

A Major Lipid Raft Protein Raftlin Modulates T Cell Receptor Signaling and Enhances Th17-Mediated Autoimmune Responses¹

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The membrane microdomains known as lipid rafts have been shown to act as platforms for the initiation of various receptor signals. Through proteomic analysis, we have identified a novel protein termed Raftlin (*raft-linking* protein) as a major protein in lipid rafts. To determine the physiological and immunological functions of Raftlin in mammals, we generated Raftlin-deficient mice, as well as Raftlin-transgenic (Tg) mice. Although Raftlin was originally identified in B cells, we observe no severe abnormalities in the B cells of these mice, presumably due to a high expression of Raftlin-homologue (Raftlin-2). T cells, in contrast, expressed a substantial amount of Raftlin but no Raftlin-2. In Raftlin-deficient mice, T cell-dependent Ab production was reduced, and experimental autoimmune encephalomyelitis, a Th17-dependent autoimmune disease model, was ameliorated. In Raftlin-Tg mice, in contrast, Ab production was enhanced and experimental autoimmune encephalomyelitis was more severe. Cytokine production, especially that of IL-17, was reduced in Raftlin-deficient T cells, while it was enhanced in Raftlin-Tg T cells. We found that these changes were associated with the strength of the TCR-mediated signals. Importantly, localization of Lck protein in the lipid rafts was enhanced by Raftlin overexpression and reduced by Raftlin deficiency. These data indicate that Raftlin modulates TCR signals and is necessary for the fine-tuning of T cell-mediated immune responses. *The Journal of Immunology*, 2009, 182: 5929–5937.

BCR engagement by Ags leads to the activation of certain intracellular signaling molecules, which results in the proliferation of B cells (1) and the promotion of Ag-specific Ab secretion. TCR engagement by APC leads to the activation of other intracellular signaling molecules, which results in the proliferation of T cells and the secretion of various kinds of cytokines. These events are thought to occur in microdomains of the plasma membrane known as lipid rafts (2, 3).

Lipid rafts are rich in cholesterol and sphingolipids (4) and are insoluble in certain nonionic detergents such as Triton X-100 (5, 6) because of the closely packed structure. Certain lipid-modified proteins, such as the GPI-linked proteins, dually acylated Src family kinases, and isoprenylated small G proteins, are enriched in lipid rafts on steady state cells. In addition, some transmembrane proteins, such as TCR and BCR, associate with lipid rafts following stimulation by multivalent ligands (7, 8). Cellular activation

causes small lipid rafts to group into clusters, and large lipid rafts are thought to sustain efficient signal transduction (9). Several reports suggest another possibility, however: that lipid rafts are necessary for the termination of signaling by accumulating negative regulators such as Csk and Cbp (10). The precise role of lipid rafts in lymphoid cells is still unclear.

To clarify the function of lipid rafts, we used a strategy in which we first identified raft-resident proteins comprehensively by proteomic analysis, then generated knockout cells lacking the genes encoding each of these proteins. We characterized a novel major raft protein called Raftlin (*raft-linking* protein), which we found in Raji B cells. Raftlin is localized in lipid rafts by fatty acylation of N-terminal Gly2 and Cys3, as are the Src family kinases. Disruption of the Raftlin gene in DT40 B cells resulted in a marked reduction in the quantity of lipid raft components, including Lyn, and a reduction in BCR-mediated tyrosine phosphorylation and calcium mobilization (11). However, the role of Raftlin in mammalian lymphoid cells and in vivo immune responses has not been clarified.

To investigate the role of Raftlin in mice, we generated Raftlin-deficient mice as well as Raftlin-transgenic (Tg)³ mice that express Raftlin excessively in B cells and T cells. We could not find any obvious difference in BCR signaling between wild-type (WT) and Raftlin-deficient B cells, but T cell-mediated immune responses were impaired in Raftlin-deficient mice. In Raftlin-Tg T cells, in contrast, TCR signaling was potentiated, Ab production was enhanced, and experimental autoimmune encephalomyelitis (EAE)

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³ Abbreviations used in this paper: Tg, transgenic; Raftlin, *raft-linking* protein; WT, wild type; EAE, experimental autoimmune encephalomyelitis; DC, dendritic cell; BMDC, bone-marrow-derived DC; MOG, myelin oligodendrocyte glycoprotein; iTreg, inducible regulatory T cell; TNP, trinitrophenyl; KLH, keyhole limpet hemocyanin; EGFP, enhanced GFP.

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was more severe. These data indicate that Raftlin positively regulates TCR signaling, and that excessive Raftlin expression might be involved in the development or progression of T cell-mediated immune diseases.

Materials and Methods

Mice

To generate Raftlin-deficient mice, we constructed a targeting vector by replacing the genomic fragment containing the second exon that corresponds to Raftlin amino acid residues 1–48 with a PGK-NEO cassette. The cassette was flanked by 4.4 and 2.3 kb of genomic sequences on the 5' and 3' sides, respectively. The PGK-TK and the MC1-DTA genes were inserted for negative selection. Homologous recombination in murine embryonic stem cells was performed as described elsewhere (12) and was confirmed by Southern blot analysis. The chimeric mice were backcrossed to C57BL/6J mice at least six times.

To generate Raftlin-Tg mice that expressed Raftlin excessively in B and T cells, we constructed an Lck- E μ Raftlin transgene. Murine Raftlin cDNA was inserted into the p1026x vector, which consists of the murine *lck* proximal promoter, the Ig intronic H chain enhancer E μ , and the human nontranslatable growth hormone gene cassette; the fragments containing the transgene were injected into the zygote pronuclei of C57BL/6J mice.

All experiments using mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Kyushu University, Fukuoka, Japan.

Abs and cells

Anti-murine Raftlin Abs were obtained by immunizing rabbits with bacterially expressed GST fusion protein containing full-length murine Raftlin. We obtained anti-STAT5b, anti-ERK2, and anti-I κ B α Abs from Santa Cruz Biotechnology; anti-pERK, anti-p I κ B α , anti-pAKT, and anti-AKT from Cell Signaling Technology; anti-actin Ab and HRP-conjugated cholera toxin B subunit from Sigma-Aldrich; 4G10, an anti-phosphotyrosine Ab, from Upstate Biotechnology; anti-Lck Ab from Transduction Laboratories; and anti-CD28, biotinylated, and fluorochrome-conjugated Abs from eBioscience. Anti-CD3 Ab was purified from serum free culture supernatant of hybridoma (145-2C11) using Protein G Sepharose 4FF (GE Healthcare). Murine splenic T cells, murine splenic B cells, and the murine EL4 T cell-line were cultured in RPMI 1640 supplemented with 10% FCS, 50 μ M 2-ME, penicillin, and streptomycin. Splenic T cells were prepared using biotinylated anti-CD4 Ab (CD4⁺ cells) or biotinylated anti-Thy1.2 Ab (Thy1.2⁺ cells), streptavidin MACS microbeads, and MACS MS columns (Miltenyi Biotec) according to the manufacturer's protocol. Splenic naive CD4⁺ T cells were prepared by the negative selection method using biotinylated anti-Ter119, anti-DX5, anti-CD8 α , anti-CD11b, anti-CD11c, anti-B220, anti-CD25 Abs, streptavidin MACS microbeads, and MACS LD columns. Splenic B cells were prepared as described elsewhere (13). Bone-marrow-derived dendritic cells (BMDCs) were prepared from bone marrow suspension from murine femurs and tibias. Bone marrow cells were differentiated for 7 days using RPMI 1640 supplemented with 10% FCS, 50 μ M 2-ME, penicillin, streptomycin, and the culture supernatant from J558L cells transfected with the murine GM-CSF gene.

