice used for the analysis of ocular inflammation were (FVB/N×B10.BR) F1 hybrids, transgenically expressing either HEL in their eyes (“HEL-Tg”), or HEL-specific TCR by their T cells (“3A9”) (43). T cell specific TRAF6-deficient mice were previously described (44).

T cell differentiation assays

Lymph nodes and spleens were harvested from mice of the appropriate genotypes and cells were passed through a 40 μm strainer. Red blood cells were lysed with Ack lysis buffer, and cells were sorted for CD4+ T cells using the EasySep Mouse CD4+ T Cell Enrichment Kit (Stemcell Technologies), according the manufacturer’s protocol. CD4+ T cells were then stained with anti-mouse CD4 PerCP-Cy5.5, anti-mouse CD44 APC, anti-mouse CD62L PE, and anti-mouse CD25 FITC (eBioscience and BD Biosciences). Naïve CD4+ T cells identified as CD4+, CD44lo, CD62Lhi, and CD25lo were separated by fluorescence-activated cell sorting on a FACSAria Flow Cytometer (BD Biosciences). For certain experiments, cells were CFSE-labeled. Cells were cultured in complete RPMI medium (RPMI with 10% fetal calf serum, 10 mM HEPES, 1 mM sodium pyruvate, 10 U/ml penicillin, 10 U/ml streptomycin, 2 mM glutamine and 0.05 mM β-mercaptoethanol). Cells were plated at 50,000-100,000 cells per well on 96-well plate or 100,000-400,000 cells per well on a 48-well plate. For activation and costimulation, plates were either coated with anti-CD3 (clone
BE0001-1) and anti-CD28 (clone 37.51) or cells were cultured in the presence of T-depleted splenic antigen-presenting cells (APC) (5:1, APC:T cells) and soluble anti-CD3 and anti-CD28. APC were prepared from total mouse splenocytes that were strained, treated with Ack lysis buffer, and depleted of T cells by staining with biotin-conjugated Thy1.1 and using magnetic biotin beads in an AutoMACS Depletes sort (Miltenyi Biotec). APC were then irradiated at 1000 rad to prevent growth. Polarization conditions were as follows: for Th0, 10 μg/mL anti-IFNγ (clone XMG1.2) and 10 μg/mL anti-IL-4 (clone 11B11); for Th1, 20 ng/mL murine IL-12 and 10 μg/mL anti-IL-4 (clone 11B11); for Th2, 20 ng/mL murine IL-4 and 10 μg/mL anti-IFNγ; for Th9, 20 ng/mL murine IL-4 and 5 ng/mL human TGFβ; for Th17, 20 ng/mL murine IL-6, 5 ng/mL human TGFβ, 10 μg/mL anti-IFNγ (clone XMG1.2), 10 μg/mL anti-IL-4 (clone 11B11), and 10 μg/mL anti-IL-2 (clone S4B6); and for iTreg, 100 units/mL human IL-2, 10 ng/mL human TGFβ, 10 μg/mL anti-IL-4 (clone 11B11), and 10 μg/mL anti-IFNγ (clone XMG1.2). In presence of APC, anti-IL-12 was added for Th0, Th2 and iTreg conditions. Additional conditions included the following as indicated: 10 ng/mL TL1A, 10 ng/mL OX40L, 10 μg/mL anti-IL-9 (clone 222622), 10 μg/mL anti-IL-13 (ratIgG1) obtained from Centocor/Johnson and Johnson (Horsham, PA), 10 μg/mL anti-IL-2 (clone S4B6), 10 μg/mL anti-CD25 (clone PC61), 20 ng/mL murine IL-6, or 100 units/mL human IL-2. Cells were cultured for 3 days. For Ova-specific Th9 cells, OT-II Rag-deficient T cells were purified and cultured with T-depleted APC with 10 ng/mL murine IL-4, 2 ng/mL human TGFβ, 0.5 μg/ml anti-CD28 (clone 37.51) 10 μg/ml of anti-IFNγ (clone XMG1.2), and 1 μM ovalbumin in complete IMDM medium for 3 days. To generate HEL-specific TCR transgenic Th9 cells we used the culture system described in detail elsewhere (45).

Surface DR3 was stained with anti-mouse DR3 biotin (R&D Systems) followed by streptavidin-conjugated eFluor 450 (eBioscience). For analysis of cytokine production, cells were restimulated with 10 nM PMA, 1 μM ionomycin, and monensin for 4 hours, harvested and washed for staining. After labeling with surface antibodies, cells were fixed overnight with Cytofix/Cytoperm (BD Biosciences) or FoxP3 Fixation/Permeabilization buffer (eBioscience). Cells were blocked and stained with the following intracellular antibodies as indicated: anti-mouse/rat FoxP3 eFluor 450 (eBioscience), anti-mouse IL-9 APC (BioLegend), anti-mouse IL-17A FITC (eBioscience), anti-mouse IL-13 PE (eBioscience), anti-mouse IFNγ PE (BD Biosciences) and/or anti-mouse IL-4 FITC (eBioscience). Fluorophore-conjugated isotype control antibodies were used to check for non-specific binding. Cells were then washed and analyzed by flow cytometry. For phosphorylated STAT5 staining, cells were fixed in 4% paraformaldehyde, washed in PBS, and permeabilized with methanol overnight at -20 °C. Cells were stained in PBS with 0.5% Triton X-100 and 0.1% bovine serum with anti-phospho-STAT5 PE (BD Biosciences) and anti-CD4 at room temperature in the dark for 60 minutes. Fluorophore-conjugated isotype control antibodies were used to check for non-specific binding. Cells were then washed and analyzed by flow cytometry.

### Induction and quantification of Th9-mediated ocular inflammation

HEL-specific Th9 cells generated in culture, at the indicated numbers were injected via the tail vein into syngeneic mice transgenically expressing HEL in their lens. The recipient eyes
were collected at day 7 (or an alternative indicated time point) and analyzed by histological examination for inflammatory changes, using a scale of 0-9, as previously described (46). To test the effect of local TL1A, mice were intraocularly injected with TL1A (2 μg/eye) or PBS on days 2 and 3 post cell transfer, and eyes were examined for histological changes on day 4. To test TL1A blockade, mice were intraperitoneally injected with 20 mg/kg of the blocking anti-mouse TL1A monoclonal antibody 5.4G6 or hamster Ig control on days -1 and 3 post cell transfer. To test the effect of adding IL-10, recipient mice were treated intraperitoneally with a daily dose of 50 μg/kg (2 experiments) or 100 μg/kg (2 experiments) murine IL-10 starting 3 hours before cell injection until day 6 post cell injection. For measuring proliferation of transferred cells, Th9 cells were labeled with Cell Proliferation Dye eFluor 670, as recommended by the manufacturer (eBioscience). Five million labeled cells were injected into HEL-Tg recipient mice and spleens of these recipients were collected for flow cytometric analysis of the proportion of donor (1G12+) cells, as indicated.

