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Cloning, Expression, and Functional Characterization of TL1A-Ig

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TNF superfamily member 15 (TL1A) is the ligand for TNFR superfamily (TNFRSF)25. We previously reported that TNFRSF25 stimulation with an agonist Ab, 4C12, expands pre-existing CD4+Foxp3+ regulatory T cells (Tregs) in vivo. To determine how the physiological ligand differs from the Ab, we generated a soluble mouse TL1A-Ig fusion protein that forms a dimer of TL1A trimers in solution with an apparent molecular mass of 516 kDa. In vitro, TL1A-Ig mediated rapid proliferation of Foxp3+ Tregs and a population of CD4+Foxp3− conventional T cells. TL1A-Ig also blocked de novo biogenesis of inducible Tregs and it attenuated the suppressive function of Tregs. TNFRSF25 stimulation by TL1A-Ig in vivo induced expansion of Tregs such that they increased to 30–35% of all CD4+ T cells in the peripheral blood within 5 d of treatment. Treg proliferation in vivo was dependent on TCR engagement with MHC class II. Elevated Treg levels can be maintained for at least 20 d with daily injections of TL1A-Ig. TL1A-Ig-expanded Tregs expressed high levels of activation/memory markers KLRG1 and CD103 and were highly suppressive ex vivo. TL1A-Ig-mediated Treg expansion in vivo was protective against allergic lung inflammation, a mouse model for asthma, by reversing the ratio of conventional T cells to Tregs in the lung and blocking eosinophil exudation into the bronchoalveolar fluid. Thus, TL1A-Ig fusion proteins are highly active and tightly controllable agents to stimulate Treg proliferation in vivo, and they are uniquely able to maintain high levels of expanded Tregs by repeated administration. The Journal of Immunology, 2013, 190: 1540–1550.

Several members of the TNF superfamily expressed by T cells and other immune and nonimmune cells regulate T cell function. TNFR signaling on T lymphocytes is usually TCR dependent and regulates polarization, proliferation, effector function, contraction, memory, and death (1). TNFR superfamily (TNFRSF)25, a member of the TNFR superfamily, is a type I transmembrane protein that has a cytoplasmic death domain with homology to TNFR1 (2–7). TNFRSF25 is expressed primarily by T lymphocytes, including CD4+, CD8+, and NKT cells (3–8) and is efficiently upregulated after TCR stimulation. The natural ligand for TNFRSF25 was identified as TNF superfamily member 15 (TL1A) (9), which is a trimeric type II transmembrane protein that can be cleaved by metalloproteases and released as a functional and soluble trimer (10). Cloned mouse TL1A translates to a 252–aa protein and shares 63.7% homology to the human counterpart (9). TL1A-Ig fusion proteins are highly active and tightly controllable agents to stimulate Treg proliferation in vivo, and they are uniquely able to maintain high levels of expanded Tregs by repeated administration. The Journal of Immunology, 2013, 190: 1540–1550.

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Abbreviations used in this article: alum, aluminum potassium sulfate; BALF, bronchoalveolar lavage fluid; CHO, Chinese hamster ovary; DN, dominant negative; ECD, extracellular domain; GS, glutamine synthetase; HT, hypoxanthine and thymidine; iTreg, induced/inducible regulatory T cell; LNC, lymph node; m, murine; MHC II, MHC class II; MSX, methionine sulfoxamine; PAS, period acid-Schiff; TCR, CD62L+CD44hi central memory CD4+ cell; Tconv, conventional T cell; Treg, CD62L+CD44hi effector memory CD4+ cell; TIAA, TNF superfamily member 15; TNFRSF, TNFR superfamily; Treg, regulatory T cell.

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the fusion protein induced the proliferation of CD4+Foxp3+ Tregs in vitro and could be repeatedly administered to maintain elevated Treg levels in vivo. Although there have been studies reporting methods that expand endogenous Tregs (22–24), to our knowledge we provide the first description of a molecule and method to sustain prolonged in vivo Treg expansion. The TL1A-Ig fusion protein may provide both ex vivo and in vivo strategies for Treg-based therapies in humans.

Materials and Methods

Mice

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. The Foxp3 reporter mice on C57BL/6 background (FIR mice) were bred in our animal facility (provided by R. Flavell, Yale University, New Haven, CT). Mice were used at 6–12 wk age and were maintained in pathogen-free conditions at the University of Miami Animal Facilities. The University of Miami Animal Care and Use Committee approved all the animal procedures used in this study.

Flow cytometry, Abs, and reagents

Intracellular Ki-67 and Foxp3 staining was performed using mAbs and fixation/permeabilization reagents from eBioscience (San Diego, CA). The mlgG1 isotype control was purchased from BioLegend (San Diego, CA). All commercial Abs used for flow cytometry staining were purchased from R&D systems (Minneapolis, MN). Cytokines were purchased from R&D systems. Transfected cells were subjected to selective pressure when placed in serum-free OptiCHO media (Invitrogen) using PCR strategy (Fig. 1A). Briefly, the fragments were obtained by PCR amplification and the PCR products were sequentially ligated into the TOPO cloning vector (Invitrogen). The mlgG1 κ-chain leader sequence was ligated to the 5′ end of the cDNA encoding the Fc portion of mlgG1, containing the hinge, CH2, and CH3 regions. IgG1 had been mutared to replace the three cysteines of the hinge portion to serines (26), with the aim of reducing the Ab-dependent cellular cytotoxicity that can occur due to binding of IgG1 to Fcγ receptors (27). The mTL1A DNA was purchased from OriGene and the extracellular domain (ECD), containing amino acids 68–252, was PCR amplified using primers from Calbiochem/EMD Millipore. Armenian hamster hybridoma producing Ab to mTLNFRSF25 (agonist 4C12) was maintained as described previously (8). 4C12 (anti-TNFRSF25 agonist) and L4G6 (anti-mTL1A blocking mAb) was produced in hollow fiber bioreactors (FiberCell Systems) and purified from serum-free supernatants on a protein G column using PBS for elution.

