INTRODUCTION

After antigen recognition, T lymphocytes require costimulatory signals for their optimal activation, differentiation, and survival to promote adaptive immune responses. A number of costimulatory receptors can contribute to T-cell responses, but TNF receptor superfamily (TNFRSF) proteins are crucial for the generation of long-lived effector and memory T cells. IQGAP1 restrains T-cell cosignaling mediated by OX40

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IQGAP1 restrains T-cell cosignaling mediated by OX40

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Abstract
A costimulatory signal from the tumor necrosis factor receptor (TNFR) family molecule OX40 (CD134), which is induced on activated T cells, is important for T-cell immunity. Aberrant OX40 cosignaling has been implicated in autoimmune and inflammatory disorders. However, the molecular mechanism by which the OX40 cosignaling regulates the T-cell response remains obscure. We found that OX40 associated with a scaffold protein, IQ motif-containing GTPase-activating protein 1 (IQGAP1) after ligation by its ligand OX40L. Naïve CD4+ T cells from Iqgap1−/− mice displayed enhanced proliferation and cytokine secretion upon receiving OX40 cosignaling. A C-terminal IQGAP1 region was responsible for its association with OX40, and TNFR-associated factor 2 (TRAF2) bridged these two proteins. The enhanced cytokine response in Iqgap1−/− T cells was restored by the expression of the C-terminal IQGAP1. Thus, the IQGAP1 binding limits the OX40 cosignaling. Disease severity of experimental autoimmune encephalomyelitis (EAE) was significantly exacerbated in Iqgap1−/− mice as compared to wild-type mice. Additionally, recipient mice with Iqgap1−/− donor CD4+ T cells exhibited significantly higher EAE scores than those with their wild-type counterparts, and OX40 blockade led to a significant reduction in the EAE severity. Thus, our study defines an important component of the OX40 cosignaling that restricts inflammation driven by antigen-activated T cells.

KEYWORDS
autoimmunity, CD4+ T cells, cosignaling, T-cell inflammation, TNFRSF
cells, and consequently they have been implicated in driving multiple inflammatory and autoimmune diseases.\textsuperscript{1,2} OX40 (CD134, TNFRSF4) is one typical TNF costimulatory receptor that is induced on the surface of activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Its ligand OX40L (CD252, TNFSF4) is found on activated antigen-presenting cells, such as dendritic cells, B cells, and macrophages. After ligation by OX40L and acting in synergy with the T-cell receptor (TCR) and CD28, the cosignaling from OX40 plays a dominant role in regulating proliferation, cytokine production, and survival of antigen-activated T cells.\textsuperscript{3,4} Signals from OX40 can promote the aberrant T-cell responses in various autoimmune and inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, and allergic asthma.\textsuperscript{3,7} In addition, OX40 agonists can augment antitumor T-cell immunity.\textsuperscript{8,10}

OX40, after ligation by OX40L, recruits TNF receptor-associated factor 2 (TRAF2), an adaptor common to all of the TNF costimulatory receptors, and assembles a signaling complex of inflammatory kinases such as IkB kinase α/β/γ and protein kinase C-θ that drive the activation of downstream T-cell signaling pathways.\textsuperscript{1,11} Whereas knowledge of TNF costimulatory receptors has been gained over the past decades, it is not clear how the activity of these receptors is regulated, knowledge of which might be crucial with regard to control of T-cell immunity.

To identify novel OX40-binding proteins that could regulate OX40 cosignaling, we performed mass spectrometry analysis and found a scaffolding-type protein, IQ motif-containing GTPase-activating protein 1 (IQGAP1). IQGAP proteins are evolutionary conserved proteins, and the mammalian IQGAP protein family consists of three homologous isoforms: IQGAP1, IQGAP2, and IQGAP3. While IQGAP1 is expressed ubiquitously, IQGAP2 and IQGAP3 are restricted to specific tissues.\textsuperscript{13} IQGAP1 contains multiple functional domains that mediate protein interactions, and it has been suggested to participate in several essential cellular functions, including cell migration, proliferation, and differentiation in many different cell types.\textsuperscript{14-16} For instance, IQGAP1 interacts with Cdc42 and Rac1 and inhibits GTPase activity by stabilizing their GTP-bound form.\textsuperscript{17} IQGAP1 binds to receptors for VEGF, EGF, and TGF-β\textsuperscript{18-20} and to signaling molecules such as ERK1/2, MEK1/2, and PI3K.\textsuperscript{21-23} IQGAP1 regulates cytoskeletal networks and cell adhesion through binding to F-actin, N-WAPS, and E-cadherin.\textsuperscript{24-26} Moreover, IQGAP1 may participate as an oncogenic molecule and be involved in promoting tumorigenesis in epithelial cells.\textsuperscript{13,27,28} An intriguing mystery is how this ubiquitously expressed and multifunctional molecule controls T-cell immunity mediated by OX40 signaling.

Here, we found that IQGAP1 restrained the costimulatory function of OX40. IQGAP1-deficient CD4\textsuperscript{+} T cells receiving OX40 cosignaling produced greater amounts of inflammatory cytokines and displayed more pathogenic function in experimental autoimmune encephalomyelitis (EAE). Our study has important implications for inflammatory diseases driven by TNF costimulatory receptors expressed by activated T cells and for how T-cell inflammation is controlled by the activated TNFR family molecule.