Ovalbumin and Papain Induced Lung Inflammation

For ovalbumin-induced lung inflammation, mice were sensitized systemically on days 0 and 7 via a 200 μl intraperitoneal (i.p.) injection containing either 100 μg Chicken Ova (Sigma) or PBS emulsified in an equal volume mixture with alum (Thermo Scientific). 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 24-72 hours after the final challenge to evaluate cell infiltration, cellular inflammation in the lung, and cytokine levels in the sera and bronchoalveolar lavage (BAL). 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 with 750 μl 1% FBS in PBS (for analysis of cellular infiltration). Samples for cellular analysis were prepared as a cytospin (Thermo-Shandon, Pittsburgh, PA) for differential cellular analysis after staining with HEMA 3 stain set (Fisher Scientific), and a portion was used to determine total cell counts. Lung tissues were fixed in 4% neutral buffered formaldehyde, embedded in paraffin, sectioned, and stained with hematoxilin and eosin (H&E) or periodic acid-Schiff (PAS) stain. Cells were isolated from lungs by incubating lung fragments with 100U collagenase for 1 hour. Cells were stained for surface antigens and intracellular cytokines after stimulation with PMA/ionomycin for 4 hours with LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies) and the following antibodies: TCRβ APC-eFluor 780 (eBioscience), CD45.2 PeCy7 (BioLegend), CD44 AlexaFluor 700 (eBioscience), CD4 V500 (BD Biosciences), IL-9 PE (BioLegend), IL-13 eFluor 660 (eBioscience), IL-17 FITC (eBioscience), IL-10 PerCP-Cy5.5 (eBioscience), IFNγ eFluor 450 (eBioscience). Cells from the BAL were stained using antibodies against CD45, SiglecF, F4/80, Ly6G and CD11b. Eosinophils were identified as CD45+ F4/80+ Ly6G+ CD11b+ SiglecF+. For RNA extraction, lung tissue was stored in Trizol and homogenized with a Precellys 24 (Bertin Laboratories) before chloroform extraction and purification with the PureLink RNA Mini Kit (Life Technologies). mRNA transcripts were measured by Taqman gene expression assays described below. For analysis of the pathogenic potential of Th9 in allergic lung disease, ovalbumin-specific wild-type or *Tnfrsf25*−/−Th9 cells (10⁶ T cells in 200 μl PBS) were adoptively transferred into CD45.1 congenic wild-type recipient mice via tail vein injection. Mice were challenged twenty-four hours after cell transfer i.t. and forty-
eight hours after cell transfer i.n. with 100 μg Ova. Mice were then sacrificed 12 hours after last challenge for further analyses described above.

For papain-induced lung inflammation, mice were anaesthetized with isoflurane and exposed intranasally to 25 μg papain (Calbiochem) in 30 μL PBS on day 0, 3, 6 and 14. 12-16 hours after the last challenge, bronchoalveolar lavage was performed as described above. Lung-isolated cell analyses, RNA extraction and gene expression assays were performed as above.

Lung histology was scored on H&E and Periodic Acid Schiff (PAS) stained sections by a reader with experimental conditions masked, using a scoring system modified from that described previously (47). Perivascular and peri-bronchiolar cuffing (PVC and PBC) were each scored as follows: 0: No visible infiltrate. 1: Patchy infiltrate in <25% of bronchioles or vessels. 2: Patchy infiltrate in <50% of bronchioles or vessels. 3: Widespread infiltrate >50% of bronchioles or vessels with circumferential infiltrates in most bronchioles or vessels. 4: Criteria for score of 3 plus vascular obliteration (for PVC) or bronchiolar plugging (for PBC). Interstitial inflammation was graded from 0-3 depending on the extent of cellular infiltrate into alveoli. Goblet cell hyperplasia was scored for small airways as follows: 0: No visible hyperplasia or mucous production, 1: patchy hyperplasia and/or PAS staining in <25% of bronchioles or vessels, not circumferential, 2: patchy hyperplasia and/or PAS staining of <50% of bronchioles, 3: widespread hyperplasia and >50% PAS staining in most bronchioles, 4: criteria for 3 plus bronchiolar plugging or obliteration. Scores reported were the total score for each lung (0-15).

**Lung immunohistochemistry**

Lungs were perfused with PBS and filled with OCT via the trachea. Lungs were subsequently embedded in OCT and frozen using an iso-pentane bath cooled with dry ice. Sections were cut to 5 microns using a cryostat (Leica CM1850) cooled to -23 °C and mounted on superfrost plus slides (Fisher). All subsequent steps were carried out at room temperature. Sections were dried for at least 1 hour and fixed in acetone for 10 minutes. Sections were blocked with 2.5% (v/v) normal goat serum (Vector laboratories) for 30 minutes and stained with 10 μg/ml Tan 2-2 (Rat anti mouse TL1A, was generated as described previously (9)) in PBS plus 0.05% Tween-20 (Sigma) for 2 hours. Slides were incubated with in 0.3% H₂O₂ in MeOH for 20 minutes to suppress endogenous peroxidases. Sections were incubated with ImmPRESS anti Rat Ig peroxidase (Vector laboratories) for 30 minutes and developed using DAB peroxidase substrate kit (Vector laboratories) for 7 minutes. Tissue was counterstained using Hematoxylin (Vector laboratories) for 2.5 minutes, washed with 2% glacial acetic acid and blued with 0.45% NaOH in 70% ethanol for 30 seconds. Slides were mounted using VectaMount AQ (Vector laboratories) and imaged using a Keyence microscope (Digital Microscopes). Image backgrounds were adjusted to white using Adobe Photoshop.

**Measurement of allergic airway reactivity**

Bronchial reactivity was determined 12h after the last challenge of Ova in the Ova-induced lung inflammation model or of papain in the papain-induced model. Mice were anesthetized
by i.p. administration of ketamine/xylazine mixture (1 ml ketamine [100 mg/ml], 0.5 ml xylazine [20 mg/ml], and 8.5 ml PBS). A 18-gauge blunt-end needle was inserted into the trachea, and the animals then were ventilated mechanically. Baseline measurements were recorded after the aerosol administration of saline, followed by doubling doses of methacholine (6.25–100 mg/ml) using flexiVent (Scireq Scientific Respiratory Equipment).

**Cytokine measurement and analysis**

Supernatants from CD4\(^+\) T cell differentiation cultures were collected at day 3 after activation and analyzed for cytokines by a multiplex, bead-based protein detection assay. Supernatants were probed for 23 different cytokines and chemokines using BioRad's Bio-Plex Pro™ Mouse Cytokine 23-plex Assay according to the manufacturer's protocol. Protein concentrations were detected and calculated using a Bio-Plex 200 instrument and Bio-Plex Manager 6.0 (BioRad). Ratios were calculated for each cytokine produced in the presence of TL1A versus in the absence of TL1A. The natural logarithms of these ratios were hierarchically clustered using uncentered correlation in Cluster 3.0 (Michael Eisen) and visualized in Java TreeView (Alok Saldanha).

For measurement of IL-9 from the supernatant of wild-type and PU.1-deficient T cells, cells were stimulated with plate bound anti-CD3 (2 µg/ml) for 24 hours. Cell-free supernatant was used to detect IL-9 by ELISA using anti-IL-9 capture antibody (D8402E8; BD Biosciences) and biotin-labeled anti-IL-9 detection antibody (D9302C12; Biolegend). For measurement of IL-9 and IL-10 in HEL-specific T cell cultures, supernatant cytokine concentrations were measured with ELISA kits from RayBiotech and R&D Systems, respectively.

For cytokine measurements in lung homogenates, the middle right lung lobes were snap frozen in liquid nitrogen and stored at -80 until analysis. The frozen lungs were weighed and immediately transferred to tubes containing 500 uL Tissue Protein Extraction Reagent (ThermoScientific) with 5 uL HALT Protease Inhibitor Cocktail (ThermoScientific, 100X). Lung tissues were homogenized using a Precellys® instrument, and then centrifuged at 9,000 \(\times\) g for 10 minutes at 4 degrees. Supernatants were transferred to clean microcentrifuge tubes. Total protein concentrations in the lung tissue homogenates were determined using a BCA kit. Cytokines were measured using the MAGPIX ® multiplexing system with BioPlex reagents (BioRad).

**Retroviral transduction**

Naïve CD4\(^+\) T cells were activated with plate-bound anti-CD3, anti-CD28 and anti-IFN\(\gamma\) for 24 hours. The activated cells were transduced with supernatants containing hNGFR retrovirus or hNGFR-caSTAT5 retrovirus in the presence of polybrene (8 µg/ml) by centrifuge at 2500 rpm for 90 minutes at 30°C. The same procedure of transduction was repeated 24 hours later and differentiation cytokines were added for 2 days. Flow cytometry analysis gated live, NGFR+ cells before examining cytokine production.