Plasmid construction

An expression plasmid of the mTL1A-Ig (TL1A-Ig) and murine glutamine synthetase (mGS) was constructed based on the plasmid pVitro2-hygro (InvivoGen, San Diego, CA) using PCR strategy (Fig. 1A). Briefly, the fragments of each gene were obtained by PCR amplification and the PCR products were sequentially ligated into the TOPO cloning vector (Invitrogen). The mlgG1 κ-chain leader sequence was ligated to the 5′ end of the cDNA encoding the Fc portion of mlgG1, containing the hinge, CH2, and CH3 regions. IgG1 had been mutared to replace the three cysteines of the hinge portion to serines (26), with the aim of reducing the Ab-dependent cellular cytotoxicity that can occur due to binding of IgG1 to Fcγ receptors (27). The mTL1A DNA was purchased from OriGene and the extracellular domain (ECD), containing amino acids 68–252, was PCR amplified using primers that were located upstream and downstream of the cloning sites. The final PCR product was ligated to the 3′ end of the cDNA encoding the Fc mlgG1 fragment, resulting in the final construct containing the leader sequence, hinge-CH2-CH3, and mTL1A ECD (Fig. 1B) that was cloned (SIGRAI/NHE1 restriction sites) into the second multiple cloning site of pVitro2-mgs, which had the mgs cDNA inserted in the first multiple cloning site (Fig. 1A), resulting in the final pVitro2-mgs-TL1A-Ig expression plasmid. The mgs cDNA was purchased from OriGene, and the open reading frame was PCR amplified with primers containing the MLP1/SAL1 restriction sites.

Cells and culture conditions

Chinese hamster ovary (CHO)-K1 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in IMDM supplemented with 10% FBS and 0.5 mg/ml gentamicin (Invitrogen). Transfected cells were subjected to selective pressure when placed in glutamine-free OptiCHO media (Invitrogen) supplemented with GS and hypoxanthine and thymidine (HT) and 1 mM methionine sulfoximine (MSX; Sigma-Aldrich).

Transfection and selection of CHO cells

The double-cassette plasmid pVitro2-mgs-TL1A-Ig was first transfected using electroporation into adherent CHO-K1 cells supplemented with 10% FBS. The following conditions were applied while using the Gene Pulser apparatus for transfection: 0.4 cm cuvette gap, 0.75 kV, 25 μF, and 0.5 ms time constant. After electroporation the cells were cultured in 10% FBS/IMDM media and equally distributed into a six-well plate (Costar). At 48 h, the cells were retransfected with the same plasmid using standard procedures for the Effectene reagent (Qiagen) and subjected to selection pressure when cultured in 10% FBS/IMDM supplemented with 1 mg/ml hygromycin. Transfectants were single cell cloned in 96-well round-bottom plates (Costar) and subjected to hygromycin selection to generate supernatant samples for analysis by mlgG ELISA. The highest producing clone (secreting 40 μg/ml TL1A-Ig) was expanded to a 10 L bioreactor and subjected to sequential adaptation from 10% FBS/IMDM to serum-free OptiCHO media supplemented with 8 mM l-glutamine and GS and HT supplements (Invitrogen). Five separate CHO clones were established in this manner and subjected to further selection when cultured in serum-free OptiCHO media lacking l-glutamine. Another mlgG ELISA was used to pick the highest producing clone, which was chosen for the third round of selection under varying concentrations of MSX (25 μM, 100 μM, 1 mM, and 2 mM). The surviving clone was passaged and ELISA samples were collected as described previously. A single clone expressing TL1A-Ig at ~200 μg/ml was chosen for generation of protein.

Production and purification conditions

The highest yielding TL1A-Ig CHO cells were placed into hollow fiber cell culture cartridges (FiberCell Systems) and cultured in serum-free media containing GS and HT Supplements. About 50% of the cells were harvested when the TL1A-Ig supernatant reached 40 μg/ml TL1A-Ig was expanded to a 10 L bioreactor and subjected to sequential adaptation from 10% FBS/IMDM to serum-free OptiCHO media supplemented with 8 mM l-glutamine and GS and HT supplements (Invitrogen). The surviving clone was passaged and ELISA samples were collected as described previously. A single clone expressing TL1A-Ig at ~200 μg/ml was chosen for generation of protein.

