Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment

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T cell helper type 2 (Th2) differentiation is driven by a source of IL-4 receptor (IL-4R) that mobilizes IL-4R signaling pathways and the transcription factor GATA-3. Naive CD4 cells can secrete IL-4 independently of IL-4R signals, but how this secretion is regulated is not understood. Here we demonstrate that costimulation through the tumor necrosis factor receptor family molecule OX40, in synergy with CD28, is essential for high levels of nuclear factor of activated T cells c1 to accumulate in the nucleus of a recently activated naive T cell. This action is not dependent on either IL-4R or IL-2R signals and results in OX40 controlling initial naive T cell IL-4 transcription. OX40 signals subsequently enhance nuclear GATA-3 accumulation through an IL-4R-dependent action, leading to Th2 differentiation. These data show that, in the absence of an exogenous source of IL-4, OX40 provides a critical synergistic and temporal signal with other noncytokine receptors to modulate nuclear factor of activated T cells c1 and to promote optimal Th2 generation.

Results

Early IL-4 Production and Th2 Differentiation Are Impaired in OX40-Deficient Naïve CD4 T Cells. To examine whether OX40–OX40L interactions control IL-4 production under physiological conditions, we cultured naïve wild-type or OX40-deficient CD4 cells from OT-II T cell antigen receptor (TCR) transgenic mice with wild-type APCs and various doses of ovalbumin (OVA) peptide for 7 days. Strong Th2 development (IL-4 shown and IL-5 not shown) occurred with a moderate/low dose of antigen (Ag), whereas the Th2 response was lost as the Ag dose was increased (Fig. L4). In contrast, Th2 differentiation was impaired in the absence of OX40, such that 100- to 1,000-fold lower recall IL-4 levels were detected (Fig. L4), correlating with OX40 and OX40L expression on CD4 cells and APCs, from 12 h to 2 days after T cell–APC interaction (Fig. 1B). Similarly, an OX40L blocking antibody (Ab) with wild-type T cells mimicked the OX40-deficient phenotype (Fig. 1C). IFN-γ recall responses were unaltered or enhanced, suggesting a selective effect on the Th2 lineage.

To show a similar role of OX40 in vivo, wild-type or OX40−/− OT-II T cells were adoptively transferred into syngeneic mice, followed by immunization with Ag. Transferred OT-II T cells...

Conflict of interest statement: No conflicts declared.

Abbreviations: Ab, antibody; Ag, antigen; APC, antigen-presenting cell; CN, calcineurin; CsA, cyclosporin A; HRPT, hypoxanthine phosphoribosyltransferase; IL-4R, IL-4 receptor; LY, LY294002; NFAT, nuclear factor of activated T cells; OVA, ovalbumin; OX40L, OX40 ligand; PI3K, phosphatidylinositol 3-kinase; TCR, T cell antigen receptor; Th1, T cell helper type 1; Th2, T cell helper type 2.

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with wild-type T cells under neutral conditions (Fig. 3). Exogenous IL-4 restored Th2 differentiation to levels seen with wild-type CD4 and CD11b+CD11c+ APCs after stimulation with 0.1 μM Ag for 36 h. The blank histograms indicate isotype control. The shaded histograms indicate positive staining. (C) Effect of OX40L blocking on recall cytokine responses of wild-type T cells cultured with 0.1 μM Ag. aOX40L, anti-OX40L Ab. All data are representative of at least three individual experiments.

were recovered 2–4 days after immunization. The number of OX40-deficient T cells was similar to that of wild-type cells, suggesting no survival defect at this time point (Fig. 2A). IL-4 mRNA levels in OX40+/− OT-II cells were reduced compared with wild-type OT-II cells, whereas no difference in IFN-γ mRNA was found (Fig. 2B and C). This finding confirms a critical role for OX40 in Th2 lineage commitment, correlating with defective Th2 immunity in the absence of OX40–OX40L interactions (25). To address whether IL-4 synergized with defective Th2 immunity in the absence of OX40–OX40L interactions during naïve T cell encounters with APCs, was a crucial factor for Th2 development. Correlating with this conclusion, we observed IL-4-independent IL-4 mRNA up-regulation in wild-type T cells responding to Ag 36 h after initial activation (Fig. 3D), as well as IL-4 secretion within 2 days of naïve T cell activation (data not shown), and this response was strongly reduced in the absence of OX40.