**Chromatin Immunoprecipitation (ChIP)**

ChIP for STAT5 binding was performed as described previously (48). Naïve CD4\(^+\) T cells were activated and polarized for 3 days followed by cross-linking for 8 minutes with 1%...
formaldehyde. The cells were harvested and lysed by sonication. After pre-clearing with protein A agarose beads (Upstate, VA), cell lysates were immunoprecipitated with anti-STAT5A/STAT5B antibody (R&D Systems) overnight at 4°C. After washing and elution, crosslinks were reversed at 65°C for 4 hours. The eluted DNA was purified, and samples were analyzed by quantitative-PCR with customer-designed primers and probes (Supplemental Table 1) using a 7500 real time PCR system (both Applied Biosystems, CA). The Ct value for each sample was normalized to corresponding input value. ChIP for PU.1 and IRF4 binding was performed as previously described (25).

Gene expression analysis
Where indicated, mRNA levels were measured by Taqman Gene Expression Assays (Life Technologies): IL-9 (Mm00434305_m1), IL-13 (Mm00434204_m1), IL-2 (Mm00434256_m1), IL-10 (Mm00439614_m1), IRF4 (Mm00516431_m1), B2M (Mm00437762_m1), Tbx21 (Mm00450960_m1), Gata3 (Mm00484683_m1), Rorc (Mm01261022_m1). Cytokine RNA expression measurements were made using the iTaq Universal Probes One-Step Kit (BioRad) on a CFX96 Real-Time PCR Detection System (BioRad). For transcription factor expression measurements, RNA was first converted to cDNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) and then qPCR reactions using Taqman Gene Expression Master Mix (Life Technologies) were run on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). All reactions were run in triplicate. For low-expressed genes, RNA input was increased until detectable and consistent Ct values were obtained across technical replicates. Several PBS-treated mice expressed no detectable IL-9 in the lung even with increased RNA input, and these were excluded from analysis. Replicate Ct values were normalized to replicate reference gene (B2M) Ct values (deltaCt), and fold-changes were calculated with respect to the indicated reference sample (2 raised to the power of delta-deltaCt).

Results
DR3 is required for optimal production of IL-9 and IL-13 in allergic airway disease
DR3 knockout (Tnfrsf25<sup>-/-</sup>) mice or animals treated with blocking anti-TL1A antibodies are resistant to allergic disease induced by sensitization and airway challenge with ovalbumin (Ova), and less IL-13 mRNA is detected in the lung (10, 14). We examined the kinetics of IL-9 and IL-13 production in the lung of wild-type (WT) and Tnfrsf25<sup>-/-</sup> mice in this T-cell-dependent model (Figure 1A). We found that in WT mice IL-9 mRNA was elevated on day 16, one day after the last challenge with Ova, whereas IL-13 production was higher at day 18. Strikingly, in Tnfrsf25<sup>-/-</sup> mice, IL-9 mRNA was not induced at either time point, and IL-13 was significantly reduced at day 18 (Figure 1B).

To determine whether T cells infiltrating the lung also depend on DR3 for efficient IL-9 and IL-13 secretion, we isolated cells from the lung at day 16 and determined the frequency of IL-9 and IL-13 producing T cells after brief restimulation in vitro. Although total lung IL-13 mRNA is very low at this time-point, IL-13 detection by flow cytometry in individual T cells enables examination of differences in this small population of infiltrating cells. As shown in Figure 1C, numbers of IL-9 and IL-13 secreting cells increased approximately 10-fold in
Ova-sensitized WT mice compared to PBS-sensitized controls, whereas no such changes in cells secreting IL-9 nor IL-13 could be seen in Tnfrsf25−/− mice. This was not due to a general failure of the T response in the lung, because total numbers of CD4+CD44hi cells were still significantly increased by Ova priming in Tnfrsf25−/− mice. Despite correlated changes in IL-9- and IL-13-producing T cells, no double-producers were observed, and CD4+CD44hi cells did not secrete IL-10, IL-17, or IFNγ (Supplemental Figure 1A).

Histological examination revealed reduced goblet cell hyperplasia and cellular infiltrates of Tnfrsf25−/− lungs compared to those from WT Ova-sensitized mice at day 16 (Figure 1D). Lung pathology in Ova-challenged Tnfrsf25−/− mice was significantly reduced compared to that in WT mice at day 16 (Figure 1D, lower panel), similar to what we previously observed at day 18 (10). Measurements of airway resistance in response to methacholine challenge revealed less airway hyperreactivity (AHR) in Tnfrsf25−/− mice challenged with Ova as compared to WT mice (Figure 1E). Bronchoalveolar lavage (BAL) of Tnfrsf25−/− mice challenged with Ova also showed reduced numbers of eosinophils and neutrophils at day 16 (Figure 1F), and as expected, IL-5 protein from whole lung homogenates paralleled eosinophil reduction (Supplemental Figure 1B). These data suggest that TL1A-DR3 interactions are important for IL-9 and IL-13 production by T cells early in the lung hypersensitivity response, which may shape the ensuing allergic pathology.

To test whether DR3 is required to support Th9 development and lung inflammation in the setting of a local allergic response, we exposed WT or Tnfrsf25−/− mice to inhaled papain, an environmental protease known to induce pulmonary allergic responses and induce IL-9 along with other Th2 cytokines (Figure 2A). We chose to analyze responses after two weeks of exposure to papain, the time at which T cells play a role in disease (49-51). As with mice sensitized and challenged with Ova, the BAL fluid of Tnfrsf25−/− mice sensitized to papain contained reduced numbers of eosinophils and neutrophils compared to WT mice (Figure 2B), and lung IL-5 protein expression was again reduced (Supplemental Figure 2A). Of note, immunohistochemical staining revealed that papain induced the expression of TL1A on cells localized near blood vessels (Figure 2C). WT mice treated with papain developed severe immunopathology with goblet cell hyperplasia and cellular infiltration in the perivascular, interstitial and peribronchial areas, while Tnfrsf25−/− mice were substantially less affected (Figure 2D). Similar to Ova-induced lung disease, papain-exposed Tnfrsf25−/− mice showed less AHR than WT in response to methacholine challenge (Figure 2E).

Transcription of IL-9 and IL-13 was highly induced after 2 weeks of papain exposure, and DR3-deficient mice were defective in induction of both IL-9 and IL-13 mRNA, with the reduction in IL-9 even more pronounced than that in IL-13 (Figure 2F). Because ILC2s have been identified as the main producers of papain-induced IL-9 in the early allergic response (51), we restimulated total lung cells with PMA and ionomycin and analyzed protein production by flow cytometry to specifically determine whether T cell production of allergic cytokines was dependent on DR3. We found that numbers of T cells expressing either IL-9 or IL-13 were strikingly reduced in DR3 deficient mice (Figure 2G). Similar to ovalbumin-induced lung disease, there were no IL-9 and IL-13 double-producing T cells, and we found very little induction of IL-10 and IFNγ in response to papain. However, in contrast to the ovalbumin model, papain exposure did induce IL-17-producing effector T cells separate from IL-9-producing cells, which were also reduced in antigen-challenged Tnfrsf25−/− mice.
These results show that DR3 also governs the production of cytokine secreting T cells and disease pathology after exposure to an inhaled allergen.