ELISA quantification assays

Three days after refueling the cartridges with serum-free OptiCHO media, supernatant was collected for 1 h at 37°C, following by blocking (10% FBS in PBS) and three washes with washing buffer (PBS containing 0.1% Tween 20). One hundred microliter standard (mlgG1, supernatant, or blank) was added to each well (with serial dilutions) and incubated for 2 h. After washing, 100 μl secondary Ab peroxidase-conjugated anti-mlgG was added to each well (1:1000 dilution in PBS) and incubated for 1 h. After five washes, 100 μl ABTS substrate was added to each well, incubated, and analyzed using a Benchmark Plus microplate spectrophotometer at 405 OD. The same procedure was used to pick the highest producing transfectants during the selection process.

SDS-PAGE, Western blot, and gel filtration chromatography analysis

Purified TL1A-Ig was denatured by boiling for 5 min in the absence or presence of 2-ME and Laemmlil sample buffer (2% SDS, 5% glycerol, 15 mM Tris [pH 6.8]) and separated by electrophoresis on 4–15% SDS-PAGE. One gel was stained with Coomassie blue stain followed by incubation with destaining buffer (50% v/v methanol and 10% v/v acetic acid) to visualize the protein. Another SDS-PAGE gel was transferred to nitrocellulose membranes for analysis by Western blot. The membrane was treated with 2% TBS for 20 min and the TL1A-Ig fusion proteins were detected with anti-mTL1A mAb (0.5 μg/ml; Jackson Immunoresearch) or anti-mlgG (1:1000 dilution; Jackson Immunoresearch), HRP-coupled secondary Ab (1:1000 dilution; Jackson Immunoresearch), and the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). The glycosylation studies were performed according to the protocol provided with enzymatic deglycosylation kit GSK0110 (ProZyme, Hayward, CA). Briefly, four samples of reduced purified TL1A-Ig (2 μg) were incubated in the absence or presence of 1 μl each of N-Glycanase, Sialidase A, and O-Glycanase (ProZyme) in the appropriate tube. The first control replicate received no enzymes, the second tube received only N-Glycanase, the third tube received only O-Glycanase, and the fourth tube received all three enzymes. The tubes were incubated for 3 h at 37°C. Samples were loaded on SDS-PAGE, subjected to electrophoresis, and stained with Coomassie blue. For gel filtration chromatography, purification was analyzed by size exclusion chromatography on a Superdex 200 gel filtration column equilibrated with Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA). The fractions were collected at flow rate of 0.5 ml/min (200
mobilized). The column was calibrated with a mixture of standards including bovine thyroglobulin (670 kDa), aldolase (148 kDa), and OVA (43 kDa) (GE Healthcare). The correct primary structure was verified by sequencing. Upon induction of polyclonal Tregcs, FcR TcSs were seeded in 24-well flat-bottom plates pretreated with plate-bound anti-CD3 (2 μg/ml) in the presence of TGF-β (5 ng/ml) and IL-2 (100 U/ml), and retinoic acid (100 nM) in the absence or presence of anti-CD4 (2 μg/ml; 2C11), mIL-2 (10 U/ml), 4C12 (10 μg/ml), or purified TL1A-Ig (0.1 μg/ml). Cultures were incubated for 72 h at 37˚C and pulsed with [3H]thymidine (1 μCi/well; PerkinElmer) for the last 6 h. The incorporated isotope was measured by liquid scintillation counting (MicroBeta TriLux counter; PerkinElmer).

### Induction of allergic lung inflammation

Mice were sensitized by i.p. injection of 66 μg OVA (crystallized chicken egg albumin, grade V; Sigma-Aldrich) adsorbed to 6.6 mg aluminum potassium sulfate (alum; Sigma-Aldrich) in 200 μl PBS on day 0, with an i.p. boost on day 5. On days 11, 12, and 13 mice were injected i.p. with 100 μg TL1A-Ig or mLG (Jackson ImmunoResearch) in 200 μl PBS. On day 16, mice were aerosol challenged with 0.5% OVA (Sigma-Aldrich) in PBS for 1 h using a BANG nebulizer (CH Technologies) into a Jaeger-NYU nose-only directed-flow inhalation exposure system (CH Technologies). On day 19, mice were sacrificed, lungs were perfused with PBS, and bronchoalveolar lavages were harvested. Lung lobes and spleens were harvested for flow cytometry analysis, and lung lavages were also analyzed for lung histology as described previously (8). Quantification of periodic acid-Schiff (PAS)—stained lung sections was performed using MacBio-photonics Imageal software by color deconvolution (using the H PAS vector) followed by thresholding of images (color 2, set to 95) and counted using the nucleus counter (limits set between 400 and 7000).

### Results

#### Biochemical characterization of TL1A-Ig

A double-cassette plasmid, based on the pVitro-2–hygro vector, expressing TL1A-Ig fusion protein and mGS was constructed and is presented schematically (Fig. 1A). The cDNA for the fusion protein was comprised of the mIgG κ-chain peptide leader sequence, followed by the hinge, CH2 and CH3 domains of mLG1, and then the ECD of mTL1A (amino acid 68–252) (Fig. 1B). Four fusion protein constructs were compared that differed by the length of the ectodomain next to the transmembrane domain and by the presence or absence of the putative protease cleavage site. The correct primary structure was verified by sequencing. Upon transfection into NIH-3T3 cells and analysis, all four constructs were functional; the TL1A-Ig construct ECD 68–252 containing the cleavage site was selected for purification and further analysis owing to its high level of expression.