Our previous data showed that phosphatidylinositol 3-kinase (PI3K) is a downstream mediator of OX40 signals (26). LY294002 (LY), an inhibitor of PI3K, was added 12 h after stimulation to coincide with OX40 expression but also to allow initial signaling to proceed normally. LY greatly inhibited IL-4 transcription in wild-type T cells (Fig. 4A) and resulted in dramatically impaired generation of effector cells producing IL-4 in recall responses (Fig. 4B). These results collectively indicate that OX40 signals enhance IL-4 transcription at an early stage, and this then leads to a feedback loop through the IL-4R in vivo (Fig. 5A).

Exogenous IL-4 synergized with IL-4 for optimal Th2 generation in both wild-type and OX40−/− T cells (Fig. 3C). This finding suggested that early IL-4, produced after OX40–OX40L interactions during naïve T cell encounters with APCs, was a crucial factor for Th2 development. Correlating with this conclusion, we observed IL-4-independent IL-4 mRNA up-regulation in wild-type T cells responding to Ag 36 h after initial activation (Fig. 3D), as well as IL-4 secretion within 2 days of naïve T cell activation (data not shown), and this response was strongly reduced in the absence of OX40.

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**Fig. 1.** OX40–OX40L interactions are required for Th2 differentiation. Naïve CD4 T cells from wild-type (filled circles) or OX40-deficient (open circles) OT-II mice were cultured with APCs and indicated doses of OVA-323–339. (A) IL-4 and IFN-γ recall responses measured at day 7 after Ag/APC restimulation of equal numbers of effector T cells for 24 h. (B) OX40 and OX40L expression on wild-type CD4 and CD11b+CD11c+ APCs after stimulation with 0.1 μM Ag for 36 h. The blank histograms indicate isotype control. The shaded histograms indicate positive staining. (C) Effect of OX40L blocking on recall cytokine responses of wild-type T cells cultured with 0.1 μM Ag. aOX40L, anti-OX40L Ab. All data are representative of at least three individual experiments.

**Fig. 2.** Defective Th2 differentiation in vivo in OX40-deficient CD4 T cells. Naïve wild-type or OX40−/− OT-II CD4 cells were transferred into Thy1.1 B6.PL hosts. Mice were immunized with OVA/alum, and draining lymph node cells were taken at 4 days. (A) The number of OT-II T cells measured by staining for Thy1.2. Data are the mean ± SE of three mice. (B and C) IL-4 and IFN-γ mRNA levels in purified Thy1.2+ cells measured by quantitative RT-PCR, normalized to hypoxanthine phosphoribosyltransferase (HPRT). Data are relative values with SD of triplicate PCR wells. Similar results were obtained at day 2.
Cytoplasmic NFATc1 was either unaltered or reduced, indicating that OX40 specifically promoted nuclear accrual. NFATc1 accumulation after antigen triggering was not reduced by anti-IL-4, indicating that this was an IL-4R-independent event (Fig. 5A). GATA-3 is also a critical transcription factor for Th2 differentiation (4, 5), and the accumulation of GATA-3 in the nucleus was decreased in the absence of OX40 (Fig. 5B). However, this costimulation-induced nuclear GATA-3 expression was inhibited by anti-IL-4 (Fig. 5B), distinguishing it from the IL-4-independent increase in NFATc1. Excluding a role for IL-2, blocking IL-2R, IL-4R, and, in this case, calcium entries (Fig. 5D) of specific protein, is shown. (C–F) Naive wild-type CD4 cells were stimulated with anti-CD3/CD28 and IL-2 for 24 h. (C) OX40 expression at 24 h. (D) Nuclear NFATc1 at 24 h. Anti-IL-2Rα and anti-β blocking mAbs were added from the beginning of culture. (E and F) Nuclear NFATc1 at 28 h. At 24 h, CD4 cells were recultured without stimulation or with anti-OX40 (100 μg/ml) for 4 h in the presence of blocking Abs, CsA, or LY. Data are representative of at least two independent experiments.

These results demonstrate that OX40 signals synergistically cooperate with TCR and CD28 pathways to induce NFATc1 to accumulate in the nucleus to drive initial IL-4 transcription from naive T cells.