2 | MATERIALS AND METHODS

2.1 | Animals

C57BL/6 mice were from Japan SLC (Hamamatsu, Japan). \textit{Iqgap1}\textsuperscript{−/−} mice (a gift from WF Bahou) on C57BL/6 background have been described previously.\textsuperscript{29,30} OX40L-transgenic (OX40L-Tg) B6 mice that constitutively express OX40L on T cell by the \textit{lck} proximal promoter have been described.\textsuperscript{31} We have tried to obtain enough numbers of \textit{Iqgap1}\textsuperscript{−/−} \times OX40L-Tg mice by crossing \textit{Iqgap1}\textsuperscript{−/−} mice with OX40L-Tg mice, but could have only one mouse. Thus, we used this one \textit{Iqgap1}\textsuperscript{−/−} \times OX40L-Tg mouse for the study. B6.SJL-\textit{PtprcaPepcb/BoyJ} (SIL) mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Littermates at 7-12 weeks of age were used in experiments. All mice were bred and maintained under specific pathogen-free conditions, and experiments were performed according to the guidelines of the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.
anti-phospho-p65 (93H1, 3033), anti-p65 (L8F6, 6956), anti-phospho-IkBa (SA5, 9246), anti-IkBa (44D4, 4812), anti-phospho-Akt (9275), anti-Akt (9272), and anti-IQGAP1 (2293) were from Cell Signaling Technology (Danvers, MA, USA). Anti-c-Myc (9E10, sc-40) and anti-IQGAP1 (H-109, sc-10792) were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Flag (DYKDDDDK, 01822381) and anti-V5 (6F5, 01123591) were from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Anti-OX40 (AF1256) was from R&D Systems (Minneapolis, MN, USA). Anti-His (34660) was from QIAGEN (Hilden, Germany). Anti-β-actin (C4, MAB1501) was from MilliporeSigma. Recombinant mouse IL-6 (216-16), human IL-6 (200-06), soluble human IL-6R (200-06R), human IL-2 (200-02), mouse IL-4 (214-14), mouse IL-12 (210-12), and human TGF-β1 (100-21C) were from PeproTech (Rocky Hill, NJ, USA).

2.4 | Plasmids and transfection

Vectors containing cDNA encoding mouse Ox40, Traf2, and Traf2-targeting shRNA were previously described.11,34 Mouse Ox40l inserted into the KpnI and XhoI sites of pREFl vector (Invitrogen) was kindly provided from Dr AD Weinberg. Based on cDNA sequence of mouse Iqgap1 (NM_016721.2), cDNA of the entire coding region was amplified with PCR using primers that added a 5′-HindIII site and a 3′-NotI site and ligated into pcDNA3.1/V5-His A vector (Thermo Fisher Scientific). Mutant DNA constructs of IQGAP1 were generated with a PrimeSTAR mutagenesis basal kit (Takara Bio, Otsu, Japan) according to the manufacturer’s instructions. The correct DNA sequence of the newly generated constructs was verified with a 3100 genetic analyzer and a BigDye terminator kit (Thermo Fisher Scientific). IQGAP1 retroviral vector was constructed by insertion of IQGAP1-encoding cDNA into pMX-IRES-EGFP. Polyethyleneimine (408727, MilliporeSigma) was used for transient transfection of HEK293T cells, as well as Plat-E retroviral packaging cells.

2.5 | Immunoprecipitation and immunoblot analysis

For immunoprecipitation, T cells were stimulated with OX40L± DCEK cells in the absence of MCC peptide or with plate-bound anti-OX40 (OX86, produced in-house). T cells and HEK293T cells were lysed in ice-cold 1% Nonidet P-40 (NP-40) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 1% NP-40, 50 mM NaF, 1 mM Na3VO4 and 10 mM N-ethylmaleimide, containing protease-inhibitor mixture [P8340; MilliporeSigma]) or RIPA buffer (1% NP-40 buffer containing 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Insoluble material was removed by centrifugation at 15 000 g for 10 minutes at 4°C. Proteins were immunoprecipitated from lysates overnight at 4°C with primary antibodies immobilized on Dynabeads protein G. After being washed extensively with ice-cold lysis buffer, beads were boiled for 5 minutes at 100°C in 4 × lithium dodecyl sulfate sample buffer (NP0007, Thermo Fisher Scientific). Eluted sample was further reduced for 10 minutes at 70°C with dithiothreitol (DTT) or 2-ME for immunoblot analysis. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, MilliporeSigma), and analyzed by immunoblot with the appropriate antibodies. All blots were developed with Immobilon Western HRP substrate (MilliporeSigma), and detected with VesaDoc 5000MP (Bio-Rad) or ChemiDoc Touch (Bio-Rad).
2.6 | Mass spectrometry

After stimulation of OX40+ T-cell hybridoma cells with plate-bound anti-OX40, OX40 was immunoprecipitated from lysates with anti-cMyc mAb. Immunoprecipitated samples were separated by SDS-PAGE, and Coomassie-stained SDS gels were cut into three slices and submitted to The Scripps Research Institute Center for Mass Spectrometry (La Jolla, CA, USA). The gels were destained, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin overnight before analysis by nano-LC/MS/MS.