Gene amplification is an efficient method for generation of high producer cell lines. mGS was used for this purpose (28). CHO-K1 cells were transfected with the double-cassette plasmid expressing TL1A-Ig and mGS. The transformants were single cell cloned under hygromycin selection and supernatants were analyzed by ELISA for mLG as a readout for TL1A-Ig. The highest producing clone (40 μg/ml × 106 cells × 24 h) was subsequently passaged in standard cell culture media and 10% FBS. FBS was slowly reduced and substituted with increasing proportions of serum-free media. Serum-free subclones were subjected to further selective pressure in glutamine-free media and with increasing concentrations of MSX, an inhibitor of GS, resulting in gene amplification. After the MSX selection, the subclone producing the highest level of TL1A-Ig (200 μg/ml × 106 cells × 24 h) was identified and maintained for production of TL1A-Ig using a hollow-fiber cell bioreactor system.
cartridge and culture in serum-free medium. mTL1A-Ig was purified to homogeneity by affinity chromatography by binding to protein A and elution with diethylamine.

TL1A is a trimeric protein (10, 29–31) whereas the IgG H chain forms dimers. The theoretical molecular mass of monomeric TL1A-Ig is 47 kDa, with the IgG contributing 26 kDa and TL1A part of 21 kDa. Upon SDS-PAGE analysis, untransfected TL1A-Ig migrated as a dimer of 100 kDa, indicating that the IgG1 H chain did not dissociate. Reducing conditions resulted in an apparent molecular mass band of ~50 kDa (Fig. 2A). The low–molecular mass band does not stain with either anti-IgG or anti-TL1A Ab and represents a minor contaminant. By Western blot analysis, both anti-TL1A and anti-IgG Ab detected only the 50-kDa band, indicating that all TL1A is in association with IgG and no cleavage occurred (Fig. 2A). Multiple bands in nonreduced conditions may be explained by thiol–disulfide exchange or posttranslational glycosylation. N-Glycanase treatment indicates posttranslational glycosylation in CHO cells whereas O-Glycanase and Salidase had no effect (Fig. 2B). Gel filtration revealed an apparent molecular mass of 516 kDa (Fig. 2C), which suggests a hexamer with a high frictional coefficient as depicted in the schematic drawing of the quaternary structure (Fig. 2D).

In vitro functional characterization of TL1A-Ig

Both in supernatant and as purified fusion protein, TL1A-Ig bound to TNFRSF25 transfected, but not untransfected (not shown), P815 cells as determined by flow cytometry and with a similar profile to TNFRSF25 transfected, but not untransfected (not shown), P815 cells. In supernatant and as purified fusion protein, TL1A-Ig bound to the TNFRSF25-specific mAb 4C12 (Fig. 3A). Additionally, binding of TL1A-Ig to P815-TNFRSF25 cells was competitively inhibited by preincubation with the TL1A-specific mAb L4G6 (not shown). Tumor cells overexpressing TNFRSF25 underwent apoptosis upon binding of the agonistic TNFRSF25 Ab (clone 4C12) as measured in caspase activation assays. Binding of purified hexameric TL1A-Ig–mediated caspase activation at a 100-fold lower concentration by weight than 4C12 (Fig. 3B), suggesting high-affinity binding and the ability to crosslink two TNFRSF25 trimers resulting in enhanced TNFRSF25 signaling.

Anti-CD3 Ab–activated CD4+ T cells, including Tconvs and Tregs, express elevated levels of TNFRSF25 (Fig. 3C) (2). Incubation of Tconvs with TL1A-Ig, but not 4C12, costimulated proliferation of Tconvs in an anti-CD3 Ab– and IL-2–dependent fashion (Fig. 3D). The fusion protein, unlike 4C12, also stimulated proliferation of purified CD4+Foxp3+ Tregs in vitro in the presence of low (10 U/ml) IL-2, even without the addition of anti-CD3 to induce TCR signaling (Fig. 3E). The sorting of live CD4+ Foxp3+ Tregs was made possible by utilizing FJR mice (25) expressing a red fluorescent protein under the Foxp3 promoter. Addition of cyclosporine in vitro inhibited TL1A-Ig–triggered proliferation of Tregs (Fig. 3F), suggesting that freshly isolated Tregs maintain residual TCR signals emanating from binding to self Ags or commensal Ags normally present in vivo. Others have reported that freshly isolated Tregs have a higher level of phosphorylation of the TCR ζ-chain compared with Tconvs, which is a biochemical consequence of TCR signaling (32). Activation of naïve CD4+ T cells by anti-CD3 or specific Ag in the presence of TGF-β, retinoic acid, and IL-2 results in the induction of Fox3 in a substantial proportion of cells. Fox3 induction is partially suppressed by the TNFRSF25 agonist 4C12 and more completely by TL1A-Ig (Fig. 3G), similar to OX40L (18, 19, 33).

In vivo expansion of Tregs by TL1A-Ig

Before determining functional effects in vivo, we determined the half-life of both TNFRSF25 agonists by i.p. injection of 100 μg 4C12 or TL1A-Ig and analyzing timed serum samples by ELISA specific for Armenian hamster IgG or TL1A (Fig. 4A). The observed half-life of TL1A-Ig is 13.5 h and for the 4C12 Ab is 5 d. The shorter half-life of TL1A-Ig requires daily injection but allows easy adjustment of desired serum levels.