Ag-specific Ab production assay

For the T cell-independent response, mice were immunized i.p. with 10 μ g of trinitrophenyl (TNP)-Ficoll (Biosearch Technologies). Mice were bled before immunization and on day 7 after immunization. The titers of Ag-specific Abs in these sera were analyzed using ELISA plates coated with TNP-BSA (Biosearch Technologies) and ELISA quantitation kits (Bethyl Laboratories). For the T cell-dependent response, mice were immunized s.c. with 100 μ g of DNP-keyhole limpet hemocyanin (KLH) (LSL Co.) in CFA on day 0 and were boosted i.p. with 50 μ g of DNP-KLH in saline on day 21. Mice were bled on days 7 and 28 after immunization. The titers of Ag-specific Abs in these sera were analyzed using ELISA plates coated with DNP-BSA (LSL) and ELISA quantitation kits. One month after the latest immunization, the splenocytes (1×10^6 cells/well) from these mice were cultured with or without 10 μ g/ml DNP-KLH for 2.5 days in a 96-well plate, and the amounts of IFN- γ contained in the supernatants were determined using an ELISA kit (eBioscience).

B cell proliferation assay

Splenic B cells (2×10^5 cells/well) were cultured with or without 10 μ g/ml anti-IgM Abs for 2 days in a 96-well plate, the final 20 h of the incubation being performed in the presence of BrdU. The incorporation of BrdU was evaluated using a Cell Proliferation ELISA, BrdU kit (Roche).

OVA-induced asthma model

Mice were sensitized with i.p. injections of 20 μ g of OVA (Sigma-Aldrich) plus 2.25 mg of aluminum hydroxide (Pierce) on days 1 and 14. On days 26–28, mice received an aerosol challenge containing 1% OVA for 20 min/day. On day 30, 36 h after the last aerosol challenge, mice were ventilated to measure airway hyperresponsiveness to acetylcholine aerosol as described elsewhere (14). Airway opening pressure was measured with a differential pressure transducer and continuously recorded. Stepwise increases in the acetylcholine dose were given with an ultrasonic nebulizer.

EAE induction

Myelin oligodendrocyte glycoprotein (MOG)_{35–55} peptide (MEVGWYRSPFSRVVHLYRNGK) (BEX) was used to induce EAE. In brief, mice were injected s.c. with 200 μ g of MOG peptide in 100 μ l of PBS emulsified in 100 μ l of CFA that was further enriched with 5 mg/ml *Mycobacterium tuberculosis* (H37Ra; Difco/BD Pharmingen). In addition, 500 ng of pertussis toxin (Calbiochem) was injected i.p. on days 0 and 2. Paralysis was evaluated according to the following scores: 0 = no disease, 1 = tail limpness, 2 = hind limb weakness, 3 = hind limb paralysis, 4 = forelimb weakness, 5 = quadriplegia, 6 = death. We also recorded relative weight, meaning the ratio of that day's weight to the weight measured on day 2. After 11 days, splenocytes (5×10^5 cells/well) from these mice were cultured with MOG peptide at the indicated concentrations in a 96-well plate and the amounts of IL-17 in the culture supernatants were measured with an ELISA kit (eBioscience).

Cytokine production assay

Splenic CD4⁺ cells (4×10^5 cells/well for Raftlin-deficient mice and 2×10^5 cells/well for Raftlin-Tg mice) were stimulated with various concentrations of plate-bound anti-CD3 Ab for 4 days in a 24-well plate. Splenic OTII CD4⁺ cells (1×10^5 cells/well) were cocultured with BMDCs (2×10^4 cells/well) in the presence of 20 μ g/ml OVA and 10 μ g/ml *M. tuberculosis* for 6 days in a 96-well plate. The amounts of IL-17, IFN- γ , and IL-4 in the culture supernatants were measured with ELISA kits. For the induction of Th17 differentiation, splenic naive CD4⁺ T cells (5×10^5 cells/well) were cultured with various concentrations of plate-bound anti-CD3 Ab, 10 μ g/ml anti-IFN- γ Ab, 10 μ g/ml anti-IL-4 Ab, 1 ng/ml human TGF- β (R&D Systems), and 20 ng/ml human IL-6 (R&D Systems) in the absence or presence of 0.5 μ g/ml anti-CD28 Ab for 4.5 days in a 24-well plate, and restimulated with 50 ng/ml PMA and 1 μ g/ml ionomycin for 4 h in the presence of brefeldin A, a protein transport inhibitor (eBioscience). These cells were stained with PerCP-conjugated anti-CD4 Ab and allophycocyanin-conjugated anti-IL17 Ab using a fixation and permeabilization kit (eBioscience).