2.7 | In vitro culture of primary CD4+ T Cells

Naïve (CD44lo CD62Lhi) CD4+ T cells were purified from spleens of wild-type or Iqgap1−/− mice with a naïve CD4+ T-cell isolation kit II (130-093-227, Miltenyi Biotec, Bergisch Gladbach, Germany) and an AutoMACS Pro cell separator (Miltenyi Biotec) or a FACSAria II cell sorter (BD Biosciences). CD4 microbeads (L3T4; 130-049-201, Miltenyi Biotec) and CD8a microbeads (Ly-2, 130-049-401; Miltenyi Biotec) were used for the separation of splenic CD4+ and CD8+ T cells, respectively. Naïve CD4+ T cells were plated at a density of 2.5 × 10^5 cells/mL, in the presence or absence of anti-OX40, 1 ng/mL IL-12, 50 ng/mL plate-bound anti-CD3 and 10 μg/mL soluble anti-OX40. T-cell proliferation was assessed with a water-insoluble MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay. The water-insoluble MTT formazan was solubilized with dimethyl sulfoxide, and the absorbance was measured with a plate reader at 570 nm. For Th1 polarization, CD4+ T cells were cultured with anti-CD3 in the presence or absence of anti-OX40, 50 ng/mL plate-bound anti-CD3 and 1 μg/mL anti-IL-4. For Th2 polarization, CD4+ T cells were cultured with anti-CD3 in the presence or absence of anti-OX40, 1 ng/mL IL-12, 1 μg/mL anti-IL-4. For Th17 polarization, CD4+ T cells were cultured with anti-CD3 in the absence of anti-OX40, 50 ng/mL IL-6, and 0.2 ng/mL TGF-β.

2.10 | Real-time PCR

SYBR Premix Ex Tag (Takara Bio) and a 7500 real-time PCR system (Thermo Fisher Scientific) were used for quantitative RT-PCR. Total RNA was extracted with TRizol (Thermo Fisher Scientific), and cDNA was then synthesized with SuperScript III Reverse Transcriptase and oligo(dT)20 (Thermo Fisher Scientific). Each transcript was analyzed concurrently on the same plate with the gene encoding Gapdh, and results are presented relative to the abundance of transcripts encoding Gapdh. Primers were as follows: Gapdh (forward primer, 5′-CCAGGTTGCTCTCCTGCAGACTT-3′; reverse primer, 5′-CCTGTGGCTTAGCCGTATCCA-3′), Il-2 (forward primer, 5′-CCTGACAGGATGGAGAATTACA-3′; reverse primer, 5′-TCCAGAACATGGCCGAGAGG-3′); Il-13 (forward primer, 5′-CTCCCCCTCTGACACCCTACAGCT-3′; reverse primer, 5′-GGTCCACACTCCCATACCAGC-3′).

2.11 | EAE induction

EAE was induced by subcutaneous injection of 150 μg MOG peptide (amino acids 35-55), in 0.1 mL of complete Freund’s adjuvant (CFA) emulsion containing 400 μg Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA), into wild-type or Iqgap1−/− B6 mice on day 0. The mice received intraperitoneal injection of 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA, USA) on days 0 and 2. Clinical signs of EAE were assessed according to the following score: 0, no sign of disease; 1, limp tail or hind limb weakness; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, complete hind limb paralysis and partial front leg paralysis; 5, moribund or dead.

IfN-γ in culture supernatants was assessed by a sandwich enzyme-linked immunosorbent assay (ELISA) protocol with R4-6A2 (551216) and biotin-XMG1.2 (554410) from BD Biosciences. IL-17A ELISA kits, 88-7371-88 and 432505, were from Thermo Fisher Scientific and Biolegend, respectively. IL-2 was measured with anti-IL-2 antibodies (JES6-1A12 and JES6-5H4) from Thermo Fisher Scientific. IL-4 was measured with anti-IL-4 antibodies (11B11 and BVD6-24G2) from Biolegend.
For the isolation of mononuclear cells from the CNS, anesthetized mice were perfused with 20 mL of phosphate-buffered saline (PBS) for the removal of blood from internal organs. The spinal cords were isolated and cut into several small pieces, then were placed in 10 mL of 2 mM EDTA in PBS and incubated for 45 minutes at 37°C. The cell suspension was mixed by pipetting and was passed through a 70-µm cell strainer. Samples were enriched for mononuclear cells by centrifugation through a two-layer Percoll gradient (37% and 70%) at 700 g for 30 minutes and were washed twice before further analysis.

For evaluation of the ability of CD4+ T cells to induce EAE, irradiated syngeneic SJL recipient mice (6 Gy) were given intravenous injection of 5 × 10⁶ donor CD4+ T cells from wild-type or Iqgap1−/− B6 mice. Three days after cell transfer, mice were given subcutaneous immunization of MOG peptide and CFA followed by intraperitoneal injection of pertussis toxin. To evaluate the role of OX40-OX40L interactions in EAE disease development, 100 µg of anti-OX40L blocking antibody (MGP34, produced in-house) or control IgG were intraperitoneally injected into recipient mice every 3 days from days 0 to day 18.

2.12 | Statistical analysis

Statistical significance was assessed with Student’s t test with two-sided distributions. In Figure 6G, one-way ANOVA with Tukey’s multiple comparison test was used for statistical comparison. P value < 0.05 was considered as statistically significant.