We previously reported that TNFRSF25 stimulation by 4C12 rapidly expands pre-existing Tregs in vivo (21). To determine whether the fusion protein similarly increases Treg proliferation in vivo, naïve FJR mice were injected i.p for 3 consecutive days with 100 μg TL1A-Ig or mlgG control (Fig. 4B). The mice were continuously monitored for the frequency and phenotype of FJR Tregs. The fusion protein induced a rapid and highly reproducible expansion of Tregs in vivo, which had similar kinetics to Treg expansion observed with one dose of 4C12. The requirement for more than one i.p injection of the fusion protein versus 4C12 is due to the short half-life of 13.5 h for TL1A-Ig (Fig. 4A). Similar to 4C12, TL1A-Ig–mediated Treg expansion was maximal at days 4 and 5, with Tregs comprising 30–35% all CD4+ T cells in peripheral blood and then contracting to baseline levels by 14 d. Daily administration of TL1A-Ig was able to sustain high Treg levels for >20 d (Fig. 4D).

To determine whether in vivo TNFRSF25-mediated Treg expansion is dependent on TCR signaling, we adoptively transferred purified CD4+ T cells from FJR mice into MHC class II (MHC II)–deficient (C747/−) mice or CD4+− mice. CD74/− mice lack MHC II Ag presentation and, as a consequence, also lack CD4+ T cells, whereas CD4+− mice can present Ag via MHC II to CD4+ cells including CD4+ Tregs. Three days after transfer (day 0), mice were treated from days 0 to 2 with 100 μg TL1A-Ig or
isotype control IgG. Analysis on day 6 revealed no TL1A-Ig–mediated CD4+Foxp3+ Treg expansion in MHC II–deficient mice. In contrast, TL1A-Ig–mediated potent Treg expansion in CD4+2/2 mice that are able to present MHC II Ags. Tregs expanded even in the absence of extraneous TL1A-Ig, suggesting TCR signaling primarily at intestinal mucosal sites (Fig. 4D). These experiments indicate that MHC II is required for costimulation of Treg proliferation by TNFRSF25. These data imply that TCR signaling is a prerequisite for TNFRSF25 costimulation, which is similar to the requirements that we observed for TNFRSF25 signaling for in vitro Tconv and Treg proliferation assays and the known requirement for TNFRSF25 signaling in Tconvs (9, 13).

**Enhanced absolute numbers and formation of memory CD4+ T cells in TL1A-Ig–treated mice**

In the course of examining the effect of TL1A-Ig on Tregs in vivo, we observed slightly enlarged spleens and lymph nodes (LNs) in TL1A-Ig–treated mice as compared with age-matched IgG-treated control mice (Fig. 5A). The total cellularity increased slightly in the spleen, whereas we observed a significant increase in total cell numbers harvested from inguinal, mesenteric, axillary, and brachial pooled LNs on day 4 after the first dose of TL1A-Ig. Flow cytometric analysis revealed increased numbers of CD4+ T cells in the LNs and to a lesser degree in the spleen, whereas CD8 T cell yields were only slightly higher in LNs and normal in the spleen (Fig. 5B). TL1A-Ig–treated mice had normal frequencies of B cells, macrophages, dendritic cells, NK cells, and NKT cells (data not shown). The percentage of proliferation Ag Ki-67 expressing CD4+Foxp3+ Tregs increased from ∼20 to 80% in the spleen and LNs. Only ∼5% of CD4+Foxp3+ Tconvs and CD8+ T cells in IgG-treated mice were Ki-67+, and this percentage increased to 25 and 17% after TL1A-Ig treatment (Fig. 5C). The frequency of memory CD4+ T cells, including Foxp3+ CD4+CD62Lhi central memory CD4+ cells (T_{CMs}) and Foxp3+ CD4+CD44hi effector memory CD4+ cells (T_{EMs}), increased in spleen and LNs (Fig. 5D). Because the intensity of an immune response is associated with the balance of Tconvs to Tregs, the Tconv/Treg and (T_{CM} + T_{EM})/Treg ratios were determined (Table I). There was no significant difference in the expression of the activation marker CD69 on either memory CD4+ cell subsets in the spleens
and LNs of IgG- or TL1A-Ig–treated mice (data not shown). No differences in memory/activation markers were detected in the CD8+ T cell subset between the IgG- and TL1A-Ig–treated mice (data not shown). Taken together, these data suggest that treatment with TL1A-Ig in the absence of exogenous Ag resulted in the proliferation of Foxp32CD4+ Tconvs that may have previously