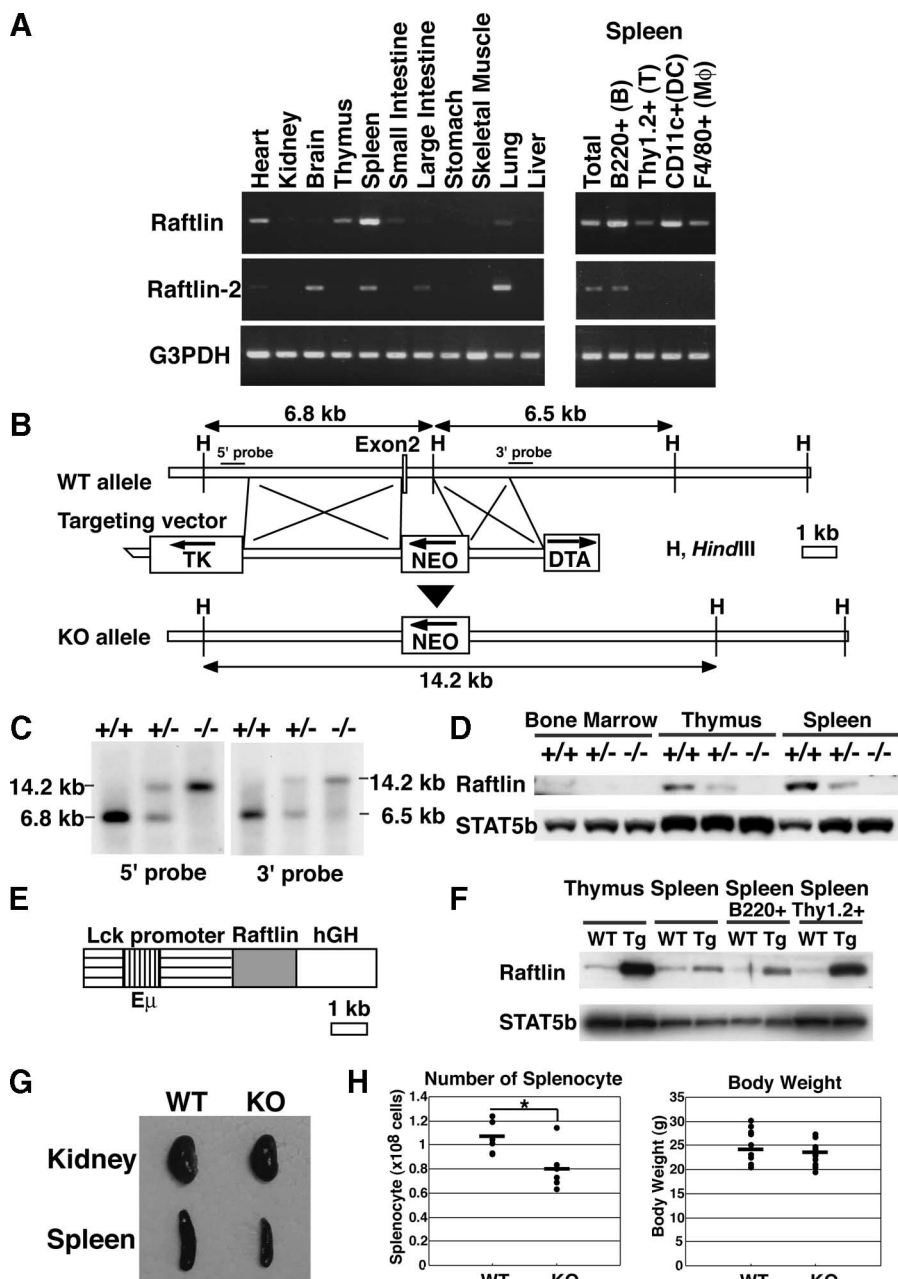
Microscopic observation of T cells

Anti-CD3 and anti-CD28 Abs immobilized on beads and OVA_{323–339} peptide (ISQAVHAAHAEINEAGR) (Sigma-Aldrich) presented on BMDCs were prepared for the EL4 T cell and splenic OTII CD4⁺ T cell stimulations, respectively. The mixture of anti-CD3 and anti-CD28 Abs was immobilized with NHS-activated Sepharose 4FF (GE Healthcare) according to the manufacturer's protocol. Immature BMDCs that were cultured in medium containing GM-CSF for 7 days were incubated with 1 μ g/ml OVA peptide at 37°C for 3 h and activated by 10 ng/ml LPS overnight. These mature BMDCs were washed with the medium and immobilized on glass-bottom dishes coated with anti-Fc γ R Ab. To express murine Raftlin-enhanced GFP (EGFP) and murine CD3 ζ -DsRed monomer in T cells, murine Raftlin and murine CD3 ζ cDNAs were cloned into pEGFP-N1 and pDsRed Monomer-N1 vectors (Clontech Laboratories), respectively. Before the transfection, primary splenic OTII CD4⁺ T cells were maintained in medium supplemented with 1 μ g/ml soluble anti-CD28 Ab on plates precoated with 2 μ g/ml anti-CD3 Ab for 1 day. EL4 T cells and splenic OTII CD4⁺ T cells were transfected by these plasmids using the Amaxa electroporation system (Amaxa Biosystems). After 16–24 h of electroporation, these T cells were observed using the DeltaVision RT system (Applied Precision). EL4 T cells and splenic OTII CD4⁺ T cells expressing Raftlin-EGFP and CD3 ζ -DsRed monomer were incubated with the anti-CD3 and anti-CD28 Ab-beads and the BMDCs presenting OVA peptide, respectively, at 37°C for 30 min to observe the immune synapse.

Measurement of the intracellular Ca²⁺ mobilization

Splenic CD4⁺ T cells were incubated with HBSS/0.5% BSA containing 3 μ M Indo-1/AM (Molecular Probes) (1×10^7 cells/ml) for 45 min at 37°C and washed with HBSS/0.5% BSA. One $\times 10^6$ cells were resuspended in 1 ml of HBSS/0.5% BSA and prewarmed for 15 min at 37°C. Cell suspension was stimulated with anti-CD3 Ab, and the Ca²⁺ flux (Ca²⁺-bound

FIGURE 1. Generation of Raftlin-deficient mice and Raftlin-Tg mice. **A**, RT-PCR analysis of murine Raftlin, Raftlin-2, and control G3PDH expression in total RNA of murine tissues and cells isolated from spleen. **B**, Schematic representation of WT and mutant (KO) loci of the *Raftlin* gene together with the targeting vector. The neomycin-resistance gene driven by a PGK promoter, the herpes simplex virus thymidine kinase gene driven by a PGK promoter, and the gene coding the diphtheria toxin fragment A driven by an MC1 promoter are indicated by NEO, TK, and DTA, respectively. **C**, Representative Southern blot analysis with *HindIII*-digested DNA. The positions of probes are shown in **B**. **D**, Western blotting of bone marrow, thymus, and spleen with anti-Raftlin Abs and control anti-STAT5b Abs. **E**, The construct of the Lck-E μ Raftlin transgene. The murine *lck* proximal promoter, the Ig intronic H chain enhancer, the murine Raftlin and hGH, respectively, cDNA, and the human nontranslatable growth hormone gene are indicated by Lck promoter, E μ , Raftlin, and human nontranslatable growth hormone, respectively. **F**, Western blotting of thymus, spleen, B220⁺ cells of spleen, and Thy1.2⁺ cells of spleen from WT and Tg mice with anti-Raftlin Abs and control anti-STAT5b Abs. **G**, Comparison of spleens and kidneys between WT and Raftlin-deficient mice. **H**, Number of splenocytes and body weights of 12- to 16-wk-old mice. Each dot represents a mouse, while bars represent the mean. *, $p < 0.05$ by Student's *t* test.



Indo-1 (FL5, 400 nm emission)/Ca²⁺-free Indo-1 (FL4, 510 nm emission)) was monitored using a BD-LSR FACS machine equipped with a UV laser and CellQuest software (BD Biosciences).

Subcellular fractionation of splenic T cells

Splenic Thy1.2⁺ cells (6×10^7 cells or 4×10^7 cells) were lysed in 150 μ l of Triton X-100 lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM NaF, 1 mM Na₃VO₄, 0.1% Triton X-100, 5 mM 2-ME, 5% glycerol, and protease inhibitor mixture) and the lysate was mixed with an equal volume of 80% sucrose in buffer A (50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 1 mM Na₃VO₄, and protease inhibitor mixture). The mixture was transferred to a centrifuge tube and sequentially overlaid with 800 μ l of 35% sucrose in buffer A and 300 μ l of 5% sucrose in buffer A. After centrifugation at $100,000 \times g$ at 4°C for 16 h, 135 μ l fractions were collected from the top of the tube.