3 | RESULTS

3.1 | OX40 recruits IQGAP1

To analyze a molecular complex of OX40 that imparts a survival signal to activated T cells, we previously established an MCC-specific T-cell hybridoma cell, derived from effector T cells from OX40−/− AND Vα11/Vβ3 TCR-transgenic mice, and this T cell was stably transfected to express cMyc-tagged OX40 to prepare OX40+ T cells.11,12 To extend our understanding of the OX40 signaling, OX40 was pulled down from OX40+ T cells that had been stimulated with plate-bound anti-OX40 agonistic antibody (OX86). Immunoprecipitates were analyzed by mass spectrometry to identify novel OX40-associated proteins in the molecular complex. We found a protein IQGAP1 as a potential binding partner for OX40 (Figure S1A,B). A conventional immunoprecipitation-immunoblot analysis revealed that the association between OX40 and IQGAP1 occurred after stimulation of the OX40+ T cell with a fibroblast antigen-presenting cell (APC) expressing OX40L33 (Figure 1A,B) or with anti-OX40 antibody (Figure 1C). We could not detect the binding between OX40 and IQGAP1 under anti-CD3 or anti-CD3/CD28 stimulation (data not shown). We also detected a low level of interaction between OX40 and IQGAP1 in HEK 293T cells that had been transduced with vectors encoding c-Myc-tagged OX40 and V5-tagged IQGAP1 (Figure 1D, lane 4), but additional expression of OX40L strongly enhanced the binding between OX40 and IQGAP1 (Figure 1D, lane 5). Furthermore, after stimulation with OX40L+ APCs, an endogenous interaction between OX40 and IQGAP1 could be detected in primary mouse effector CD4+ T cells (Figure 1E). Therefore, these results convincingly show that the activated OX40 receptor recruits IQGAP1.

3.2 | IQGAP1-deficient CD4+ T cells exhibit enhanced proliferation and cytokine production mediated by OX40 cosignaling

Iqgap1−/− deficient mice develop normally and show no obvious abnormality for most of adulthood as previously reported.35 In Iqgap1−/− mice, the development of CD4+ and CD8+ T cells in the thymus and peripheral lymphocyte compartments is normal, and the expression level of TCR and CD28 on CD4+ and CD8+ T cells is comparable between Iqgap1−/− and wild-type groups.36 However, mature Iqgap1−/−/CD8+ T cells produced higher amounts of IL-2 and IFN-γ in response to PMA/ionomycin or anti-CD3/CD28,36,37 suggesting that IQGAP1 controls TCR-related T-cell signaling.

The main aim of this study is to explore functional and structural insights into the interaction between OX40 and IQGAP1. To investigate the role of IQGAP1 in OX40 signaling, sorted naïve CD4+ T cells from wild-type or Iqgap1−/− mice were stimulated with anti-CD3 in the presence or absence of anti-OX40 (OX86). Proliferative responses of Iqgap1−/−/CD4+ T cells against anti-CD3 was significantly higher than that of wild-type CD4+ T cells (Figure 2A), confirming previous results.36,37 Importantly, anti-OX40 significantly enhanced the cell proliferation mediated by anti-CD3, and Iqgap1−/−/CD4+ T cells showed a more pronounced cell proliferation against anti-CD3/OX40 as compared to wild-type CD4+ T cells (Figure 2A). Similarly, when the expression of IQGAP1 in CD4+ T cells from wild-type OT-II mice (which have transgenic expression of an ovalbumin-specific TCR) was reduced by a retrovirus vector encoding shRNA that targets Iqgap1 (shIQGAP1), the cell division mediated by antigen and anti-OX40 was significantly increased (Figure S2A,B). These results show that IQGAP1 negatively regulates T-cell proliferation mediated by the TCR/CD3 and OX40, and thus this means that IQGAP1 limits OX40 cosignaling.
In a similar experimental setting, we evaluated cytokine production from \textit{Iqgap1}\textsuperscript{−/−} CD4\textsuperscript{+} T cells. After culturing naïve CD4\textsuperscript{+} T cells under Th1 or Th17 polarizing conditions for 72 hours in the presence of anti-CD3 without antigen peptide for 1 h, and OX40 were immunoprecipitated from the cell lysate with anti-c-Myc antibody or control immunoglobulin G (IgG), followed by immunoblot analysis with anti-IQGAP1 and anti-c-Myc antibodies. IP, immunoprecipitation. B, cMyc-OX40 T-cell hybridoma cells were stimulated as in (A), and endogenous IQGAP1 was immunoprecipitated with anti-IQGAP1 antibody or control IgG, followed by immunoblot with anti-c-Myc and anti-IQGAP1 antibodies. C, cMyc-OX40 T-cell hybridoma cells were stimulated with plate-immobilized anti-OX40 agonistic antibody (OX86) for 15 min, and OX40 was immunoprecipitated with anti-c-Myc antibody or control IgG, followed by immunoblot with anti-IQGAP1 and anti-c-Myc antibodies. D, HEK293T cells were transiently transfected with indicated plasmid vectors encoding cMyc-tagged OX40, V5-tagged IQGAP1, and His-tagged OX40L, followed by immunoprecipitation of proteins from cell lysates with anti-c-Myc antibody or control IgG, and immunoblot analysis with anti-c-Myc, anti-V5, and anti-His antibodies. The asterisk indicates the light chain of immunoglobulin. E, Wild-type primary effector CD4\textsuperscript{+} T cells were stimulated with OX40L\textsuperscript{+} DCEK APCs without antigen for indicated times, and endogenous IQGAP1 was immunoprecipitated from the cell lysate with anti-IQGAP1 or control IgG antibodies, followed by immunoblot analysis with anti-OX40 and anti-IQGAP1 antibodies. The asterisk indicates the heavy chain of immunoglobulin. Data are from one experiment representative of at least two independent experiments with similar results.