**FIGURE 4.** mTL1A-Ig stimulates rapid proliferation of CD4+Foxp3+ Tregs in vivo. (A) The time-related serum concentrations of 4C12 and TL1A-Ig in C57BL/6 mice (n = 3–5) was determined after a single i.p injection of 100 μg 4C12 or TL1A-Ig. Protein concentration was measured in serum samples collected at indicated time points by a sandwich ELISA specific for Armenian hamster IgG or TL1A. Concentration at each time point was calculated as a percentage of the average initial serum concentration (stable agonist concentration after equilibration between blood and tissues). Unique symbol represents each 4C12 treated (solid) mouse or TL1A-Ig (hollow) mouse. (B) The kinetics and dose-dependent expansion of Tregs in peripheral blood were determined after i.p injection of 100 μg IgG (n = 3) or TL1A-Ig (n = 4) in FIR mice for 3 consecutive days as indicated by the arrows. Mice were bled daily and the percentage of peripheral Tregs relative to total CD4+ cells was determined by flow cytometry. (C) Treg expansion was monitored in the peripheral blood while administering daily i.p injections of 100 μg IgG (n = 2) or TL1A-Ig (n = 4) starting on day 0 and ending on day 20. (D) Ten million CD4+ cells were highly purified by FACS sorting from FIR mice and adoptively transferred into CD742/CD42/2 mice. After 3 d (day 0), recipient mice were treated with 100 μg TL1A-Ig or IgG followed by two consecutive doses on days 1 and 2. The percentage of Foxp3+ cells was analyzed in the spleen and pooled lymph nodes on day 6. Data are representative of two independent experiments, with two or more mice per group. Statistical analysis was performed by an unpaired two-tailed Student t test (B, C). All data are means ± SEM (B–D). *p < 0.05, **p < 0.01, ***p < 0.001 versus control.

**FIGURE 5.** Characterization of T cell subsets in TL1A-Ig–treated mice. (A) Spleen and pooled LNs (inguinal, mesenteric, axillary, and brachial pooled LNs) harvested on day 4 from FIR mice that were injected i.p with 100 μg IgG or TL1A-Ig on days 0, 1, and 2. (B) Absolute numbers of total cells, CD3+ T cells, CD4+ T cells, and CD8+ T cells from spleen and pooled LNs from 8- to 12-wk-old age-matched IgG- and TL1A-Ig–treated mice as described in (A). The line represents the average absolute number from mice analyzed in seven independent experiments. (C) Increased numbers of T cell subsets in TL1A-Ig–treated mice as depicted by the frequency of proliferating (Ki-67+) Tconvs, Tregs, and CD8+ T cells in the spleen and pooled LNs. (D) Representative flow cytometry plots and compilation of percentage of memory markers on CD4+FIR Tconvs harvested from spleen and pooled LNs from IgG- and TL1A-Ig–injected mice treated as in (A) (8- to 12-wk-old mice; the data are compiled percentage averages of five to seven mice per group from two independent experiments). Statistical analysis was performed by unpaired two-tailed Student t test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars indicate means ± SEM.
encountered environmental and/or commensal Ags. H&E staining of tissue sections from the heart, kidney, lungs, liver, and intestine harvested from 3 d (data not shown) or 21 d (Supplemental Fig. 1) TL1A-Ig–treated mice did not indicate any cellular infiltration or tissue inflammation. Furthermore, similar to 4C12, TL1A-Ig also significantly increased the frequency of the CD25\textsuperscript{int} Treg population (Fig. 6A). Flow cytometric analysis revealed a 3-fold increase in the expression of Ki-67 on the CD25\textsuperscript{int} Treg populations in TL1A-Ig–treated mice compared with IgG controls (Fig. 6B). No differences were observed in the expression of CD127, CTLA-4, OX40, GITR, and CD39 on Foxp3\textsuperscript{+} Tregs between IgG- and TL1A-Ig–treated mice. An increase in \( \alpha_5 \beta_7 \) integrin (CD103) and KLRG1 indicating activation was observed in Foxp3\textsuperscript{+} Tregs in spleen and LNs of TL1A-Ig–treated mice (Fig. 6C) (34–36), whereas the expression was unchanged in Tconvs and CD8\textsuperscript{+} T cell subsets (data not shown). We also found an increase in the frequency of splenic and LN Foxp3\textsuperscript{+} Tregs with increased expression of T cell memory markers in the TL1A-Ig–treated mice as compared with IgG-treated controls (Fig. 6D).

Foxp3\textsuperscript{+} Tregs from TL1A-Ig–treated mice are highly suppressive ex vivo

TL1A-Ig, in the presence of cognate Ag or TCR triggering, costimulates both Tconvs and Tregs. To study TL1A-Ig–mediated effects on Treg suppression of CD4\textsuperscript{+} Tconvs and to distinguish the effect from Tconv costimulation by TL1A-Ig, we used transgenic Tconvs expressing a DN mutant of TNFRSF25 (DN-TNFRSF25) (8) that is not costimulated by TL1A-Ig. Tregs expanded in vivo with TL1A-Ig and analyzed ex vivo are highly suppressive for Tconv proliferation by TCR signaling, including DN-TNFRSF25 Tconvs (Fig. 7A). The addition of TL1A-Ig attenuated the suppressive potency of TL1A-Ig Tregs, allowing transgenic Tconvs to proliferate even in the presence of Tregs (Fig. 7B). Similar abrogation of suppressive activity was observed when TL1A-Ig was added in a suppression assay with Tregs from IgG-treated mice (data not shown). Taken together, these data demonstrated that the inhibition of Treg suppressive activity by TL1A-Ig, as shown

| Table I. TL1A-Ig decreases Tconv/Treg ratio and (T_{CM} + T_{EM})/Treg ratio |
|-----------------|-----------------|
|                  | IgG             | TL1A-Ig         |
| Tconvs per spleen (no.) | 8,048,067       | 7,201,228       |
| T_{CM} per spleen (no.)  | 299,608         | 575,196         |
| T_{EM} per spleen (no.)  | 1,074,847       | 2,369,402       |
| Tregs per spleen (no.)   | 1,350,559       | 5,121,313       |
| Tconv/Treg ratio        | 6.0             | 1.4             |
| (T_{CM} + T_{EM})/Treg ratio | 1.0           | 0.6             |