Results

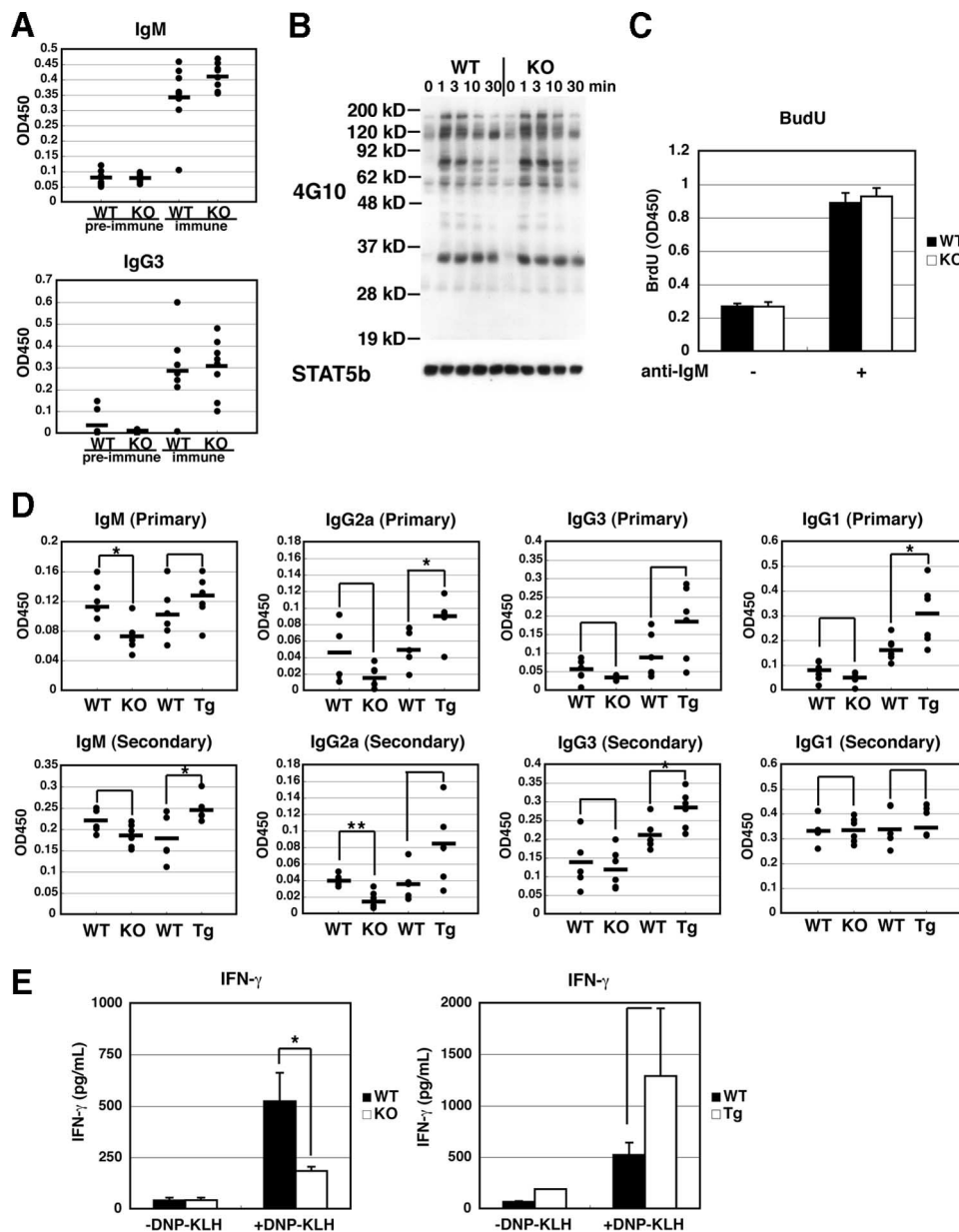
Generation of Raftlin-deficient and Raftlin-Tg mice

The tissue and cell distribution of murine Raftlin RNA was examined by RT-PCR analysis (Fig. 1A). Compared with the other tis-

sues we examined, Raftlin mRNA was expressed abundantly in the spleen and thymus; it was detected in the various immune cells, such as B cells, T cells, dendritic cells (DCs), and macrophages. The expression of Raftlin-2 mRNA, in contrast, was restricted to B cells in the spleen.

To examine the function of Raftlin in lymphocytes, we generated mice lacking the *Raftlin* gene by homologous recombination techniques (Fig. 1B) and Tg mice expressing Raftlin excessively in T and B cells (Fig. 1E). Disruption of Raftlin in embryonic stem cells was achieved by replacing exon 2 of the *Raftlin* gene with a PGK-NEO cassette. Successful recombination was confirmed by Southern blotting of murine livers (Fig. 1C), and the complete absence of the Raftlin protein in the Raftlin-deficient mice was confirmed by Western blotting of tissue extracts (Fig. 1D). The excessive expression of Raftlin in the splenic T cells (Thy1.2⁺) and B cells (B220⁺) of our Raftlin-Tg mice was also confirmed by Western blotting (Fig. 1F). The excessive expression of Raftlin did not affect either the number of splenocytes or the size of the spleen (data not shown).

FIGURE 2. Raftlin enhanced T cell dependent Ag-specific Ig production. **A**, Humoral immune responses to T cell independent Ag. Wild-type and Raftlin-deficient mice were immunized with TNP-Ficoll. The serum titers of TNP-specific Igs of the indicated isotypes before (pre-immune) and on day 7 after immunization (immune) were determined by ELISA. Each dot represents a mouse and bars represent the mean serum titer of Ag-specific Ig. **B**, Splenic B cells from WT and Raftlin-deficient mice were stimulated with 10 μ g/ml anti-IgM Abs for the length of time indicated. The whole-cell lysates were loaded onto SDS-PAGE and analyzed by Western blotting with anti-phosphotyrosine Ab (4G10). **C**, Splenic B cells from WT and Raftlin-deficient mice were cultured with or without anti-IgM Abs for 2 days, and the cell proliferations were evaluated by BrdU incorporation during DNA synthesis. **D**, Humoral immune responses to T cell dependent Ag. DNP-KLH/CFA was administered to WT and Raftlin-deficient mice or to WT and Raftlin-Tg mice. Mice were boosted with DNP-KLH on day 21 to elicit secondary responses. The serum titers of DNP-specific Igs of the indicated isotypes on day 7 (primary) and on day 28 (secondary) after the first immunization were determined by ELISA. Each dot represents a mouse and bars represent the mean serum titer of Ag-specific Ig. *, $p < 0.05$; **, $p < 0.01$ by Student's t test. **E**, One month after the last immunization with DNP-KLH, the splenocytes from these mice were cultured with or without DNP-KLH for 2.5 days, and IFN- γ levels in the supernatants were determined by ELISA. Data are represented as mean $\pm$ STD. *, $p < 0.05$ by Student's t test. One representative data performed triplicates are shown.



Raftlin-deficient offspring were born at the expected Mendelian ratio from intercrosses of heterozygotes and were fertile. These data indicate that Raftlin is not necessary for fertility and development. Adult Raftlin-deficient mice in the SPF condition appeared to be healthy and showed no apparent abnormalities in most organs; the one exception was their smaller spleens (Fig. 1G).

Raftlin-deficient mice had significantly fewer splenocytes than WT mice (Fig. 1H), although their T cell development in the thymus and B cell development in bone marrow were both normal (data not shown). The ratio of B cells/T cells and that of CD4⁺ T cells/CD8⁺ T cells in the spleens of Raftlin-deficient mice were similar to those of WT mice (data not shown).

Raftlin is involved in T cell-dependent but not T cell-independent Ab production

To investigate the role of Raftlin in vivo, we first examined the influence of Raftlin-expression level on the induction of Ag-specific humoral immune responses using a group of T cell-independent and T cell-dependent Ags (Fig. 2). The serum Ig levels of all isotypes examined in nonimmunized mice were the same in

Raftlin-deficient and Raftlin-Tg mice as in WT littermates (data not shown). When mice were immunized with TNP-Ficoll, a T cell-independent Ag, Raftlin-deficient mice showed normal levels of Ag-specific response (Fig. 2A). In our previous report, we found that BCR signaling of Raftlin-deficient B cells was reduced compared with that of WT cells. Therefore, we measured BCR-induced proliferation and tyrosine phosphorylation of cellular proteins in splenic B cells in vitro (Fig. 2, B and C). There was no difference in the levels of tyrosine phosphorylation between WT mice and Raftlin-deficient mice (Fig. 2B). BCR-mediated proliferation was also similar between WT mice and Raftlin-deficient mice (Fig. 2C). These findings are consistent with our observation of similar T cell-independent Ag-specific Ab production between WT mice and Raftlin-deficient mice (Fig. 2A). One possible explanation for the unexpectedly small effect of Raftlin-deficiency on B cells is the compensation by Raftlin-2 (Fig. 1A). WT T cells, in contrast, expressed only a small amount of Raftlin-2 (Fig. 1A).