**TL1A promotes Th9 differentiation in the presence of TGFβ and either IL-4 or IL-2**

TL1A may exert the effects we observed in the lung through enhancing differentiation of naïve T cells or promoting proliferation or survival of activated T cells. To investigate the effects of TL1A on T cell differentiation, we activated T cells under conditions optimized for differentiation into various T cell subsets in the presence or absence of TL1A. We previously found that DR3 was not required for generation of Th1, Th2 or Th17 cells, but that TL1A-DR3 signaling suppressed generation of FoxP3-expressing iTreg (10, 13). Consistent with previous reports (52), TL1A increases the percent of IFNγ-producing T cells in Th1 differentiation conditions to a moderate extent, but we do not see any consistent effects on Th2 or Th17 differentiation (Supplemental Figure 3A). Expression of lineage-defining transcription factors generally paralleled flow cytometry measurements of signature cytokines (Supplemental Figure 3B). When T cells were cultured under iTreg-inducing conditions in the presence of IL-2 and TGFβ, we found that in addition to reducing the percentage of FoxP3-expressing cells, TL1A promoted the production of IL-9 (Figure 3A). The effect was particularly strong in the presence of antigen presenting cells (APC), which enhanced T cell costimulation as evidenced by increased secretion of many cytokines and chemokines (Supplemental Figure 3C). Suppression of FoxP3 and induction of IL-9 was dependent on DR3, as Tnfrsf25−/− T cells were unaffected by TL1A treatment (Figure 3A). TL1A did not induce IL-9 production in T cells cultured for 3 days under non-polarizing Th0, Th1 or Th2 conditions, and TL1A did not enhance the production of cells expressing IL-4 or IL-13 under Th2 conditions (Supplemental Figure 3A), suggesting that the TGFβ and IL-2 included in iTreg polarization conditions are important for the ability of TL1A to induce IL-9 production. Interestingly, under Th17 conditions (TGFβ, IL-6 and blocking antibodies to alternative lineages), TL1A-induced IL-9 production was suppressed compared with that of iTreg cultures, which differ only in their lack of IL-6 and presence of IL-2 (1.6 % vs. 17.5 % in Figure 3B), while IL-17 production remained largely unaffected. This suggests that IL-6-derived signals antagonize the ability of TL1A to promote Th9 differentiation, or that IL-2 signaling enhances it.

In the presence of IL-4 and TGFβ, conditions previously found to direct T cells to become IL-9-producing effector cells (23, 24, 53), TL1A significantly enhanced the development of IL-9-producing cells, both in the presence and absence of APC. In the presence of APC, some cells producing both IL-13 and IL-9 were induced by TL1A (Figure 3C). As with iTreg conditions, the enhancement of IL-9 production by TL1A was completely dependent on DR3. Although DR3 deficient T cells differentiated normally into Th9, endogenous TL1A produced under these culture conditions may not be sufficient to influence Th9 differentiation, as the bulk of TL1A is produced by APC induced by inflammatory stimuli such as TLR ligands, TNF and IL-1b (54). Titration of TGFβ showed that TL1A could enhance Th9 differentiation across a wide range of concentrations of TGFβ above 0.1 ng/ml, and TL1A lowered the amount of TGFβ necessary to induce Th9 differentiation by approximately 10-fold (Figure 3D). TL1A was extremely potent at enhancing Th9 differentiation, doubling the percentage of IL-9-producing T cells at concentrations as low as
0.1 ng/ml, and sharply augmenting IL-9 production with increasing concentration before plateauing between 1 and 10 ng/ml (Figure 3E).

T cell subset-specific responses to TL1A may reflect differential expression of the TL1A receptor DR3. To test this possibility we measured DR3 surface expression under various polarization conditions (Figure 3F). DR3 expression was upregulated under all conditions with TGFβ (Figure 3F), indicating that TGFβ signaling enhances cellular responsiveness to TL1A. However, DR3 expression on Th9-polarized cells decreased with addition of TL1A (Figure 3G), suggesting downregulation of the receptor by its ligand. These results show that TL1A potently promotes differentiation of IL-9-producing T cells in a TGFβ-dependent manner but does not induce a positive feedback loop by inducing DR3 expression.

Since TL1A can costimulate T cell proliferation, the increase in IL-9-producing T cells we observed might be due to enhanced proliferative responses. To explore this possibility, we examined IL-9 production in cultures of CFSE-labeled T cells to simultaneously track cell division and IL-9 production. Addition of exogenous TL1A only slightly enhanced proliferation in the absence of APC (data not shown), while no difference was detected in the presence of APC (Figure 3H). This is consistent with previous data showing that TL1A affects proliferation mainly under suboptimal stimulatory conditions (10). TL1A enhanced IL-9 production in all cells that had divided at least once, increasing IL-9 production in the first division by a factor of eight whether APC were absent or present (data not shown, and Figure 3H). TL1A enhanced the percentage of IL-9 producing cells at least 10-fold in cells that had divided twice. These data show that the TL1A primarily induces Th9 differentiation and IL-9 production as opposed to increasing T cell proliferation.

TL1A can co-stimulate cytokine production and may therefore promote Th9 differentiation by inducing production of other cytokines that act in an autocrine manner. We measured cytokines in the supernatants of T cells activated in the presence or absence of TL1A under a number of polarization conditions by multiplex cytokine detection assays. In addition to IL-9, TL1A enhanced the production of IL-2, IL-3, IL-10, IL-13, and GM-CSF by purified T cells activated under Th9 conditions (Figure 3I, top panel). In the presence of APC, IL-5 and IL-6 were also upregulated by TL1A (Figure 3I, bottom panel). Unsupervised clustering of the TL1A-induced changes in cytokines secreted by various T helper subset differentiation cultures showed that while TL1A may be generally considered a costimulator, its effect depends on the polarization culture conditions. For example, IL-9 and IL-13 upregulation appeared strongest in Th9 and iTreg conditions in purified T cell culture. It is also interesting to note that the cytokines induced by TL1A under Th2 and Th9 conditions were closely correlated, which may reflect a common differentiation pathway of these two subsets (24).

**Induction of Th9 by TL1A depends on IL-2 and STAT5**

To determine which cytokines may be responsible for enhanced Th9 differentiation, we considered those cytokines upregulated by TL1A that activate JAK/STAT signaling, which is well known to program T cell differentiation. IL-2, IL-3, IL-5, IL-6, IL-9, and GM-CSF fit these criteria and are upregulated by TL1A in both iTreg and Th9 conditions. To test whether IL-9 itself might positively influence Th9 differentiation, we added an IL-9
blocking antibody to T cells cultured under Th9 conditions in the presence and absence of TL1A. As shown in Figure 4A, blocking IL-9 did not reduce the frequency of IL-9-producing cells (Figure 4A, left panel). As in Th17 differentiation cultures containing IL-6 (Figure 3B), addition of IL-6 suppressed the ability of TL1A to induce Th9 (Figure 4A, middle panel), whereas blocking IL-6 antibodies enhanced Th9 differentiation in the presence of TL1A (Figure 4A, left panel). Addition of exogenous IL-2 did not increase Th9 producing cells over the TGFβ and IL-4 condition (Figure 4A, middle panel). However, a combination of anti-IL-2 and anti-CD25 antibodies, which completely blocks IL-2 signaling, dramatically suppressed Th9 differentiation in T cell activation cultures containing TGFβ and IL-4 and potently blocked the ability of TL1A to enhance Th9 differentiation (Figure 4A, right panel). Thus IL-2 appears to be a key autocrine factor in Th9 differentiation and its enhancement by TL1A, while IL-6 appears to antagonize this process, possibly though competition between IL-2-induced STAT5 and IL-6-induced STAT3 (48).

To probe the relevant signaling pathways downstream of candidate cytokines induced by TL1A that might aid Th9 polarization, we used T cells deficient in the relevant STAT transcription factors that mediate their action. STAT6 is responsible for IL-4 and IL-13 signal transduction and is required for Th9 polarization induced by IL-4 (23-25). As expected, Stat6-deficient T cells were defective in IL-9 production under Th9 conditions, but, importantly, TL1A still enhanced numbers of IL-9-producing T cells by 10-fold (Figure 4B). Under iTreg conditions, deviation towards Th9 differentiation by TL1A was completely independent of STAT6 (Figure 4B). This shows that although signaling through STAT6 is required for conventional Th9 differentiation mediated by IL-4 and TGFβ, STAT6 is not required for the enhancement of Th9 differentiation mediated by TL1A.