Total splenocytes were harvested as in Fig. 5. Cell numbers were calculated by multiplying the number of cells obtained in a single-cell suspension of the spleen by the percentage of lymphoid-gated cells per total cells analyzed by flow cytometry by the percentage of total CD4\textsuperscript{+}Foxp3\textsuperscript{+} Tconvs, T_{CMs}, T_{EMs}, or CD4\textsuperscript{+}Foxp3\textsuperscript{+} Tregs within the lymphoid-gated cell population.

**FIGURE 6.** TL1A-Ig treatment mediated in vivo expansion and enhanced activation of Tregs. (A) FIR mice were treated with IgG or TL1A-Ig on days 0, 1, and 2 and splenocytes were harvested on day 4 and analyzed by flow cytometry. Representative plots are shown. (B) Representative dot plots were pregated on CD4\textsuperscript{+}FIR\textsuperscript{+} Tregs and analyzed for Ki-67\textsuperscript{+} on CD25\textsuperscript{hi} and CD25\textsuperscript{int} Treg populations in TL1A-Ig–treated mice compared with IgG controls (Fig. 6B). No differences were observed in the expression of CD127, CTLA-4, OX40, GITR, and CD39 on Foxp3\textsuperscript{+} Tregs between IgG- and TL1A-Ig–treated mice.
that were treated with TL1A-Ig, analysis of BALF showed a significant decrease in both the percentage and number of eosinophils recovered, as compared with mlgG isotype control–treated mice (17.2% and 60 × 10^5 cells; Fig. 8B).

The frequency of Foxp3^+ Tregs was significantly increased in single-cell suspensions of lung tissue and spleens of mice treated with TL1A-Ig (Fig. 8C) 8 d after the first dose of TL1A-Ig (3 d after aerosolization). Treg analysis in the lung tissue revealed that the frequency of Tregs was 66% of all CD4^+ T cells in TL1A-Ig–treated mice as compared with 25% in control IgG-treated mice. The number of CD4^+Foxp3^+ Tconvs within the lungs was similar between control and TL1A-Ig–treated mice, whereas in TL1A-Ig–treated mice, the number of Tregs was significantly increased (Fig. 8D). Several studies have reported that the balance between Tconvs and Tregs is a better determinant of disease pathogenesis than merely the absolute number of Tregs (37, 38), and thus the Tconv/Treg ratio was calculated in the lung tissue (Fig. 8E). The average Tconv/Treg ratio in the lung tissue decreased from 2.8 to 0.5 in TL1A-Ig–treated mice. PAS and H&E staining of lung tissue revealed reduced lymphocyte infiltration and airway mucus secretion in TL1A-Ig–treated animals compared with OVA aerosolized IgG-treated mice (Fig. 8F, 8G).

**Discussion**

During the past several years, TNF superfamily members have been identified as important costimulators of TCR-activated T cells and inducers of caspase-dependent apoptosis. In a previous study we showed, with the aid of the agonistic Ab 4C12, that TNFRSF25 is unique among TNFR family members in expanding T cells in vivo. Preferential expansion and proliferation of Tregs by TNFRSF25 costimulation was shown to be dependent on TCR signaling by interaction with MHC II–presented self Ag.

Heterologous Abs are antigenic and do not always precisely mimic the function of the cognate ligands on different cells (39, 40). Therefore, a comparison with the functional effects of the natural ligand, TL1A, is necessary. Consistent with the effects elicited by 4C12, transgenic overexpression of TL1A resulted in increased numbers of Tregs, but TL1A-transgenic overexpression also led to modulation of CD4^+ Tcon responses, intestinal goblet cell hyperplasia, and intestinal inflammation (14, 41–43), indicating important regulatory functions of TL1A, especially in the intestinal mucosa.

In this study we report that 4C12- and TL1A-Ig–mediated TNFRSF25 signaling have different outcomes in vitro and in vivo. The explanation may be due to qualitative differences between the agonist Ab and the natural ligand. The TNFRSF25 Ab 4C12 is an IgG molecule with two binding sites, which enables it to interact with only two subunits of the trimeric receptor. In contrast, TL1A occurs as a membrane-associated ligand able to engage and immobilize several receptor complexes on TNFRSF25–bearing cells. TL1A, upon cleavage from the membrane, also occurs as a soluble molecule able to diffuse through tissues and engage TNFRSF25 receptors on distant cells. TL1A, similar to all TNF superfamily members, is a trimer interacting with each subunit of the trimeric receptor TNFRSF25. According to our hydrodynamic analysis, the TL1A-Ig construct is a dimer of TL1A trimers. Each trimer will be able to interact with each subunit of TNFRSF25 and crosslink two receptor complexes. This difference in binding sites and ability to crosslink may account for some of the differences observed between TL1A-Ig and 4C12 in vitro and in vivo. Both TNFRSF25 agonists induced apoptosis in transfected tumor cells overexpressing TNFRSF25. mTL1A-Ig, however, is effective at a 100-fold lower concentration, suggesting higher affinity and/or effects of crosslinking. We also demonstrated that 4C12 is a
poor costimulator of anti-CD3-activated CD4+ Tconvs in vitro, whereas TL1A-Ig is quite effective. As reported previously, 4C12 does not costimulate proliferation of Tregs in vitro, whereas TL1A-Ig is quite potent, but strictly dependent on added IL-2 and TL1A-Ig. In studies investigating the suppressive activity of Tregs in vitro, both TL1A-Ig and 4C12 attenuated the inhibitory activity of Tregs for Tconv proliferation. This effect was dependent on the expression of TNFRSF25 on Tregs, and not Tconvs, as transgenic DN-TNFRSF25 Tconvs were used in the suppression assay.