When mice were immunized with DNP-KLH/CFA, a T cell-dependent Ag, Raftlin-deficient mice showed a reduction of DNP-specific IgG2a, while Raftlin-Tg mice showed an enhancement of

DNP-specific IgG2a (Fig. 2D). To confirm whether T cell responses were altered in Raftlin-deficient and Tg mice, we examined IFN- γ secretion from splenocytes. As expected, Raftlin-deficient splenocytes from mice immunized with DNP-KLH secreted less IFN- γ than WT splenocytes after *in vitro* restimulation with DNP-KLH (Fig. 2E). In contrast, Raftlin-Tg splenocytes secreted more IFN- γ than WT splenocytes (Fig. 2E). In our Tg mice, the exogenous Raftlin level was lower in the B cells than in the T cells (Fig. 1F). This may account for the similar humoral immune responses of Raftlin-Tg mice and WT mice to a T cell-independent Ag (TNP-Ficoll) (data not shown). When a T cell-dependent Ag (DNP-KLH/CFA) was used, however, we clearly observed enhanced humoral immune responses in Raftlin-Tg mice (Fig. 2D).

Raftlin was not involved in OVA-induced asthma, a Th2-mediated disease

To investigate the involvement of Raftlin in T cell dependent diseases, we examined OVA-induced asthma as a Th2-cell mediated disease model. After mice were systemically sensitized to OVA and subjected to aerosolized OVA challenges, we measured their airway responsiveness to acetylcholine aerosol using an invasive technique. Rates of airway hyperresponsiveness were the same in Raftlin-deficient mice, Raftlin-Tg mice, and WT mice (Fig. 3A).

Raftlin is deeply involved in EAE, a Th17-mediated disease

Next, we examined MOG-induced EAE to investigate the involvement of Raftlin in Th17 cell-mediated disease. We used Raftlin-deficient mice that were backcrossed at least nine times; even so, we still compared them with WT littermates to reduce the background effects of the ES strain. WT mice exhibited characteristic signs of EAE starting on day 7, whereas Raftlin-deficient mice developed less severe symptoms of EAE compared with WT mice (Fig. 3B, days 8–10). Raftlin-Tg mice, in contrast, initially developed EAE symptoms similar to those seen in littermate WT mice, but after day 12, their condition was observed to be more severe; it also lasted longer, beyond day 21.

We then examined cytokine secretion from splenocytes in mice with EAE. Splenocytes were isolated from the spleens of mice on day 11 and restimulated with MOG peptide. Restimulated splenocytes from Raftlin-deficient mice showed a reduction in IL-17 production compared with those from WT mice, while those from Raftlin-Tg mice showed an increase in IL-17 production (Fig. 3C). In addition, cytokine expression in the spinal cords on day 11 was examined by RT-PCR (Fig. 3D). Various kinds of cytokines were expressed at low levels in the spinal cords of Raftlin-deficient mice and at higher levels in the spinal cords of Raftlin-Tg mice. Histological examination revealed that the number of immune cells invading the spinal cords was increased in Raftlin-Tg mice and reduced in Raftlin-deficient mice (data not shown). These data indicate that excessive Raftlin expression leads to excessive IL-17 production, which deteriorates Th17-mediated autoimmune disease.

Raftlin modulates TCR-mediated cytokine production

Next, we measured TCR-mediated T cell responses *in vitro*. Proliferation of Raftlin-deficient T cells was not altered, while that of Raftlin-Tg T cells was slightly enhanced (data not shown). Then cytokines secreted from CD4⁺ T cells were measured after stimulation with plate-bound anti-CD3 Ab in various concentrations (Fig. 4, A and B). The CD4⁺ T cells of Raftlin-deficient mice responded less efficiently to the anti-CD3 Ab for IFN- γ and IL-4 secretion than WT cells did; they needed a higher concentration of the Ab to secrete the same amount of cytokines (Fig. 4A). The difference between WT and Raftlin-deficient T cells in the secre-

tion of IL-17 was more obvious than that in the secretion of IFN- γ or of IL-4. IL-17 levels were always lower in Raftlin-deficient T cells than in WT T cells at any anti-CD3 concentrations (Fig. 4A). In contrast, the CD4⁺ T cells of Raftlin-Tg mice responded more sensitively to the anti-CD3 Ab (Fig. 4B). Again, the most obvious difference between WT and Raftlin-Tg T cells in terms of cytokine secretion was in the secretion of IL-17. We also investigated the whether there was a difference in the cell survivability between WT and Raftlin-deficient T cells or WT and Raftlin-Tg T cells under stimulation by anti-CD3 Ab with or without anti-CD28 Ab. We observed no differences in the cell death between WT and Raftlin-deficient T cells or between WT and Raftlin-Tg T cells under either set of conditions (supplemental Fig. 1).⁴

Next, we examined Ag-specific T cells responses by using OTII transgenic mice that have MHC class II restricted OVA_{323–339}-specific TCR transgene (Fig. 4, C and D). Raftlin-deficient or Raftlin-Tg mice were crossed with OTII-Tg mice, and CD4⁺ T cells were stimulated with OVA-loaded BMDCs. As we might have expected given the results obtained through anti-CD3 stimulation, the amount of IL-17 secreted from Raftlin-deficient OTII CD4⁺ T cells was less than that secreted from WT T cells (Fig. 4C), while that secreted from Raftlin-Tg OTII CD4⁺ T cells was greater than that secreted from WT T cells (Fig. 4D). IFN- γ production, however, did not change markedly (Fig. 4, C and D), and IL-4 was not detectable under these conditions (data not shown). These data indicate that Raftlin has a more profound effect on IL-17 production than it has on IFN- γ or IL-4 production. Th17 differentiation seemed to require stronger TCR signaling than Th1 or Th2 differentiation did (Fig. 4, A and B), suggesting that Raftlin is involved in determining the strength of TCR signaling.

To investigate the involvement of Raftlin in Th17 differentiation from naive T cells, naive CD4⁺ T cells were differentiated to Th17 with TGF- β and IL-6 in the absence or presence of anti-CD28 Ab (Fig. 4, E and F). Th17 differentiation from Raftlin-deficient naive T cells was insufficient compared with that from WT naive T cells, especially under weak stimulation conditions (0.1–1 μ g/ml anti-CD3 Ab without anti-CD28 Ab and 0.1 μ g/ml anti-CD3 Ab with anti-CD28 Ab). In contrast, under strong TCR signaling conditions, there was little difference between WT and Raftlin-deficient T cells in terms of Th17 differentiation. These data suggest that Raftlin is necessary for the enhancement of signals when the amount of Ag is limited. On the other hand, naive CD4⁺ T cells from Raftlin-deficient mice differentiate to Th1, Th2, and inducible regulatory T cells (iTreg) normally (supplemental Fig. 2).