**OX40 Signals Up-Regulate IL-4 Through NFATc1 in Th2 Cells.** Finally, to assess whether OX40 targets the IL-4 transcriptional machinery in differentiated T cells, we generated effector Th2 cells and ligated OX40 with antibody in the presence of anti-CD3. A differentiation period of 6 days in the presence of IL-4 leads to effector T cells that retain surface OX40 (Fig. 6A), have only low levels of nuclear NFATc1 (Fig. 6C and F), and do not secrete IL-4 without further stimulation (Fig. 6B and E). Upon cross-linking of both OX40 and CD3, substantially elevated levels of IL-4, mRNA (Fig. 6D and G), and protein (Fig. 6B and E) were induced compared with CD3 alone. Cross-linking OX40 in the absence of CD3 had no effect (data not shown). Thus, with more differentiated T cells, unlike naive T cells, CD28 signals were not required for OX40 to signal effectively.

Consistent with naïve T cells (Fig. 5), the level of NFATc1 preferentially increased in the nucleus of effector cells when OX40 was engaged (Fig. 6C and F), and this increase depended on PI3K (Fig. 6F). In contrast to naive T cells that were previously activated for 24 h (Fig. 5E and F), effector Th2 cells did not already express high levels of nuclear NFATc1, suggesting that, here, OX40, in combination with CD3, signals pro-
molecules necessary for later events in lineage commitment and is that signaling through the TCR is important both for inducing These data have implied that TCR signal strength might be a
differentially promote Th1 versus Th2 differentiation (32–35).

so sources of IL-4 or IL-12, the affinity or dose of antigen can
hence instruct Th2
control, or at least synergize with, these cytokine signals and
providing strong evidence of factors that might precede and
Th2
GATA-3, c-maf, and T-bet, which can regulate commitment to
as IL-4 and IL-12, and their transcription factors, such as
Major strides have been made in delineating the cytokines, such
Discussion
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Fig. 6. OX40 triggering up-regulates IL-4 and nuclear NFATc1 in effector Th2
cells. CD4 T cells were cultured in Th2 skewing conditions. At day 6, live
effector cells were restimulated with beads coated with anti-CD3 and anti-
OX40. (A) OX40 expression at day 6. (B and E) IL-4 protein and (D and G) IL-4
mRNA, 4 h after stimulation. (C and F) Nuclear NFATc1 levels at 4 h. Th2 cells
were preincubated for 2 h with cycloheximide (O), LY (E and F), CsA, VIVIT
peptide, or VEET peptide (G), then restimulated with Ab-coated beads for 4 h.

motivated nuclear import and potentially suppressing export. Accordingly, the CN/NFAT inhibitor peptide VIVIT inhibited
production of IL-4 protein (data not shown) and mRNA (Fig.
6G). CsA also inhibited IL-4 (mRNA shown and protein not
shown) (Fig. 6G). IL-4 mRNA up-regulation was further blocked by
cycloheximide (Fig. 6D). These data confirm a positive
regulatory role for OX40 signals in controlling both nuclear
accumulation of NFATc1 and IL-4 transcription.

for allowing other signals from distinct T cell membrane recep-
tors. Our data now show that OX40 triggering is absolutely
critical for Th2 development where antigen presentation deter-
mines the Th2/Th1 balance.

The OX40 signal was transmitted through increasing the level of
NFATc1 that accumulates and/or persists in the nucleus,
thereby promoting early IL-4 transcription by naive T cells. At
the same time, the level of nuclear NFATc2 (NFATp/NFAT1)
was low and not increased, consistent with a counteracting role
for this molecule on IL-4 (36). This finding correlates with data
showing that presentation of low-affinity peptide, leading to Th2
development, also results in early induction of high levels of
nuclear NFATc1 but low levels of NFATc2 (37). Collectively,
these results suggest that regulation of the NFATc1/NFATc2
balance in naive T cells is thus central to Th2 programming. In
line with these results, NFATc2-deficient T cells show sustained
IL-4 mRNA expression after CD3 triggering (38). Interestingly,
we found that nuclear accumulation of NFATc1 was terminated
at 48–60 h after stimulation of naive cells (data not shown),
suggesting this termination could be an important late regulatory
event that additionally controls T cell differentiation. This event
might be significant in light of surprising data that showed that
a retroviral vector containing constitutively active NFATc1, and
hence sustaining nuclear NFATc1 after its normal time of
export, resulted in Th1 and not Th2 differentiation in wild-type
cells (39). Thus, high-level, early but transient accumulation
of NFATc1 in the nucleus appears to be critical for ultimately
producing a Th2 cell.