In vivo we find that both 4C12 and TL1A-Ig potently costimulate Treg expansion with similar kinetics, which is dependent on the presence of MHC II and can be blocked by cyclosporine A, indicating TCR signaling by self Ag. Tconvs are not costimulated in vivo by 4C12 in the absence of Ag. A population of conventional T memory cells, which previously had encountered non-self environmental/commensal Ags, was costimulated with TL1A-Ig. Unlike 4C12, TL1A-Ig has a relatively short biological half-life of ~13.5 h, which requires daily injection but offers great flexibility in dosing studies. Daily injections of TL1A-Ig lead to Treg expansion in vivo, which can be sustained for >20 d. Hamster Abs, similar to 4C12, generate Ab responses in mice, resulting in rapid clearance after the first week. Finally, both TL1A-Ig and 4C12, when given to OVA sensitized mice d prior to OVA aerosol exposure, expand Tregs that protect mice from allergic lung inflammation upon migration to the lung.

To take advantage of natural Treg expansion by TNFRSF25 signaling, it is important to inject the agonist at a time when no exogenous Ag is present. Treg--TCR signaling via MHC II-presenting self Ag is sufficient for TNFRSF25 costimulation and Treg expansion. Once expanded, the massive quantity of Tregs, which includes Ag-specific Tregs (44–46), inhibits T cell effector responses. In this way it may be possible to reset overactive immune responses typical to allergic responses, to normal immune reactivity. Indeed, TNFRSF25-mediated expansion of endogenous Treg population prior to transplantation results in prolongation of cardiac allograft survival in a mouse model of fully MHC-mismatched ectopic heart transplants (47). Similarly, TNFRSF25 stimulation prior to infection in a model of corneal infection with HSV reduced the severity of subsequent stromal keratitis lesions (48), which was attributed to the expansion of the Treg population that additionally expressed activation markers such as CD103 needed to access inflammatory sites.

We demonstrate that TL1A-Ig costimulates a portion of Tconvs that appears to be a population that recently encountered non-self Ag (i.e., environmental and/or commensal). One possibility is that the encountered non-self Ag is absent in vivo at the time of TNFRSF25 signaling, allowing Ag-independent memory CD4+ T cells to proliferate without harmful activation. Another possibility is that the cognate Ag is present (in the case of commensal bacteria), where we would expect to see inflammation due to activated effector CD4+ T cells. The absolute numbers of Tregs, total Tconvs, TCMs, and TEMs in response to TL1A-Ig stimulation in mice clearly reveal that Tregs expand more extensively than do Tconvs, resulting in a change in the (TCM + TEM)/Treg and Tconv/Treg ratios in favor of Tregs. Although memory CD4+ T cells proliferate owing to TL1A-Ig, this effect is more than offset by the larger Treg proliferation and expansion resulting in an overall immunosuppressive effect of TL1A-Ig administration in vivo as shown in the acute lung inflammation model. Additionally, we did not observe any physical signs of inflammation, such as weight...
loss, diarrhea, or dermatitis in naive mice that were treated with TL1A-Ig with three daily consecutive doses or daily for a 20-d period. Histopathology also failed to detect cellular infiltration or inflammation in any organ. Moreover, the asthma studies demonstrate that TNFRSF25 stimulation with 4C12 or TL1A-Ig protects against lung inflammation upon airway Ag challenge of sensitized mice, without inducing disease pathology. However, caution should be practiced when considering using TL1A-Ig in treating autoimmune diseases because pathogenic T effector cells might be costimulated in addition to Tregs. In fact, in a clinical trial in 2006 testing the CD28 superagonistic Ab, which was expected to preferentially activate and expand immunosuppressive Tregs, this Ab induced a rapid and massive cytokine storm due to activation of other T cells subsets (49).

The functional efficiency of TL1A-Ig in murine models can easily be translated for human disease by constructing human TL1A-Ig in an analogous fashion. Rigorous experimental verification to that effect is ongoing in mice with a human immune system to determine whether human TL1A-Ig is sufficiently Treg-specific in vivo to warrant further clinical development. Because TNFRSF25 signaling has the unique ability to expand and transiently inhibit Treg suppressive function, we are also investigating the potential use of this reagent in modulating immune responses in disease models of transplantation (47), autoimmune disease, chronic infection, and cancer to benefit clinical outcome.

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Disclosures

E.R.P., S.Q.K., T.H.S., D.W., and V.V.D. have patent applications relevant to material described in this manuscript. M.S.T. has no financial conflicts of interest.

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