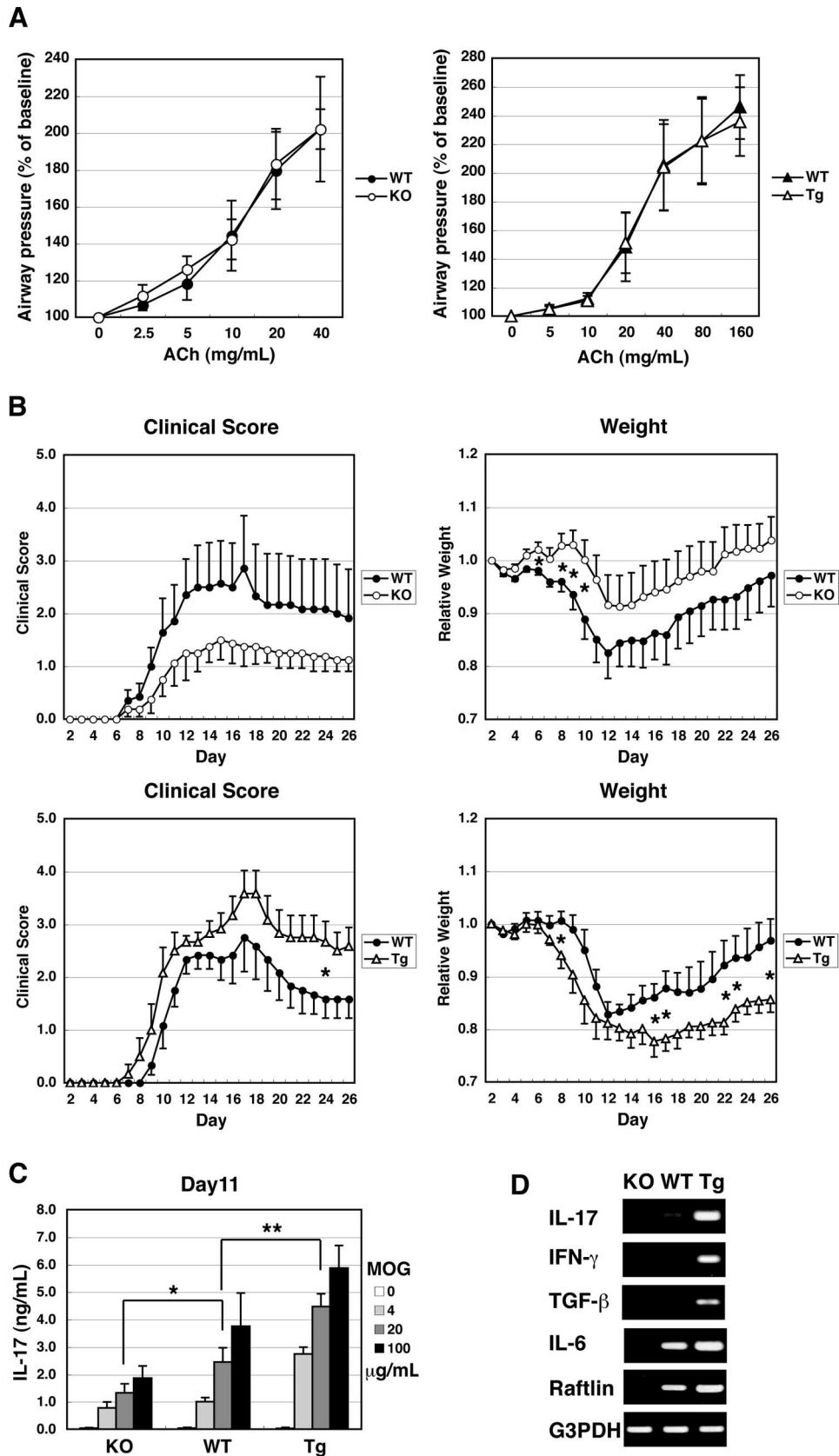
Raftlin affects TCR signaling

We identified Raftlin in lipid rafts fractions of B cells. To investigate the intracellular localization of Raftlin in T cells, EL4 T cells, and OTII CD4⁺ T cells were transfected with CD3 ζ -DsRed Monomer and Raftlin-EGFP cDNAs. Raftlin-EGFP was uniformly localized in the plasma membrane without stimulation (data not shown). Immune synapse was formed by treating EL4 T cells with the beads immobilizing anti-CD3 and CD28 Abs or by culturing OTII CD4⁺ T cells with BMDCs presenting OVA_{323–339} peptide (Fig. 5A). CD3 ζ -DsRed Monomer assembled on the contact side in these T cells at 30 min after stimulation. Raftlin-EGFP was concentrated at the same position, suggesting that Raftlin is accumulated in the immunological synapse of T cells.

Next, we investigated TCR-induced cellular protein phosphorylation by Western blotting (Fig. 5, B and C). Tyrosine phosphorylation of some proteins (for example, some bands stained with

⁴ The online version of this article contains supplementary material.

FIGURE 3. The level of Raftlin expression affected Th17-dependent disease. **A**, Effect of Raftlin expression on asthmatic responses in an OVA-induced asthma as a Th2-dependent disease model. Mice were sensitized and aerosol challenged with OVA. Airway responsiveness was determined by the acetylcholine-dependent change in airway pressure. Data are represented as mean $\pm$ SEM ($n = 8$). **B**, Effect of Raftlin expression on autoimmune responses in a MOG-induced EAE as a Th17 dependent disease model. Raftlin-deficient mice and their littermate WT mice or Raftlin-Tg mice and their littermate WT mice were immunized with MOG peptide in CFA and were administered *Pertussis toxin* on days 0 and 2 ($n = 8, 7, 6$, and 6). Their clinical scores and the relative body weights (body weight/body weight on day 2) were monitored every day. Data are represented as mean $\pm$ SEM. *, $p < 0.05$ by Student's *t* test. **C**, On day 11 after immunization, the splenocytes from these mice were re-stimulated with MOG peptide for 4 days, and IL-17 concentration in the cultured medium was determined by ELISA. Data are represented as mean $\pm$ STD. *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test. **D**, RT-PCR was performed using total RNA of the spinal codes from these mice on day 11 after immunization.



anti-phosphotyrosine Ab, 4G10, including 120 kDa, 60 kDa, and 20 kDa) and phosphorylation of ERK2, I κ B α , and AKT were reduced in Raftlin-deficient T cells compared with WT T cells (Fig. 5B). In Raftlin-Tg T cells, in contrast, these phosphorylations were enhanced (Fig. 5C). We also investigated TCR-induced intracel-

lular Ca²⁺ mobilization (Fig. 5, D and E). When the T cells were stimulated with a low concentration of anti-CD3 Ab, the intracellular Ca²⁺ increase in Raftlin-deficient T cells was reduced and that in Raftlin-Tg T cells was enhanced compared with that in WT littermate T cells.