As a major signal transducer for IL-2, STAT5 is a good candidate for mediating the effects of TL1A. To determine the role of STAT5 in the enhancement of Th9 differentiation by TL1A, we activated naïve CD4⁺ T cells with conditional deletion of the STAT5A/B locus (Stat5CD4⁻/⁻) or control T cells (Stat5fl/fl) under Th9 polarization conditions with or without TL1A. Due to the requirement for STAT5 in T cell development (55), both STAT5-deficient and control T cells carried the OT-II transgenic TCR, but T cells were activated polyclonally. Under Th9 conditions, STAT5-deficient T cells were unable to differentiate into IL-9-producing cells, and importantly, IL-9 production by STAT5 deficient T cells could not be rescued by the addition of TL1A (Figure 4C). Only IL-13 production is increased by TL1A in the absence of STAT5 signaling (Figure 4C). To determine whether STAT5 is sufficient to drive Th9 development, we activated naïve T cells transduced with a retrovirus encoding a constitutively active STAT5 protein. These cells efficiently differentiated into IL-9-producing cells in the absence of TL1A or other T cell polarizing cytokines (Figure 4D), indicating that activated STAT5 is sufficient to drive Th9 differentiation. T cells expressing activated STAT5 differentiated into Th9 even in the presence of IL-6, suggesting that activated STAT5 can overcome the inhibitory effects of IL-6 acting through STAT3 on Th9 differentiation. Taken together, these data confirm that STAT5 is necessary and sufficient for Th9 differentiation, and show that TL1A, acting through autocrine IL-2 and STAT5, mediates a distinct pathway from IL-4 and TGFβ for inducing Th9 from naïve T cell precursors.
To determine whether DR3 plays a cell-intrinsic role in Th9 differentiation beyond promoting autocrine IL-2 production, we mixed CD45 congenic WT naïve CD4$^+$ T cells with DR3-deficient naïve CD4$^+$ T cells, and activated them together under Th9 and iTreg conditions. Only wild-type cells responded to TL1A with an increase in IL-9 production under either iTreg or Th9 conditions (Figure 5A), indicating that T cells must receive a signal through DR3 in addition to IL-2 for optimal Th9 differentiation. These data suggested that DR3 signaling may potentiate the action of other transcription factors on the IL-9 promoter. PU.1 (Sfpi1), an ETS-family transcription factor that binds to the IL-9 promoter, enhances the generation of Th9 both in vitro and in vivo (18). As previously described, activating PU.1-deficient T cells under Th9 conditions resulted in a lower percentage of IL-9-producing cells and reduced IL-9 in the culture supernatant. However, TL1A enhanced IL-9 production in both WT and PU.1-deficient T cells to a similar degree (Figure 5B).

Chromatin immunoprecipitation (ChIP) experiments also showed that TL1A did not significantly enhance PU.1 binding to the IL-9 promoter (Figure 5C). IRF4, which is induced by STAT6 and coordinately binds the IL-9 promoter with SMAD2/3 (25, 56), has also been identified as a key transcription factor in Th9 differentiation (57). ChIP experiments revealed a trend toward increased binding of IRF4 to the IL-9 promoter in the presence of TL1A (Figure 5D), but addition of TL1A did not increase IRF4 expression at the mRNA level (data not shown). Along with the data from STAT6-deficient T cells (Figure 4B), these results suggest that TL1A makes the IL-9 promoter more accessible to IRF4 but its effect is not directly through the STAT6-IRF4 pathway. Alternatively, we reasoned that DR3 signaling may enhance the ability of STAT5 to transactivate the IL-9 promoter. In support of this possibility, T cells activated under Th9 conditions in the presence of TL1A had higher baseline levels of phosphorylated STAT5 and increased STAT5 binding to the IL-9 promoter as assayed by ChIP (Figure 5E). These data support a role for TL1A, acting through DR3, in enhancing IL-2 signaling through STAT5 and increasing STAT5 promoter binding to promote differentiation of T cells capable of producing IL-9.

OX40L, another member of the TNF superfamily, was recently reported to enhance Th9 differentiation through its receptor OX40, signaling via the non-canonical NF-κB pathway and TNF-receptor associated factor 6 (TRAF6) (26). To determine whether TL1A may induce Th9 through a TRAF6-dependent pathway, we activated naïve Traf6$^{-/-}$ T cells with TGFβ and IL-4. Unlike OX40L, we found that TL1A was able to enhance Th9 generation with similar efficiency in WT and Traf6$^{-/-}$ T cells (Figure 5F). Th9 differentiation by OX40L was dependent on IL-4 acting through STAT6, as OX40L only slightly increased the percentage of IL-9-producing cells differentiating from STAT6-deficient naïve T cell precursors (Figure 5G). These data show that TL1A mediates Th9 differentiation through mechanisms that are distinct from those of OX40L with respect to STAT6 and non-canonical NF-κB signaling through TRAF6.

**TL1A enhances the pathogenicity of antigen-specific Th9 in ocular and allergic lung inflammatory disease**

To determine whether TL1A enhances the pathogenicity of antigen-specific Th9 cells, we turned to a model system where T cells specific for hen egg lysozyme (HEL) trigger ocular inflammation when transferred into transgenic mice expressing HEL in the eye. HEL-
specific T cells differentiated into Th1, Th17 and Th9 can all trigger ocular pathology when transferred into HEL-expressing recipients, and pathogenicity of Th9 correlates with their ability to produce IL-9 (45). We activated HEL-specific TCR transgenic T cells with antigen and APC under Th9 conditions in the presence or absence of TL1A. TL1A enhanced the differentiation of HEL-specific Th9 to an even greater extent than in polyclonal T cell cultures, increasing the percentage of T cells capable of producing IL-9 to more than 90% after 3 days of activation, the peak of IL-9 production, compared with 40% without TL1A (Figure 6A). TL1A also increased the percentage of IL-9-producing T cells after 4 days of activation (30% vs 8%), but after 6 days of activation, the percentage of IL-9-producing T cells fell to less than 3% with or without TL1A. IL-9 production was enhanced by TL1A at the RNA level as well, with a two-fold enhancement of IL-9 RNA at the peak of expression at day 3. IL-9 RNA expression steeply declined at day 4 with or without TL1A (Figure 6B). TL1A also greatly enhanced the amount of IL-9 in culture supernatants on days 3 and 4, with a ten-fold enhancement of IL-9 in the supernatant (Figure 6C). Interestingly, a significant fraction of T cells produced IL-10 after 6 days of culture, and TL1A almost completely extinguished IL-10 production by these ‘ex-Th9’ cells (Figure 6A). Measurement of IL-10 on days 3 and 4 by intracellular staining, in culture supernatants, and in RNA showed a smaller reduction with addition of TL1A, demonstrating that downregulation of IL-10 by TL1A occurred later after activation (Figures 6A-C).

To assess the effects of TL1A on the pathogenic potential of Th9 cells, HEL-specific T cells differentiated under Th9 conditions for 3 or 4 days in the presence or absence of TL1A were transferred into mice expressing HEL in the eye. Examination of ocular pathology 7 days after transfer of Th9 cells revealed that TL1A added during T cell differentiation enhanced the ability of HEL-specific cells to induce ocular inflammation, even when the increased efficiency of Th9 generation in the presence of TL1A was taken into account by transferring proportionally fewer cells (Figure 6D). Histological changes in the recipients of Th9 cells included accumulation of inflammatory cells at the optic nerve head and limbus, as well as in the anterior chamber and throughout the vitreous. These changes were markedly more severe in the recipients of Th9 cells generated with the addition of TL1A (Figure 6E, left panels). Higher magnification showed the limbus and ciliary body area, with higher numbers of invading cells as compared with the control Th9 eye section (Figure 6E, right panels). In addition to intra-ocular changes, transferred Th9 cells uniquely invaded the conjunctiva, evoking changes reminiscent of allergic conjunctivitis. These changes were remarkably more severe in recipients of TL1A-stimulated donor cells (Figure 6F).