![Figure 1](image)

**FIGURE 1** OX40 binds to IQGAP1. A, cMyc-tagged OX40 transduced T-cell hybridoma cells were unstimulated (NON) or stimulated with OX40L\textsuperscript{+} DCEK antigen-presenting cells (APC) without antigen peptide for 1 h, and OX40 were immunoprecipitated from the cell lysate with anti-c-Myc antibody or control immunoglobulin G (IgG), followed by immunoblot analysis with anti-IQGAP1 and anti-c-Myc antibodies. IP, immunoprecipitation. B, cMyc-OX40 T-cell hybridoma cells were stimulated as in (A), and endogenous IQGAP1 was immunoprecipitated with anti-IQGAP1 antibody or control IgG, followed by immunoblot with anti-c-Myc and anti-IQGAP1 antibodies. C, cMyc-OX40 T-cell hybridoma cells were stimulated with plate-immobilized anti-OX40 agonistic antibody (OX86) for 15 min, and OX40 was immunoprecipitated with anti-c-Myc antibody or control IgG, followed by immunoblot with anti-IQGAP1 and anti-c-Myc antibodies. D, HEK293T cells were transiently transfected with indicated plasmid vectors encoding cMyc-tagged OX40, V5-tagged IQGAP1, and His-tagged OX40L, followed by immunoprecipitation of proteins from cell lysates with anti-c-Myc antibody or control IgG, and immunoblot analysis with anti-c-Myc, anti-V5, and anti-His antibodies. The asterisk indicates the light chain of immunoglobulin. E, Wild-type primary effector CD4\textsuperscript{+} T cells were stimulated with OX40L\textsuperscript{+} DCEK APCs without antigen for indicated times, and endogenous IQGAP1 was immunoprecipitated from the cell lysate with anti-IQGAP1 or control IgG antibodies, followed by immunoblot analysis with anti-OX40 and anti-IQGAP1 antibodies. The asterisk indicates the heavy chain of immunoglobulin. Data are from one experiment representative of at least two independent experiments with similar results.

OX40L-transgenic (OX40L-Tg) mice that constitutively express OX40L in T cells under the control of the lck proximal promoter were previously established.\textsuperscript{31} Spleens from OX40L-Tg or OX40L-Tg × \textit{Iqgap1}\textsuperscript{−/−} mice contained higher frequencies of CD4\textsuperscript{+} T cells (Figure S3A), and these CD4\textsuperscript{+} T cells displayed the phenotype of CD4\textsuperscript{4}CD62L\textsuperscript{−} effector or memory (effector/memory) T cells (Figure S3B). In T cells derived from OX40L-Tg or OX40L-Tg × \textit{Iqgap1}\textsuperscript{−/−} mice, OX40-OX40L signaling can be activated through the T cell-T cell interaction, and thus we thought that CD4\textsuperscript{+} T cells from OX40L-Tg × \textit{Iqgap1}\textsuperscript{−/−} mice could receive a pronounced OX40 cosignaling. To evaluate this possibility, CD4\textsuperscript{+} T cells purified from wild-type, \textit{Iqgap1}\textsuperscript{−/−}, OX40L-Tg, or OX40L-Tg × \textit{Iqgap1}\textsuperscript{−/−} mice were stimulated with anti-CD3 alone. CD4\textsuperscript{+} T cells from these mice marginally expressed OX40 on the surface before stimulation (Figure S3C), and upon anti-CD3, OX40 was upregulated on the cell surface (Figure S3D). Interestingly, CD4\textsuperscript{+} T cells...
derived from OX40L-Tg × Iqgap1−/− mice expressed the highest level of OX40 after anti-CD3 (Figure S3D), suggesting that enhanced OX40 cosignaling caused by IQGAP1 deficiency promotes the expression of OX40 on activated T cells.

Next, to assess the cytokine response, splenic CD4+ T cells from wild-type, Iqgap1−/−, OX40L-Tg, or OX40L-Tg × Iqgap1−/− mice were activated in vitro with PMA and ionomycin. We found that IFN-γ, IL-17A, and IL-13 were strongly induced in CD4+ T cells from OX40L-Tg × Iqgap1−/− mice (Figure 3A,B). Although CD4+ T cells from wild-type and Iqgap1−/− mice could not produce enough amounts of IFN-γ, IL-17A, and IL-2 in response to anti-CD3 alone within 24 h, CD4+ T cells from OX40L-Tg and OX40L-Tg × Iqgap1−/− mice, which contained higher frequencies of CD62L-CD44+ effector/memory T cells (Figure S3B), secreted significantly higher amounts of these cytokines. Importantly, this cytokine response was significantly promoted by IQGAP1 deficiency in OX40L-Tg CD4+ T cells (Figure 3C-D). Moreover, OX40 blockade with MGP34 significantly suppressed the production of IFN-γ and IL-17A (Figure 3C,D), suggesting that IQGAP1 preferentially restrains cytokine responses driven by OX40 cosignaling in effector/memory T cells.

Collectively, these results demonstrate that IQGAP1 works as a negative regulator for the T-cell proliferation and cytokine production mediated by OX40 cosignaling.

3.3 | TRAF2 bridges IQGAP1 and OX40

TRAF2 directly binds to the cytoplasmic region of OX40 and regulates OX40 signaling,3,4,6 and thus we assessed whether TRAF2 was required for the interaction between OX40 and IQGAP1. In HEK 293T cells coexpressing both IQGAP1 and TRAF2, IQGAP1 was coimmunoprecipitated with TRAF2 (Figure 4A, lane 3), and the TRAF2-IQGAP1 interaction was greatly increased by additional coexpression of OX40 and OX40L (Figure 4A, lane 6). Similarly, in cells coexpressing both OX40 and IQGAP1, only a small amount of IQGAP1 was coimmunoprecipitated with OX40 (Figure 4B, lane 3), but the amount of IQGAP1 was further increased
by additional coexpression of TRAF2 (Figure 4B, lane 5) or OX40L (Figure 4B, lane 6). Furthermore, concurrent coexpression of TRAF2 and OX40L greatly promoted the amount of IQGAP1 associated with OX40 (Figure 4B, lane 8). However, in this overexpression system, OX40L did not promote the interaction between OX40 and TRAF2 (Figure 4B, lane 4 vs 7). These results show that IQGAP1 is incorporated into the activated OX40 receptor complex.