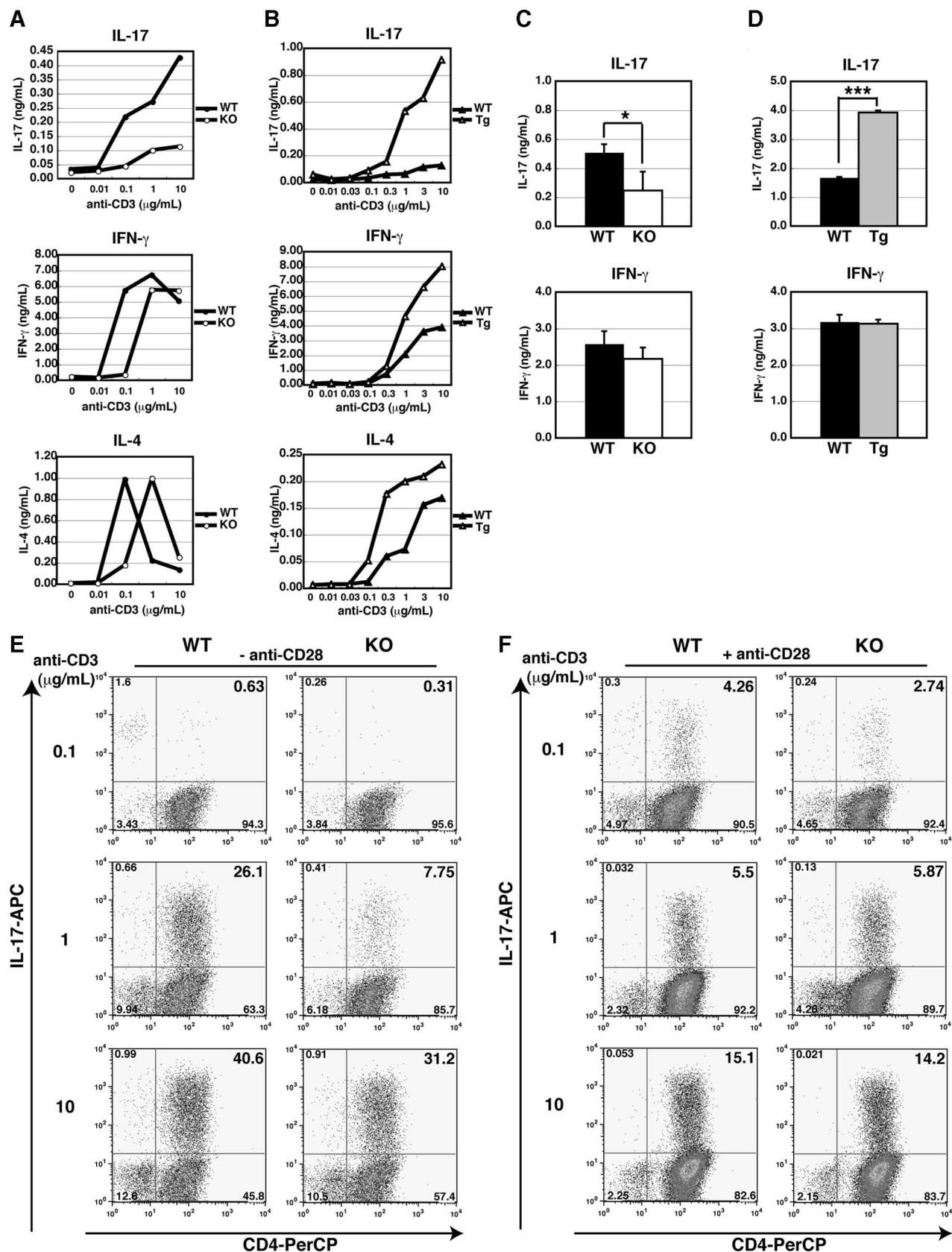


FIGURE 4. Raftlin enhanced the secretion of various cytokines from T cells. Splenic CD4⁺ cells from WT and Raftlin-deficient mice (A) or splenic CD4⁺ cells from WT and Raftlin-Tg mice (B) were stimulated with various concentrations of plate-bound anti-CD3 Ab for 4 days. The amounts of IL-17, IFN-γ, and IL-4 in the culture supernatants were calculated using ELISA. Splenic CD4⁺ cells from OTII-Tg and OTII-Tg/Raftlin-deficient mice (C) or splenic CD4⁺ cells from OTII-Tg and OTII-Tg/Raftlin-Tg mice (D) were cocultured with BMDCs in the presence of OVA for 6 days. The levels of IL-17 and IFN-γ in the culture supernatants were calculated using ELISA. Data are represented as mean ± STD. *, $p < 0.05$; ***, $p < 0.005$ by Student's *t* test. Naive CD4⁺ T cells from WT and Raftlin-deficient mice were cultured under the Th17 conditions in the absence (E) or presence (F) of anti-CD28 Ab for 4.5 days, and internal staining of IL-17 was applied. One representative data of performed triplicates are shown.

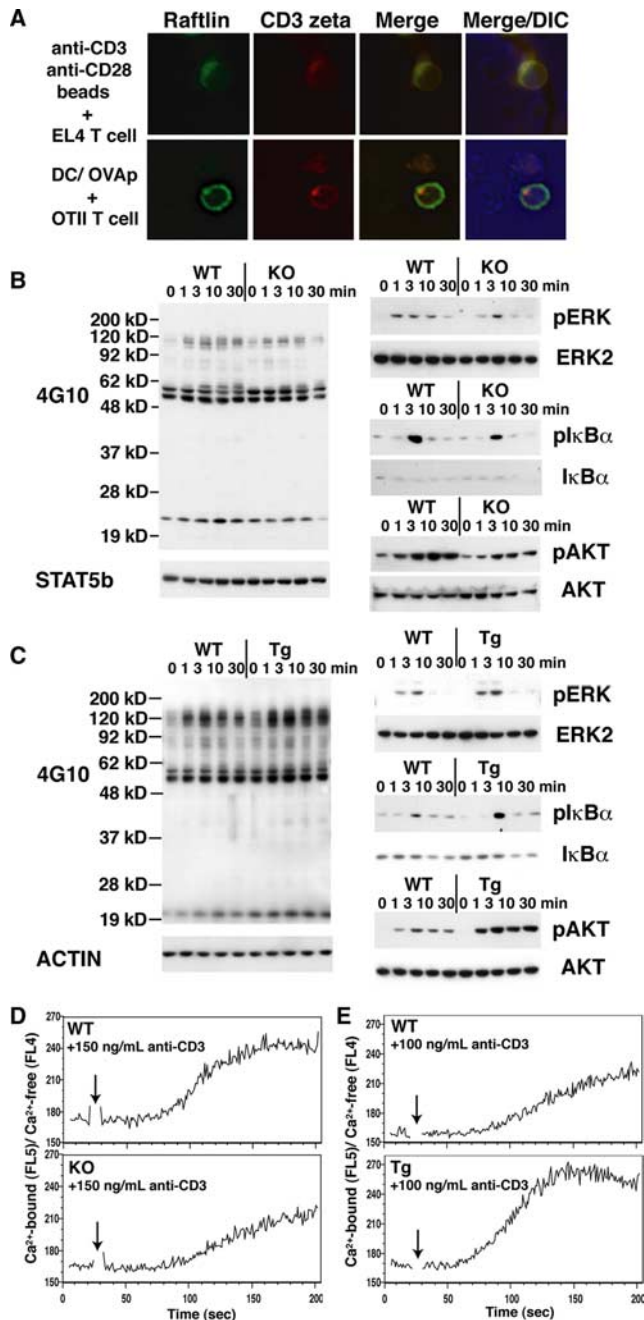


FIGURE 5. The level of Raftlin expression influenced the protein phosphorylation induced by TCR stimulation. *A*, EL4 T cells expressing Raftlin-EGFP and CD3 ζ -DsRed monomer were cocultured with the beads immobilizing anti-CD3 Ab and anti-CD28 Ab for 30 min at 37°C (upper column). CD4⁺ cells expressing Raftlin-EGFP and CD3 ζ -DsRed monomer prepared from OTII-Tg mice were cocultured with BMDCs presenting OVA peptide for 30 min at 37°C (lower column). *B*, Splenic Thy1.2⁺ cells from WT and Raftlin-deficient mice or (*C*) splenic Thy1.2⁺ cells from WT and Raftlin-Tg mice were stimulated with 5 μ g/ml anti-CD3 Ab for the length of time indicated. The whole-cell lysates were loaded onto SDS-PAGE and analyzed by Western blotting with the indicated Abs. *D*, Splenic CD4⁺ cells from WT and Raftlin-deficient mice or (*E*) splenic CD4⁺ cells from WT and Raftlin-Tg mice labeled with Indo-1/AM were stimulated with 150 ng/ml or 100 ng/ml anti-CD3 Ab, and the Ca²⁺ flux was monitored. The ratio metric analysis of Ca²⁺-bound Indo-1 (FL5, 400 nm emission)/Ca²⁺-free Indo-1 (FL4, 510 nm emission) is shown. The arrows indicate the points in time at which stimulation was applied.