To determine whether TL1A influences the proliferation of HEL-specific Th9 prior to their invasion of the recipient mouse eye, we monitored transferred cells on days 1, 2, 3, 4 and 7 post transfer in the spleens of recipient mice, where Th9 initially migrate and proliferate (45). We enumerated the percentage of TCR transgenic T cells in the CD4+ T cell pool using the anti-clonotypic monoclonal antibody 1G12. HEL-specific transgenic T cells exposed to TL1A during Th9 differentiation were present in greater numbers than control Th9 after transfer to HEL-expressing recipients (Figure 6G). Th9 cells generated in the presence of TL1A proliferated more rapidly than control Th9 cells (Figure 6H), demonstrating that while TL1A does not greatly enhance proliferation of Th9 during differentiation (as shown in
Figure 3H), it does prime cells for more rapid expansion in vivo, which may contribute to their increased pathogenicity.

To determine whether TL1A can also enhance the pathogenicity of Th9 at the site of inflammation we injected TL1A or PBS into the eyes of HEL-expressing recipient mice 2 and 3 days after transfer of HEL-specific Th9. Intra-ocular injection of TL1A into Th9 recipients slightly increased inflammation on day 4 post-transfer compared to those receiving PBS (Figure 6I). It is important to note that the ocular injection itself induced some inflammation and may have obscured the effects of TL1A, as evidenced by increased basal histological scores seen with Th9 transferred cells followed by intra-ocular PBS injection. Injection of TL1A alone induced very little ocular pathology. TL1A and DR3 have previously been found upregulated in experimental autoimmune uveitis, suggesting an endogenous role for this signaling pathway in ocular inflammation (58). To determine whether endogenous TL1A/DR3 interactions are important in vivo to enhance ocular pathology mediated by Th9, we treated recipient HEL-transgenic mice receiving HEL-specific Th9 cells with a blocking anti-TL1A antibody (13). Anti-TL1A significantly reduced ocular inflammation compared to control immunoglobulin (Figure 6J), with reduced cellular infiltration (Figure 6K). Anti-TL1A reduced the number of donor HEL-specific T cells in the eye (1.0% with isotype versus 0.6% with anti-TL1A), while the percentages of donor HEL-specific T cells in the spleen remained unchanged. Percentages of host cells in both organs only slightly decreased with anti-TL1A treatment (data not shown). We also examined the possibility that the effect of TL1A is related to the suppression of IL-10 production that we observed in vitro, but injection of IL-10 did not reduce ocular pathology induced by Th9 differentiated in the presence of TL1A (data not shown). Thus, TL1A appears to enhance Th9 pathogenicity independently of reducing IL-10 production. Taken together, these data show that in addition to improving the efficiency of Th9 differentiation, endogenously produced TL1A can increase pathology mediated by antigen-specific Th9.

To determine whether endogenous TL1A can enhance the pathogenicity of Th9 cells in Ova-induced lung inflammation, we transferred WT or Tnfrsf25−/− OT-II Ova-specific T cells activated under Th9 conditions into congenic hosts. Recipient mice were then given two inhaled challenges of Ova and responses were measured 12 hours after the last challenge, the time point at which we observed maximal IL-9 expression (Figure 7A and 7B). At this time point, neutrophils predominated in the BAL, and there was a trend towards lower neutrophil counts in recipients of Tnfrsf25−/− OT-II cells (Figure 7C). Histological analysis revealed a significant reduction in pathology in recipients of Tnfrsf25−/− OT-II Th9 cells after challenge with Ova compared to mice receiving WT OT-II Th9 cells, including reduced perivascular and peribronchial infiltrates (Figure 7D). IL-9, IL-13 and IL-2 mRNA were significantly reduced in Ova-treated recipients of Tnfrsf25−/− OT-II T cells compared with mice that received WT OT-II T cells (Figure 7E). Expansion of donor OT-II Th9 was observed locally in the lung (Figure 7F) and the mediastinal lymph nodes but not in the spleen after Ova challenge (data not shown). Expansion of the total number of donor OT-II cells, as well as the number of OT-II cells producing IL-9, was significantly reduced when the cells lacked DR3, along with reduction in a much smaller number of OT-II cells producing IL-13 (Figure 7F). The mean fluorescence of IL-9 upon restimulation and intracellular cytokine staining was lower in Tnfrsf25−/− T cells (Figure 7F), suggesting that
TL1A may potentiate their IL-9-producing phenotype. Because only T cells lacked DR3 in these experiments, these results demonstrate a requirement for interaction between endogenous TL1A and DR3 expressed on Th9 cells to promote the expansion and effector function of Th9 and immunopathology in this model of allergic lung disease.

Discussion

The importance of TL1A in animal models of allergic disease has been well characterized (10, 11, 14), but the mechanism by which TL1A promotes T cell-mediated immunopathology in allergic disease has remained elusive, as systemic Th2 polarization is unaffected in DR3-deficient mice despite reduced lung pathology. Here we find that TL1A acts on CD4+ T cells both to inhibit iTreg differentiation and to promote Th9 differentiation. The effects of TL1A are independent of IL-4 and STAT6, but require IL-2 acting through STAT5, as well as TGFβ signaling. TL1A also enhances the pathogenic potential of Th9, and TL1A-DR3 interactions in vivo are required for allergic lung inflammation and the accumulation of IL-9 secreting cells in the lung early in the inflammatory response. These findings provide a more specific mechanism by which TL1A can promote T cell mediated immunopathology and identify an alternate pathway for generation of IL-9-secreting T cells that is more efficient than TGFβ and IL-4 alone and generates higher numbers of pathogenic T cells.

TL1A/DR3 interactions are not required for generation of Th1 or Th2 cells in vitro or during primary polarized T cell responses, and although TL1A has been shown to promote or inhibit Th17 differentiation depending on culture conditions, its interaction with DR3 is not necessary for Th17 formation (10, 11, 59). We here show that TL1A can divert T cells in iTreg differentiation cultures to become producers of IL-9 and enhance differentiation of Th9 in the presence of TGFβ and IL-4, conditions previously shown to bias naïve T cells towards IL-9 production (23, 24). However, TL1A can induce Th9 in the complete absence of STAT6 signaling, indicating that the action of TL1A is mechanistically distinct from Th9 differentiation induced by TGFβ and IL-4, which depends on STAT6 (24). Other cytokines, such as the IL-17 family member IL-25 (60) and the IL-1 family members IL-1β, IL-18, and IL-33 (28), have also been shown to enhance T cell production of IL-9, but unlike these cytokines, TL1A also enhanced differentiation of IL-9 producing cells from naïve precursors. More recently the TNF-family member OX40L was shown to limit iTreg differentiation and bias T cell differentiation towards IL-9 production (26). Like OX40L, TL1A-induced Th9 differentiation does not require the transcription factor PU.1, which independently enhances the generation of Th9 (18). However, unlike OX40L, TL1A promotion of Th9 differentiation is independent of IL-4 and STAT6.

Rather than IL-4, we have found that IL-2 signaling through STAT5 is critical for the ability of TL1A to enhance Th9 differentiation. IL-2 has been previously identified as an enhancer of IL-9 production by T cells (53, 61). Mechanistically, TL1A enhances STAT5 activation and binding to the IL-9 promoter. Yao et al. have recently observed that TSLP can enhance IL-9 production by directly activating STAT5 in T cells (17), and STAT5 was necessary for increased IL-9 production by mice deficient in the SOCS protein CIS (62). The fact that TL1A was unable to induce IL-9 production under conditions with blocked IL-2 signaling
suggests that DR3 signaling alone is not sufficient to promote IL-9. Rather, IL-2-STAT5 signaling is required in addition to the signal mediated by DR3 on the same cell to promote Th9 differentiation. TL1A enhancement of Th9 differentiation in the presence of IL-2 (iTreg cultures) was most efficient when T cells were activated in the presence of APC, suggesting that APC-derived costimulatory signals or cytokines are likely required for optimal effects of TL1A. As suggested by the global increase in cytokine production in the presence of APC (Supplemental Figure 3C), it is possible that the enhanced effect of TL1A is due to extra costimulatory capacity from B cells, macrophages, and dendritic cells present in these cultures. Future studies of separated APC subsets may help to identify specific cell types and factors responsible for this effect.