To evaluate how TRAF2 is critical for the binding between IQGAP1 and OX40, the endogenous TRAF2 in OX40+ T cells was reduced by stable transfection of short hairpin RNA that targets Traf2 (shTRAF2). We found that after triggering with OX40L, a lesser amount of IQGAP1 was coimmunoprecipitated with OX40 in this TRAF2-knockdown T cell (Figure 4C), indicating that TRAF2 augments the association between OX40 and IQGAP1.

IQGAP1 is a multidomain protein (Figure 4D, below). To evaluate which regions of IQGAP1 are responsible for the binding to TRAF2, we prepared two truncated mutants of IQGAP1, namely N-terminal (1-735) IQGAP1 and C-terminal (736-1657) IQGAP1 (Figure 4D, below). HEK293T cells were cotransfected to express TRAF2, OX40, and OX40L together with either N-terminal or C-terminal IQGAP1, and then TRAF2 was immunoprecipitated from the cell lysates. The C-terminal IQGAP1 was coimmunoprecipitated with TRAF2, whereas the N-terminal IQGAP1 was not (Figure 4D), indicating that TRAF2 interacts with the C-terminal IQGAP1.

Next, to explore which domains of TRAF2 (Figure 4E, below) are essential to interact with IQGAP1, HEK293T cells were cotransfected to express the C-terminal IQGAP1 together with either full-length TRAF2 (FL) or N-terminal TRAF2 (N-TRAF2, amino acids 1-271) or C-terminal TRAF2 (C-TRAF2, amino acid 272-501) or really interesting new gene (RING) domain deleted TRAF2 (ΔRING, amino acid 99-501) (Figure 4E, below), and then TRAF2 was immunoprecipitated from the cell lysates. C-TRAF2 and ΔRING displayed considerably diminished binding to IQGAP1, whereas FL and N-TRAF2 did not (Figure 4E), indicating that the RING domain of TRAF2 is critical for binding to the C-terminal IQGAP1.

Collectively, these results suggest that the RING domain of TRAF2 interacts with the C-terminal domains of IQGAP1 and that this TRAF2-IQGAP1 complex is next incorporated into the activated OX40 receptor complex.

3.4 C-terminal IQGAP1 restricts OX40 cosignaling

When OX40+ T cells as shown in Figure 1A-C were cocultured with fibroblast APCs that coexpress CD80, OX40L, and I-Ek,33 OX40-OX40L interactions significantly increased antigen-dependent IL-2 protein and mRNA11,12 (Figure 5A,B; see control vector (Ctrl) transduced T-cell groups). On the other hand, in the absence of antigen, OX40+ T cells did not produce IL-2 (Figure 5A,B). Thus, both antigen and OX40 signals are important for the IL-2 response, and the level of IL-2 serves as an indicator of OX40 cosignaling activity.

Using this bioassay, we examined how IQGAP1 contributed to the OX40 cosignaling. We newly prepared
IQGAP1-deficient (ΔIQGAP1) OX40⁺ and OX40⁻ T-cell hybridoma cells by CRISPR-Cas9 technology. After stimulation with antigen, ΔIQGAP1 T cells produced significantly higher amounts of IL-2 than did control vector (Ctrl) transduced T cells in both OX40⁺ and OX40⁻ groups (Figure 5A,B). Thus, IQGAP1 deficiency promoted not only antigen-dependent but also OX40 cosignal-dependent IL-2 responses.

Using this OX40 signal-dependent T-cell hybridoma assay, we next evaluated how phosphorylation profiles were altered in the presence or absence of IQGAP1. Data are from one experiment representative of at least three independent experiments with similar results.
antigen. Both antigen-dependent and -independent phosphorylations of p65 and IκBα were elevated in ΔIQGAP1 OX40+ T cells as compared to control OX40+ T cells (Figure 5C). Additionally, antigen-dependent phosphorylation of Akt was increased in ΔIQGAP1 OX40+ T cells as compared to control OX40+ T cells (Figure 5C, right [+]). Thus, these results suggest that IQGAP1 inhibits NF-κB (p65 and IκBα) and Akt activity mediated by OX40.
In addition, we prepared ΔIQGAP1 T-cell hybridoma cells that were further transduced with a retrovirus vector encoding green fluorescent protein (GFP) and full-length IQGAP1 (1-1657) or N-terminal IQGAP1 (1-735) or C-terminal IQGAP1 (736-1657) (Figure S4A,B). Upon OX40 triggering, full-length or C-terminal IQGAP1, but not N-terminal IQGAP1, was communoprecipitated with OX40 (Figure 5D). Moreover, full-length or C-terminal IQGAP1 interacted with endogenous TRAF2 in the steady state (Figure 5E). Thus, the TRAF2-IQGAP1 complex is incorporated into the signaling complex of OX40 in T cells.