Although we show that OX40 cosignals with TCR signals can
regulate NFATc1 and IL-4 in an effector T cell with a naive T
cell, both TCR and CD28 signals were initially necessary for
OX40 to promote IL-4. Stimulation of naive T cells with
TCR/CD28 results, within 1–3 h, in the STAT-independent
induction of IL-4 and IFN-γ transcription (40) and in nonselective
histone modifications at both IL-4 and IFNG loci (41). In
addition, NFAT has been suggested to be a critical transcription
factor for cytokines at this stage (42). Our data now complement
these studies and demonstrate that signals from OX40 act
synergistically with these early signals, also in a STAT-
independent manner, to maintain and further up-regulate IL-4
mRNA within 24–36 h after naive T cell activation. Supporting
the notion of a combined synergistic and temporal action of
CD28 and OX40, other data have shown that a CD28 deficiency
also results in impaired Th2 responses (43) and that CD28 signals
can up-regulate nuclear NFATc1 levels above those present after
TCR triggering (44). Thus, several signals control early Th2
divergence in a naive T cell but through a common target,
NFATc1.

Ca2+/CN are required for allowing NFATc1 entry into the
nucleus (27), and we observed an absolute requirement for CN
activity for IL-4 induction in both naive and effector T cells. This
result is in agreement with previous data showing CN was
essential for early IL-4 mRNA expression in response to weak
peptide stimulation (37) and that T cells from dominant negative
CN transgenic mice were impaired in Th2 development (45).
OX40 signals did not regulate phosphorylation of phospholipase
C-γ (data not shown) or induce Ca2+ influx (Fig. 8, which is
published as supporting information on the PNAS web site) in
effector Th2 cells, suggesting that it is unlikely that OX40 directly
targets Ca2+/CN. Moreover, ionomycin could not restore de-
fective Th2 differentiation of OX40−/− T cells (data not shown),
demonstrating that although Ca2+/CN are essential for Th2
development, they are not the primary factors that dictate the
Th2/Th1 balance.

A critical requirement for Pi3K was found in blocking studies
where suppressed IL-4 mRNA and protein expression and
NFATc1 nuclear translocation were observed. These data pos-
sibly suggest that nuclear export of NFATc1 could be one target
of OX40, which is supported by data in naïve T cells previously stimulated for 24 h (Fig. 5). This type of action might be mediated through glycosyn gen synthase kinase (GSK)3β by way of PI3K-regulated activation of Akt. A pathway postulated to regulate such export. Activation of Akt and phosphorylation of GSK3β does occur after ligation of OX40 (ref. 26 and data not shown). But, in effector Th2 cells, we failed to confirm a role for Akt in OX40-controlled IL-4, in that retroviral expression of dominant negative Akt had no effect on IL-4 transcription (data not shown). This finding implies that, at least with effector cells, nuclear import of NFATc1 might be the principal function controlled by OX40 or that OX40 can control both import and export, depending on the stage of differentiation of the T cell, or perhaps that signaling pathways other than through Akt are active. In support of regulating nuclear import, a peptide that inhibits NFAT–CN interaction suppressed OX40-induced IL-4. However, further experiments will be needed to fully understand how TCR, CD28, and OX40 signals synergize to control NFATc1 nuclear accumulation and the upstream signaling pathways that are involved.