DR3 signals similarly to TNFR1 through TRADD, RIP, and TRAF2, ultimately resulting in activation of MAP-kinases and NF-κB, or alternatively through FADD and Caspase-8 to activate apoptosis (63-67). TL1A signaling through DR3 is unlikely to directly induce STAT5 activation, but may enhance STAT5 activity indirectly. MEK and ERK kinases have been found to phosphorylate STAT5 (68, 69), and NF-kB and STAT proteins can bind coordinately to the promoters of a number of genes, including TLR2 (70), FcεRII (71), and NOS2 (72). The IL-9 promoter contains binding sites for NF-κB and STAT5 making such transcriptional coordination possible (17, 73). Xiao et al. (26) have suggested that signaling through TRAF6 and the non-canonical NF-κB pathway is responsible for the ability of OX40 to bias T cell differentiation towards Th9. Interestingly, the same mechanism does not apply for DR3, suggesting that different TNF family members have unique ways to modulate T cell differentiation.

In addition to enhancing the efficiency of Th9 differentiation, TL1A also enhances the pathogenicity of Th9 in animal models of ocular inflammation and allergic lung disease. In ocular inflammation, antigen-specific Th9 generated in the presence of TL1A induced significantly more pathology than conventional Th9, even when the increased percentage of IL-9-producing cells differentiated in the presence of TL1A was taken into account. In allergic lung disease, antigen-specific Th9 cells capable of DR3 signaling exhibited significantly greater pathogenicity than DR3-deficient Th9. Detection of TL1A-expressing cells in the perivascular region of inflamed lungs, as well as the reduction of ocular pathology seen after local injection of anti-TL1A, supports the notion that endogenous TL1A is likely the trigger for enhanced expansion of antigen-specific T cells which we observed in these models. We and others have found that TL1A costimulation of ILC2s plays a role in allergic lung disease (15, 16), and it has recently been shown that ILC2s can influence T cell responses in allergic diseases (49, 74). Thus, defective ILC2 activity in DR3-deficient mice may play a role in the reduced T cell accumulation and allergic response in the T-cell dependent allergic inflammation models that we studied. However, our results with transfer of DR3-deficient T cells show that TL1A-DR3 signaling on T cells plays an independent role in amplifying allergic lung disease mediated by Th9, as shown in this work, and as shown previously for Th2 cells (10). As IL-2 produced by T cells can promote ILC2 expansion (51, 74) and IL-9 can promote ILC2 survival (75), the reduced IL-2 and IL-9 production seen in the lungs of recipients of DR3-deficient Th9 suggests a mechanism by which T cells could reciprocally activate ILC2 in a DR3 dependent manner. Taken together, these results show that TL1A-DR3 interactions coordinately amplify both innate
and adaptive immune responses in allergic disease. Our results predict that blockade of TL1A-DR3 interactions would be beneficial in diseases in which IL-9 plays an important role, including allergic asthma. Given the prominent role of STAT5 in mediating the effects of TL1A on Th9, blockade of STAT5 activity with small molecules directly targeting STAT5 or essential upstream kinases for STAT5 activation such as JAK3 (76) could also act to limit the pathological consequences of IL-9 production triggered by TL1A.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. DR3 is required for optimal pathology and IL-9 and IL-13 production in T-cell driven allergic lung inflammation

(A) Time line of antigen sensitization and challenge in this model; IP (intraperitoneal), IN (intranasal), and IT (intratracheal). (B) mRNA levels of IL-9 and IL-13 measured by qRT-PCR are shown for lung samples harvested 16 or 18 days after initial challenge with ovalbumin. Values were normalized to the average level of RNA in the PBS-treated wild-type (WT) mice at each time point (t-test statistics comparing Ova-treated WT and Ova-treated Tnfrsf25<sup>-/-</sup> mice, *p<0.05, **p<0.01). (C) Frequency of IL-9 and IL-13 producing CD4<sup>+</sup>CD44<sup>hi</sup>T cells in the lung are shown from cells harvested at day 16 from mice challenged as in (A). Right panels, absolute numbers with total number of CD4<sup>+</sup>CD44<sup>hi</sup> cells shown at the top. (D) PAS-stained sections of lungs harvested at day 16 from mice challenged as in (A); airways (aw), and blood vessels (bv) marked; scale bar = 50 μm. Bottom panel shows histopathology scores. (E) Airway resistance was measured in response to increasing doses of aerosolized methacholine in mice challenged as in (A). Results are a combination of 2 independent experiments (Two-way ANOVA comparing Ova-treated WT and Tnfrsf25<sup>-/-</sup> mice, **genotype term p<0.01). (F) Absolute numbers of eosinophils and neutrophils in the bronchoalveolar lavage (BAL) of mice treated as in (A) at day 16 are
shown (Mann-Whitney test: *p<0.05, **p<0.01). (A-D and F) are representative of at least 2 independent experiments.
Figure 2. DR3 is required for optimal pathology and IL-9 and IL-13 production by T cells in response to an inhaled allergen

(A) WT or Tnfrsf25−/− mice were given intranasal PBS or papain according to the timeline. All mice were euthanized 12 hours after the last challenge. (B) Total cell counts, neutrophils and eosinophils present in the BAL. (C) Localization of TL1A expression in the lungs of WT mice treated with PBS or papain is demonstrated by immunohistochemistry. (D) Lung histology of WT and Tnfrsf25−/− mice (PAS staining, left panel). Scale bar= 50 μm. Histopathology scores of lung sections from the indicated groups of mice (right panel). (E) Airway resistance was measured in response to increasing doses of aerosolized methacholine in mice challenged as in (A) (Two-way ANOVA comparing papain-treated WT and Tnfrsf25−/− mice, ****genotype term p<0.0001). (F) IL-9 and IL-13 mRNA expression in the lung relative to β2m in each condition. Values were normalized to the average level of RNA in PBS-treated WT mice. (G) Frequency and total number of IL-9- or IL-13-producing CD45+ TCRβ+CD4+CD44+ T cells, as well as total CD44high T cells, were measured by flow cytometry. In all panels, averages are indicated for each group and statistical significances between papain-treated WT and Tnfrsf25−/− mice are shown (Mann-Whitney, *p< 0.05, **p<0.01, ***p< 0.001, **** p<0.0001). (A-G) are pooled data from 2 experiments.
Figure 3. TL1A enhances IL-9 production by T cells differentiated under Th9 and iTreg conditions

(A) Naïve CD4+ T cells from WT and Tnfrsf25−/− mice were differentiated for 3 days under iTreg conditions with or without TL1A, in the absence or presence of T-depleted antigen presenting cells (APC). Intracellular staining shows a representative experiment, with a compilation of 4 independent experiments depicted below (Mann-Whitney test, *p<0.05).