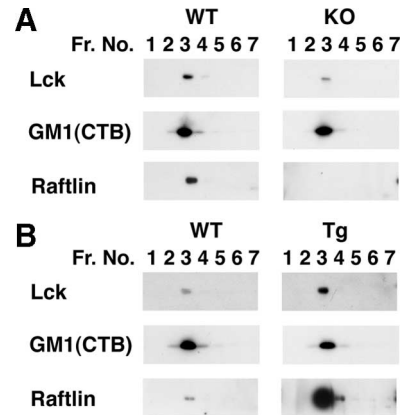


FIGURE 6. Raftlin is necessary for the localization of Lck in lipid rafts on resting T cells. Splenic Thy1.2⁺ cells (6×10^7 cells) from WT and Raftlin-deficient mice (*A*) or splenic Thy1.2⁺ cells (4×10^7 cells) from WT and Raftlin-Tg mice (*B*) were lysed and fractionated in a sucrose gradient. Western blotting was performed using anti-Lck Ab, anti-Raftlin Abs, and HRP conjugated cholera toxin B subunit (CTB) that binds ganglioside GM₁ as a marker of raft fraction. One representative data of performed triplicates are shown (*A* and *B*).

To investigate the mechanism of modulation of intracellular signaling by Raftlin in T cells, we performed fractionation using sucrose gradient centrifugation (Fig. 6, *A* and *B*). Raft fractions in Raftlin-deficient T cells contained less Lck than in WT T cells, (Fig. 6*A*, fraction 3), while raft fractions in Raftlin-Tg T cells contained more Lck (Fig. 6*B*, fraction 3). Because Lck is a kinase that is activated first by TCR stimulation, the amount of Lck that exists on the lipid rafts in a steady state might influence the intensity of downstream events including protein tyrosine phosphorylation.

Discussion

In this study, we report that the amount of Lck in resting T cells was reduced in Raftlin-deficient T cells and increased in Raftlin-Tg T cells. The intensity of TCR signaling, revealed in such processes as intracellular protein phosphorylation and cytokine production, was also reduced in Raftlin-deficient T cells and increased in Raftlin-Tg T cells. Considering that TCR signaling is initiated by the translocation of TCR into lipid rafts that include Lck, and that Lck is a kinase that initiates TCR signaling, the intensity of TCR signaling might correlate with the amount of Lck in the lipid rafts of T cells.

In our previous report, disruption of the Raftlin gene in the chicken DT40 B cell line resulted in a reduction in the quantity of lipid raft components, including Lyn and the ganglioside GM₁, while overexpression of Raftlin increased the amount of raft proteins (11). Raftlin is localized in lipid rafts by dual acylation, as are Src family kinases such as Lck and Lyn (15). Because Raftlin is so abundant in the lipid rafts, we suspected that it is involved in the integrity of the raft and that it might promote the accumulation of other membrane proteins into the lipid rafts. In an observation compatible with this theory, we showed that the amount of Lck in T cell lipid rafts changed in the absence or overabundance of Raftlin, as did the amount of Lyn in DT40 B cell lipid rafts. However, in contrast to DT-40 B cells, the amounts of the other proteins and of GM₁ in T cell lipid rafts were not changed. It is not clear at present why the amounts of GM₁ and of proteins other than Lck were not so influenced by the level of Raftlin expression in primary T cells. We noticed that the levels of Raftlin in primary T cells from Tg mice were not as high as those in Raji and DT-40

cells. Therefore, on a physiological level, Raftlin may influence the levels of specific raft proteins but not those of total raft proteins.

The remaining question is the biochemical role of Raftlin. Considering that Raftlin is conserved among vertebrates and that it has two highly conserved domains, there is a possibility that Raftlin interacts with other molecules. To test this possibility, we have searched for binding partners that were coprecipitated, and we have identified two molecules: clathrin H chain and huntingtin-interacting protein 1 (K. Saeki and A. Yoshimura, unpublished data), both of which are involved in vesicle transport (16). Despite this, the absence of Raftlin had no effect on either the amount of TCR on the cell surface or the internalization rate of TCR after stimulation. Further investigation is necessary to determine the role of Raftlin in vesicular transport.

In the present study, we investigated the cytokines produced by various concentrations of plate-bound anti-CD3 Ab. We noticed that the intensity of Raftlin's effect varies among cytokines; IL-17 was most strongly affected by the amount of Raftlin, while IFN- γ and IL-4 were modestly affected. IL-17 levels were affected by Raftlin when Ag-specific T cells were stimulated with Ag-peptide loaded DCs, while IFN- γ levels were not affected. We also confirmed that TGF- β -mediated Foxp3 induction was similar in WT and Raftlin-deficient T cells (data not shown). Therefore, Raftlin affects mostly Th17, but does not affect Th1, Th2, or iTreg polarization. These data suggest that suitable intensity of TCR stimulation is necessary for the production of each cytokine: IL-4 is saturated in response to the weakest stimulation, and IL-17 is saturated in response to the strongest stimulation. We suspect that the fact that Th17 differentiation requires stronger TCR signaling than other helper T cell lineages is the reason why Raftlin has such a profound effect on IL-17 production, as seen in the comparison between WT T cells and Raftlin-deficient T cells, over a broad range. However, we cannot rule out the possibility that Raftlin is deeply involved in an unknown pathway that is specific to IL-17 production or to Th17 differentiation. One possibility is the requirement of Raftlin for IL-2 signaling. IL-2 is known to suppress Th17 but rather enhance proliferation of other subsets. However, we did not see much difference in the iTreg population, which is strictly dependent on IL-2; this makes it appear less likely that Raftlin affects the IL-2 signaling pathway. Further investigation may uncover a novel mechanism for modulation of Th17 differentiation.

In the in vivo Th17-disease model, Raftlin-deficient mice showed less severe EAE and expressed smaller quantities of IL-6 and IL-17 in their spinal cords than WT mice did. Because IL-6 is a cytokine that induces Th17 differentiation, there was a possibility that the reduced Th17 development in Raftlin-deficient mice was also due to reduced IL-6 expression. The mechanism behind such a reduction in IL-6 levels in the Raftlin-deficient spinal cords is not clear at present, but Raftlin might have some function in monocytic cells, which causes IL-6 expression to be reduced in Raftlin-deficient spinal cords. To investigate this matter, we examined LPS-induced IL-6 production from Raftlin-deficient BMDCs, but we found no significant differences between WT and Raftlin-deficient DCs. Further study is necessary to clarify how Raftlin is involved in IL-6 production in the brain. Raftlin-Tg mice, in contrast, exhibited more severe EAE and expressed larger quantities of IFN- γ as well as IL-17 in their spinal cords compared with WT mice. A prior studies (17, 18) have suggested that the ratio of IL-17 to IFN- γ determines whether disease pathology occurs in the

spine or in the brain, with increasing levels of IL-17 associated with disease in the brain. However, a more recent study has demonstrated that lesion location is mainly controlled by the brain's response to IFN- γ (19). Thus, there is a possibility that a higher Th1 induction in Raftlin-Tg mice is also involved in determining disease severity.

Taken together, our data suggest that Raftlin might modulate the amount of Lck in lipid rafts, regulate the intensity of TCR signaling, and control immune responses in vivo.

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Disclosures

The authors have no financial conflict of interest.

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