In addition to CD28 and OX40 controlling Th2 differentiation, two other noncytokine receptors have been implicated in this process. Inducible costimulator (ICOS)-deficient mice are impaired in generating asthmatic and nematode-induced Th2 responses (46, 47), but there is some controversy regarding the target of ICOS action. Dong and colleagues (48) reported that ICOS can induce IL-4-independent early IL-4 transcription, also targeting NFATc1. However, Abe and colleagues (49) reported normal initial IL-4 transcription and nuclear NFATc1 levels in ICOS-deficient TCR transgenic T cells and suggested that ICOS enhances IL-4R sensitivity and control of STAT6 phosphorylation and GATA-3 induction. Notch signaling has also been shown to regulate IL-4R/STAT6-independent IL-4 (50). After ligand binding, Notch undergoes proteolytic processing by γ-secretase to release its intracellular signaling peptide, which translocates to the nucleus. Flavell and colleagues (50) proposed that Notch regulates the IL-4 gene by binding to RBPjk sites in the IL-4 enhancer, theoretically separating its action from CD28, OX40, and ICOS. Future studies will be important to truly understand the relationship between these molecules and the molecular basis for their apparent commonality in control of IL-4.

In conclusion, we show that OX40 signals are intimately associated with early accumulation of high levels of NFATc1 in the nucleus of recently activated naïve T cells and that this controls initial IL-4R-independent transcription of IL-4. Collectively, these data highlight the stringent control of IL-4 production by naïve T cells and its regulation by the combined action of several membrane receptors.

Materials and Methods

Mice. The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for use of animals in research. All experiments were done in compliance with the regulations and guidelines of the Association for Assessment of Laboratory Animal Care, BL/6 and B6.PL/Thy1.1 (Thy1.1) mice were from The Jackson Laboratory. OT-II TCR transgenic mice, bred on BL/6 background, were a gift from W. Heath (The Walter and Elizabeth Hall Institute, Melbourne) and used as a source of Vβ5/Vα2/Thyl.2 CD4 T cells responsive to the peptide OVA323–339. OX40−/− OT-II mice were produced by intercrossing with OX40+/− mice on the BL/6 background.

Peptides, Abs, and Cytokines. OVA323–339, VIVIT (RRRRRRRRRRR-GGG-MAGPVIVITGHPPE), and VEET (RRRRRRRRRRR-GGG-MAGPPHIHEVEETGPHVI) were synthesized by A&A Laboratories (San Diego). Abs against CD3 (145–2C11), CD28 (37N51), OX40 (OX86), OX40L (RM134L), IFN-γ (XMG1.2), and IL-4 (11B11) were produced in-house. Abs against IL-2R-α (PC61.5), IL-2R-β (TM-b1), and OX40L-PE were from eBioscience (San Diego, CA). GATA-3 (HG3-31), NFATc1 (7A6), NFATc2 (4G6-G5), and laminin B (M20) were from Santa Cruz Biotechnology. STAT5A was from R & D Systems. Phospho-STAT5 was from Cell Signaling Technology (Beverly, MA). Actin (C4) was from ICN, CD4- FITC, CD11b-FITC, CD11c-APC, biotinylated OX40, and streptavidin-PE were from BD Pharmingen. IL-2 and IL-4 were from PeproTech (Rocky Hill, NJ).

T Cells and APCs. CD4 T cells were purified from spleen and lymph nodes. Briefly, whole cells, passed over nylon columns, were subjected to complement lysis using antibodies to CD8 (3.151), heat-stable antigen (J11D), class II MHC (M5/114, Y17, and CA-4.A12), B cells (RA3.6B2), macrophage (M1/70), natural killer cells (PK136), and dendritic cells (33D1). Any residual APCs and any in vivo-activated T cells were removed by isolating high-density cells spun through a Percoll gradient. Purified T cell populations were phenotypically naïve and contained ~1% CD44highCD62Llow T cells (Fig. 9, which is published as supporting information on the PNAS web site). APCs were made by depleting T cells with complement and antibodies to Thy1.2 (F7D5 and HO.13.4), CD4 (RL172.4), and CD8 (3.151) and were irradiated with 3,000 rad before use.

Ab-Coated Beads. Ten million streptavidin-coated beads (CELLLection Biotin Binder Kit; Invitrogen) were incubated with biotinylated anti-CD3 (1 μg)/control rat IgG (2 μg), anti-CD3 (1 μg)/anti-OX40 (2 μg), or control rat IgG (3 μg) at 30 μl total volume for 30 min with a mild vortex.