(B) Naïve CD4+ T cells from WT mice were differentiated into Th17 or iTreg with and without TL1A in the presence of APC. Intracellular staining is representative of 2
independent experiments. (C) Naïve CD4+ T cells isolated from WT and Tnfrsf25−/− mice were differentiated into Th9 in the absence or presence of APC with or without TL1A. Representative intracellular staining is shown with a compilation of 8 independent experiments (-APC) and 4 independent experiments (+APC) (Mann-Whitney test, *p<0.05, ***p<0.001). (D) Th9 differentiation was performed as in (C) in the absence of APC, with varying concentrations of TGFβ. Percentage of IL-9-producing cells by intracellular staining is representative of 2 independent experiments. (E) Th9 differentiation was performed as in (C) without APC, with varying amounts of TL1A. Percentage of IL-9-producing cells by intracellular staining is representative of 2 independent experiments. (F) DR3 surface expression vs. isotype control was measured by flow cytometry in naïve CD4+ T cell cultures differentiated in the absence of APC under various polarization conditions. Results are a compilation of 2 independent experiments; error bars represent +/- SEM. (G) DR3 surface expression was measured by flow cytometry in Th9 cultures with and without TL1A. Results are representative of 2 independent experiments. (H) Naïve WT CD4+ T cells were CFSE-labeled and differentiated under Th9 conditions as in (C) in the presence of APC: top row, CFSE dilution with the indicated expansion index; bottom row, CFSE dilution versus intracellular IL-9 with the percentage of cells producing IL-9 after each division shown above each box. Results are representative of 2 independent experiments. (I) Cytokines were measured in the supernatants from Th9 differentiation cultures without (top) or with T-depleted splenocytes (bottom). Heatmaps show TL1A-induced cytokine changes within each condition (red, increased; green, decreased; black, no change; grey, undetectable in one or both conditions). Results show average changes from two independent experiments. Only those cytokines detectable with and without TL1A in at least 4 polarization conditions are shown.
Figure 4. TL1A-induced Th9 differentiation depends on IL-2 and STAT5

(A) Naïve CD4+ T cells were polarized under Th9 conditions with or without TL1A, with the indicated blocking antibodies or added cytokines; left and middle panels without APC, right panel with APC. IL-9 production assayed by flow cytometry is representative of 2 independent experiments. (B) WT and STAT6-deficient (Stat6−/−) naïve CD4+ T cells were differentiated in the presence of APC under Th9 and iTreg conditions with and without TL1A. Intracellular staining is representative of 3 independent experiments. (C) Control Stat5fl/fl and STAT5-deficient (Stat5CD4−/−) naïve CD4+ T cells were differentiated in the...
presence of APC under Th9 conditions with and without TL1A. Intracellular staining is representative of 2 independent experiments. (D) WT naïve CD4+ T cells were transfected with a constitutively active caSTAT5-retrovirus or control virus and differentiated under Th0, Th17 in the presence of IL-2, and regular Th17 (blocking IL-2) conditions. Intracellular staining is representative of 2 independent experiments.
Figure 5. Cell-intrinsic effects of TL1A in Th9 differentiation are independent of PU.1 and Traf6

(A) Naïve CD4+ T cells from CD45.1+ congenic WT mice were mixed with an equal number of naïve CD4+ T cells from CD45.1– Tnfrsf25−/− mice and polarized under Th9 conditions without APC, or under iTreg conditions with APC, with and without TL1A. IL-9 intracellular staining versus CD45.1 surface staining with the percentages of CD45.1+ and CD45.1− cells expressing IL-9 is representative of 2 independent experiments. (B) WT and PU.1-deficient (Sfpi1lck−/−) naïve CD4+ T cells were differentiated without APC under Th9 conditions with and without TL1A. Left panel shows intracellular staining. Right panel
shows IL-9 production in the supernatant of restimulated cells 5 days after polarization by ELISA. Results are representative of 2 independent experiments. (C) Naïve CD4+ T cells were differentiated without APC under Th9 conditions for 5 days and analyzed by ChIP assay for PU.1 binding to the IL-9 promoter. Error bars represent combined results from 3 mice, representative of 2 independent experiments. (D) As (C), cells were analyzed by ChIP assay for IRF4 binding to the IL-9 promoter. Error bars represent combined results from 4 mice in two independent experiments. (E) WT naïve CD4+ T cells were differentiated without APC under Th9 conditions for 3 days and stained for intracellular phospho-STAT5 (left panel), representative of 2 independent experiments. Cells were also analyzed by ChIP assay for STAT5 binding to the IL-9 promoter (right panels). Error bars represent SEM of technical qPCR replicates. Data is representative of 2 independent experiments. (F) WT and Traf6−/− naïve CD4+ T cells were polarized under Th9 conditions in the absence of APC, with or without TL1A. Intracellular staining is representative of 2 independent experiments. (G) WT and Stat6−/− naïve CD4+ T cells were polarized in the presence of APC under Th9 conditions with or without TL1A or OX40L. Intracellular staining is representative of 3 independent experiments.
Figure 6. TL1A enhances ocular inflammation induced by Th9

(A) Naïve anti-HEL CD4+ TCR transgenic T cells were activated with HEL+APC under Th9 conditions, with or without TL1A. Cells were analyzed for intracellular IL-9 and IL-10 production at the indicated time points (Student’s T test: * p<0.05, **p<0.01); combined results from 3 independent experiments. (B) IL-9 and IL-10 mRNA was measured relative to β-actin in cultures described in (A); combined results from 2 independent experiments. (C) Cytokine production was measured in the supernatants of cultures described in (A); combined results from 3 independent experiments. (D) Th9 cells generated as in (A) (5

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milion or 1.7 million to compensate for increased percentage of cells expressing IL-9 in the presence of TL1A) were adoptively transferred into syngeneic recipients expressing HEL in the lens. Inflammatory changes in recipients of Th9 cells activated for 3 or 4 days in culture were evaluated by histological analysis 7 days after transfer (Mann-Whitney *p<0.05, ****p<0.0005); combined results from 3 independent experiments. (E) H&E stained eye sections are examples of mice described in (D) receiving 5 million Th9 cells, differentiated for 3 days, and a mouse with no treatment (naïve); scale bars: 400 μM for low magnification and 50 μM for high magnification enlarged from the indicated boxes; representative of 3 independent experiments. (F) Histological sections showing conjunctivitis in recipients of Th9 and TL1A-stimulated Th9 cells are representative of 3 independent experiments. (G) As in (D), 5 million transgenic T cells were transferred into recipients after 3 days of differentiation. Recipient spleens were collected at the indicated time points and donor cells identified by flow cytometry with an anti-clonotypic monoclonal antibody (1G12), specific to the donor cell TCR. Graph shows mean +/- SEM of transgenic T cell yields from 2 independent experiments. (H) Th9 cells transferred as in (D) were labeled with Cell Proliferation Dye eFluor 670 and their division in vivo determined by dye dilution at the indicated times after transfer: black line, Th9 control; gray line and shaded graph, Th9+TL1A; representative of 2 independent experiments. (I) Naïve mice or recipients of Th9 transfer as in (D) were injected with PBS or TL1A intraocularly 2 and 3 days after transfer. Eye pathology was examined at day 4 (Mann-Whitney *p<0.05, **p<0.01); combined results from at least 2 independent experiments per condition. (J) Recipients of Th9 transfer as in (D) were administered anti-TL1A antibody or hamster Ig control on days -1 and 3. Recipient eyes were analyzed on day 7 (Mann-Whitney *p<0.05); combined results from 3 independent experiments. (K) Representative examples of ocular histological sections from transfer experiments in (J).
Figure 7. TL1A enhances allergic lung inflammation induced by antigen-specific Th9
(A) Cytokine expression was measured in restimulated Th9-polarized OT-II T cells or Tnfrsf25<sup>−/−</sup> OT-II T cells. (B) Timeline of transfer experiment: 10<sup>6</sup> T cells were transferred into CD45.1<sup>+</sup> congenic recipient mice and the recipient mice challenged with IT Ova 24 hours after transfer and IN Ova 48 hours after transfer. Control recipient mice were challenged with PBS. All mice were euthanized 12 hours after the last challenge. (C) Cell counts of neutrophils and eosinophils present in the BAL from each mouse are shown. (D) Representative lung histology of each group (left panel; H&E stain, 20x original magnification, black bar represents 50 μm), and summary of lung histopathology scores from the same experiment (right panel) are shown. (E) IL-9, IL-13 and IL-2 mRNA expression in the lung relative to β2M in each condition was measured by qRT-PCR. (F) Yields of total CD45.2<sup>+</sup> donor T cells and IL-9- and IL-13-producing donor T cells per lung (left panel) are shown with representative FACS plots of IL-9 and IL-13 expression by restimulated T cells from each group (right panel). In all graphs, each point represents one mouse with the mean as a black line and significances of differences between Ova-treated
groups indicated (Mann-Whitney *p<0.05, **p<0.01). A-D and F are representative of 2 independent experiments. E shows compiled results of 2 independent experiments.