An important question is how IQGAP1 specifically regulates OX40 signaling independently of TCR signaling. We thought that the C-terminal part of IQGAP1 might display a specific function for OX40. Thus, we next evaluated whether the elevated IL-2 response of OX40+ ΔIQGAP1 T cells could be restored by the expression of C-terminal IQGAP1. Indeed, although the C-terminal IQGAP1 (736-1657) had no role for OX40− ΔIQGAP1 T cells (Figure 5F, left), the C-terminal IQGAP1 significantly restored the elevated IL-2 response in OX40+ ΔIQGAP1 T cells (Figure 5F, right). In addition, in the presence of anti-OX40L (MGP34), this restoration was canceled (Figure 5G). Importantly, the expression of C-terminal IQGAP1 in primary Iqgap1−/− Th2 cells could also restore the elevated IL-13 expression mediated by OX40 cosignaling (Figure 5H).

Collectively, these results demonstrate that the C-terminal IQGAP1 displays a more specific effect on OX40 signaling.

3.5 The OX40-IQGAP1 cosignaling axis restrains CD4+ T cell-dependent autoimmune neuroinflammation

Signals from OX40 enhance antigen-specific T-cell responses that impact the development of autoimmune diseases. To clarify the relevance of our findings in the context of autoimmunity, we immunized groups of Iqgap1−/− and wild-type B6 mice with a peptide of amino acids 35-55 of MOG emulsified in CFA to induce EAE, whose disease activity is tightly controlled by OX40 cosignaling. In vivo via administration of anti-OX40L (MGP34) led to a significant reduction in exacerbated EAE in recipient mice with Iqgap1−/− mice 22 days after immunization (Figure 6D). These results suggest that IQGAP1 limits MOG antigen-specific effector T-cell responses during the development of EAE.

To examine whether the enhanced EAE responses in Iqgap1−/− mice were caused by IQGAP1 deficiency in CD4+ T cells, we adoptively transferred wild-type or Iqgap1−/− CD45.2+ CD4+ T cells into congenic CD45.1+ B6 recipient mice that had been sublethally irradiated, then immunized the recipient mice with the MOG peptide in CFA. Recipient mice transferred with Iqgap1−/− CD4+ T cells developed EAE with a more severe clinical score and with enhanced body-weight loss as compared with wild-type mice (Figure 6A,B). Immune cells infiltration and demyelination in spinal tissue were also increased in Iqgap1−/− mice (Fig, 6C). In accordance with the disease severity, the numbers of IFN-γ+ and IL-17A+ effector CD4+ T cells in the central nervous systems were significantly increased in Iqgap1−/− mice at 22 days after immunization (Figure 6D). These results suggest that IQGAP1 limits MOG antigen-specific effector T-cell responses during the development of EAE.
FIGURE 6  OX40 blockade dampens aberrant autoimmune neuroinflammation driven by IQGAP1-deficient CD4+ T cells.  A-D, Groups of wild-type and qgap1−/− B6 mice were immunized with MOG35-55 in CFA. Mice were monitored for clinical signs of EAE (A) and body weight changes (B) of each individual mouse for 22 days. C, Histology of spinal cord at day 21. Arrows indicate region of cellular infiltration. Scale bar, 200 mm. HE; hematoxylin-eosin staining. LFB; Luxol Fast Blue staining. D, The numbers of total, IL-17A+, IFN-γ+, and IL-17A+IFN-γ+ CD4+ lymphocytes isolated from central nervous system at day 23. Isolated cells were restimulated ex vivo with PMA and ionomycin for 5 h to measure intracellular levels of IL-17A and IFN-γ. (E, F) Wild-type or qgap1−/− CD45.2+CD4+ T cells (5 × 10^6 cells/mouse) were adoptively transferred into irradiated CD45.1+ B6.SJL hosts. Recipient mice were immunized with MOG peptide, and mice were monitored for clinical signs of EAE (E) and body weight changes (F) for 28 days. G, Wild-type or qgap1−/− CD45.2+ CD4+ T cells were transferred into irradiated recipient mice as in (E), and recipient mice were immunized with MOG peptide and injected i.p. with anti-OX40L blocking antibody (MGP34) or control IgG every 3 days from day 0 to day 18. EAE clinical scores were monitored for 44 days. Results are average values with means ± SEM P values were calculated using Student’s t tests (A-F; *P < .05; **P < .01) or one-way ANOVA test (G; *P < .05; NS, not significant). Data are from one experiment representative of two experiments (A–F; average and SEM of six [A–D] or four [E,F] mice/genotype) or are from one experiment (G; average and SEM of four mice per group)
CD4+ T cells that exacerbate EAE disease. These results demonstrate that \textit{Iqgap1}^{-/-} CD4+ T cells display enhanced inflammatory activity after receiving OX40 cosignaling and that IQGAPI1 protein incorporated into the OX40 receptor complex negatively controls the signaling strength of antigen-responding T cells.

4 | DISCUSSION

In this study, we have found that IQGAPI1 works as an important regulator of OX40 cosignaling. Genetic ablation of \textit{Iqgap1} in CD4+ T cells augmented OX40 cosignaling both in vitro and in vivo and promoted the development of pathogenic CD4+ T cells that cause autoimmune EAE. This novel mechanism of T-cell cosignaling thus impacts the extent of inflammation driven by antigen-activated CD4+ T cells.