T Cell Culture. Cells were cultured in RPMI medium 1640 (Invitrogen) with penicillin, streptomycin, glutamine, 2-mercaptoethanol, and 7% FCS (Omega Scientific, Tarzana, CA). CD4 T cells (5 × 105 cells per ml) were stimulated with 5 μg/ml plate-bound anti-CD3, 5 μg/ml soluble anti-CD28. For Th2 polarization, CD4 T cells were cultured with anti-CD3/CD28, 10 μg/ml anti-IFN-γ (XMG1.2), 10 ng/ml IL-2, and 10 ng/ml IL-4. Cells were initially stimulated for 3 days then transferred to new plates containing new Th2-skewed culture media and expanded for an additional 3 days without anti-CD3 stimulation. For stimulation of OT-II CD4 T cells, cultures were plated at 5 × 105 cells per ml with 6 × 105 cells per ml T cell-depleted splenic APCs and various concentrations of OVA323–339. At day 3, whole cells were transferred to plates containing new media and expanded for an additional 4 days. Equivalent numbers of live effector cells were restimulated at 1.5 × 106 cells per ml with 1.5 × 105 cells per ml splenic APCs and 20 μM OVA323–339 to assess differentiation into Th2/Th1 phenotypes. For blocking experiments, anti-OX40L (50 μg/ml), anti-IL-4 (10 μg/ml), anti-IFN-γ (10 μg/ml), anti-IL-2R-α plus -β (5 μg/ml each), LY (Calbiochem; 3 μM), CsA (Calbiochem; 0.1 μM), VIVIT, or control VEE7 peptide (25 μM) were added to the OT-II cell cultures, or VIVIT (25 μM), VEET (25 μM), LY (20 μM), CsA (1 μM), or cycloheximide (CHX, 10 μM; Calbiochem) were added to the Th2 cell cultures. For anti-OX40 stimulation of activated primary CD4 T cells, 200 μl of anti-OX40 in PBS (0.5 mg/ml) was added to polylysine-3 ml round-bottom tubes and incubated overnight at 4°C. Twenty million CD4 T cells in 0.8 ml of culture medium were directly added to the tubes and cultured for 4 h.

In Vivo Priming of CD4 T Cells. Nonirradiated syngeneic B6.PL recipient mice (Thy1.1) were injected i.v. with 3 × 10⁶ naïve Vβ5/Vα2/Thyl.2 CD4 T cells from wild-type or OX40–/– OT-II transgenic donors. One day after cell transfer, mice were im-
munnized s.c. with 10 µg of OVA protein (Sigma) and 2 µg of aluminum hydroxide (InjectAlum; Pierce) in PBS. Inguinal and peri-aortic draining lymph node cells were harvested 2 and 4 days after immunization. FACS staining with anti-Thy1.2-FITC was used to enumerate responding T cells, and OT-II cells were purified by magnetic cell sorting Thy1.2 microbeads (Miltenyi Biotech, Auburn, CA).

ELISA. Culture supernatants were assessed for cytokine content by enhanced sandwich ELISA protocols: JES6–1A12 and biotin-XMG1.2 for IFN-γ (BD Pharmingen). ExtrAvidin, biotinylated monoclonal anti-avidin (WC19.10), and ExtrAvidin-peroxidase conjugate were from Sigma.

Real-Time RT-PCR. Quantitative RT-PCR was performed by using SYBR green I dye and an ABI GeneAmp 5700 sequence BioDe
tector (PE Biosystems, Foster City, CA) according to the manu-
SYBR green I dye and an ABI Geninjected into parametric response T cells, and OT-II cells were

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Supporting Fig. 1

The graph shows the relationship between Ag (µM) and cpm. The graph is split into two sections: one for IL-2 (ng/ml) and the other for cpm.

For cpm, there are two lines representing different genotypes (+/+ and -/-). The +/+ line is shown with filled circles, and the -/- line is shown with open circles. The y-axis ranges from 0 to 200,000 cpm.

For IL-2 (ng/ml), the y-axis ranges from 0 to 20 ng/ml, and the x-axis ranges from 0 to 10 µM. Two lines representing +/+ and -/- genotypes are shown, with the y-axis and x-axis labels clearly indicated.
Supporting Fig. 2
Supporting Fig. 3

- BL/6 splenocytes CD4
- Purified CD4 (wild-type OTII)
- Purified CD4 (BL/6)
- Purified CD4 (OX40-/- OTII)