IQGAPI1 plays an important role for OX40-driven CD4+ T-cell proliferation and cytokine production. In polarized cytokine conditions with anti-CD3/OX40, differentiating naïve CD4+ T cells lacking IQGAPI1 produced greater amounts of effector cytokines as compared with their wild-type counterparts. Additionally, upon receiving OX40 cosignaling, differentiated \textit{Iqgap1}^{-/-} effector CD4+ T cells also produced higher amounts of cytokines, and the expression of C-terminal IQGAPI1 could restore the enhanced cytokine response of \textit{Iqgap1}^{-/-} T cells. Furthermore, spleens from \textit{Ox40L-Tg × Iqgap1}^{-/-} mice contained higher numbers of effector/memory CD4+ T cells, and upon receiving the TCR/CD3 and OX40 signaling, these effector/memory T cells produced significantly higher amounts of effector cytokines. These results demonstrate that the inhibitory activity of IQGAPI1 for OX40 cosignaling may not be restricted to a specific subset of CD4+ T cells or to a stage of T-cell development, if a T cell expresses a substantial amount of OX40.

The mechanism by which IQGAPI1 restrains OX40 cosignaling remains a key question. Although the TCR signaling is essential for induction of OX40-dependent proliferative and cytokine responses, the formation of a functional signaling complex of OX40 is dependent on OX40 engagement with OX40L and on recruitment of TRAF2 to OX40, but independent of the antigen/TCR stimulation. \textsuperscript{11,12} TRAF2 works as a key signaling adapter and plays a critical role in activating NF-κB and PI3K/Akt pathways downstream of OX40. \textsuperscript{11,12} CD4+ T cells expressing a dominant-negative form of TRAF2 are defective in antigen- and OX40-driven differentiation of effector/memory T cells. \textsuperscript{40} In this study, we showed that TRAF2 bound to IQGAPI1 in the steady state and that engagement of OX40 but not TCR was required for the recruitment of the TRAF2-IQGAPI1 complex to OX40. TRAF2 is utilized by most of the TNFR cosignaling molecules and works as a dominant signaling transducer, implying that IQGAPI1 may work as a common inhibitor for TNFR family molecules. If this is correct, it will be important to understand the generalized function of IQGAPI1 in the TNFR cosignaling pathway in T cells.

In resting T cells, IQGAPI1 is associated with a large cytoplasmic RNA-protein scaffolding complex containing nuclear factor of activated T cells (NFAT), NFAT kinases, and other proteins that limit dephosphorylation and nuclear shuttling of NFAT. \textsuperscript{36,41} IQGAPI1 bound to the inactive phosphorylated form of NFAT. \textsuperscript{37} After an increase in intracellular Ca\textsuperscript{2+} concentrations, knockdown or deficiency of IQGAPI1 led to increased NFAT dephosphorylation by calcineurin and nuclear import. In this reason, upon triggering the TCR, CD8+ T cells from \textit{Iqgap1}^{-/-} mice secreted higher amounts of NFAT-dependent cytokines IL-2 and IFN-γ. \textsuperscript{36,37,41} Interestingly, the expression of N-terminal IQGAPI1 (1-735), but not of C-terminal IQGAPI1 (744-1657), inhibited the elevated TCR-dependent \textit{Il2} gene transcription in \textit{Iqgap1} knockdown Jurkat T cells, \textsuperscript{36} suggesting that the N-terminal IQGAPI1 is critical for assembly of the large RNA-protein complex limiting NFAT nuclear shuttling and gene transcription. Thus, the N-terminal IQGAPI1 may be responsible for TCR-regulated NFAT activity in T cells. In contrast, we showed in this study that the C-terminal IQGAPI1 (736-1657) was incorporated into the molecular complex of OX40 and inhibited OX40 cosignaling. Furthermore, IQGAPI1 deficiency augmented NF-κB and Akt signaling mediated by OX40, suggesting that IQGAPI1 exhibits a specific regulatory function for OX40 cosignaling. Further studies are required to elucidate the molecular mechanism of how IQGAPI1 controls combined signals mediated by the TCR and OX40.

The OX40-IQGAPI1 axis limits EAE in a CD4+ T-cell intrinsic manner. It has been demonstrated that OX40-OX40L interactions promote the expansion of myelin-specific autoreactive CD4+ T cells producing IL-17 and IFN-γ. Eencephalitogenic T cells and APCs from the central nervous system (CNS) of mice with actively induced EAE express higher levels of OX40 and OX40L, respectively. Thus, blocking the interaction between OX40 and OX40L ameliorates EAE disease symptom. Additionally, OX40L-deficient mice displayed attenuated EAE, and OX40L-transgenic mice developed more severe EAE, demonstrating critical roles of the OX40-OX40L system in amplifying pathogenic T-cell responses in the CNS. \textsuperscript{38,39,42,43} We showed in this study that OX40 blockade corrected the aberrant activity of \textit{Iqgap1}^{-/-} CD4+ T cells and suppressed the development of EAE. This suggests that, in a situation that endogenous OX40-OX40L interactions promote the development of encephalitogenic effector T cells and facilitate their entry into the CNS, IQGAPI1 expressed by CD4+ T cells negatively regulates the cosignaling activity of OX40. In humans, SNPs in IQGAPI1 affect the incidence
of multiple sclerosis and allergic disease, although the precise molecular mechanism has been unclear.

In summary, we reveal an important regulatory function of IQGAP1 in OX40 signaling. Our results explain how the T-cell costimulatory receptor OX40 incorporates IQGAP1 and modulates inflammatory function of antigen-activated OX40+ CD4+ T cells. This study suggests an important concept that TNF costimulatory receptors contains both positive and negative modules for signal transduction in order to optimally control T-cell immunity.

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AUTHOR CONTRIBUTIONS
TS and MC conceived the project and initiated the studies; YO, HN, MC, NI, and TS designed the experiments; YO, HN, MC, NI, and TS analyzed data; MUF, MC, and NI contributed reagents and analytical tools; YO, MC, NI, and TS wrote the manuscript; TS supervised the project.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.