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. 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.

Abbreviations: Ab, antibody; Ag, antigen; APC, antigen-presenting cell; CN, calcineurin; CYA, cyclosporin A; HCPT, 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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result, exogenous IL-4 restored Th2 differentiation to levels seen in wild-type CD4 and CD11b+CD45R0 T cells after stimulation with 0.1 μg 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 μg Ag. (D) Primary IL-4 mRNA induction, in the presence or absence of anti-IL-4, 36 h after naive T cell activation. mRNA levels in purified CD4 cells by RT-PCR, normalized to HPRT, are shown. Data are relative values with SD of triplicate PCR wells. Results are representative of at least two experiments.

Exogenous IL-2 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.

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.

OX40 Signals Facilitate Nuclear Translocation of NFATc1. NFATc1 (NFATc, NFAT2) resides in the cytoplasm of resting cells. Upon appropriate stimulation, NFATc1 translocates to the nucleus, where it can activate IL-4 (27). The phosphatase calcineurin (CN) can dephosphorylate NFAT in a calcium-dependent manner, resulting in NFAT's nuclear entry, and cyclosporin A (CsA) inhibits this activity of CN. Additionally, a peptide, VIVIT, can interfere selectively with the interaction of CN with NFAT and block NFAT nuclear entry without affecting CN phosphatase activity (28, 29). Correlating with a potential role for these molecules in early IL-4 production by naïve T cells, CsA and VIVIT peptide inhibited both primary IL-4 mRNA and recall IL-4 protein expression (Fig. 4). To evaluate whether NFATc1 was targeted by OX40, we prepared nuclear extracts from naïve T cells 36 h after activation. Impaired nuclear accumulation of NFATc1 was observed in T cells lacking OX40 (Fig. 5A).
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 signals (Fig. 5A) did not affect nuclear accrual of NFATc1 (Fig. 5D).

Our previous data showed that OX40 acts on naïve T cells in combination with CD28 and that OX40 action is in large part dependent on initial CD28 signaling (15, 30). To more easily dissect the effect of OX40 away from TCR and CD28 signals, naïve T cells were stimulated for 24 h with CD3/CD28 to express OX40 (Fig. 5C) and were then recultured with agonist anti-OX40 in isolation for 4 h. OX40 triggering strongly enhanced the levels of nuclear NFATc1 (Fig. 5E and F), and this was independent of IL-2R, IL-4R, and, in this case, calcium/ CN signals (Fig. 5E) but was dependent on P13K (Fig. 5F). When analyzing the level of nuclear NFATc1 at 24 h before reculture, compared with 4 h after (Fig. 5F), it was observed that OX40 maintained NFATc1 over this time period, as opposed to inducing its entry, suggestive of an effect on suppressing nuclear export.

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 naïve 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 naïve 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 P13K (Fig. 6F). In contrast to naïve 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 factor in regulating early events of differentiation. Thus, one idea these data have implied that TCR signal strength might be a differently promote Th1 versus Th2 differentiation (32–35). Accordingly, the CN/NFAT inhibitor peptide VIVIT inhibited production of IL-4 protein (data not shown) and mRNA (Fig. 6G). CsA, VIVIT peptide, or VEEV peptide, then restimulated with Ab-coated beads for 4 h.

**Discussion**

Major strides have been made in delineating the cytokines, such as IL-4 and IL-12, and their transcription factors, such as GATA-3, e-maf, and T-bet, which can regulate commitment to Th2/Th1 lineages (31). However, there have been few reports providing strong evidence of factors that might precede and control, or at least synergize with, these cytokine signals and hence instruct Th2/Th1 divergence. In the absence of exogenous sources of IL-4 or IL-12, the affinity or dose of antigen can differentially promote Th1 versus Th2 differentiation (32–35). These data have implied that TCR signal strength might be a factor in regulating early events of differentiation. Thus, one idea is that signaling through the TCR is important both for inducing molecules necessary for later events in lineage commitment and for allowing other signals from distinct T cell membrane receptors. Our data now show that OX40 triggering is absolutely critical for Th2 development where antigen presentation determines 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 T 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 defective 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 possibly 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 thenase kinase (GSK3β) 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-4/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 RBPJκ 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.PLT/hy.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/Thy1.2 CD4 T cells responsive to the peptide OVA-323–339. OX40−/− OT-II mice were produced by intercrossing with OX40+/− mice on the BL/6 background.

Peptides, Abs, and Cytokines. OVA-323–339, VIVIT (RRRRRRRRRR-RGG-MAGPVITGPHEE), and VEET (RRRRRRRRRR-RGG-MAGPPHIVEETGPHV1) were synthesized by A&A Laboratories (San Diego). Abs against CD3 (145–2C11), CD28 (37N51), OX40 (OX86), OX40L (RM134L), IFN-γ (XM1G1.2), 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 lamin B (M20) were from Santa Cruz Biotechnology. Abs against IL-2R-α (XMG1.2), CD4 (10H7), and CD8 (3.155) and IFN-γ (XM1G1.2), CD4 (10H7), and CD8 (3.155) and IFN-γ (XM1G1.2) were from 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 (3.155), heat-stable antigen (J11D), class II MHC (M5/114, Y17, and CA-4.12), 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% CD44highCD62low 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 (FD75 and HO.13.4), CD4 (RL172.4), and CD8 (3.155) 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, gluatamine, 2-mercaptoethanol, and 7% FCS (Omega Scientific, Tarzana, CA). CD4 T cells (5 × 10⁶ 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 (XM1G1.2), 10 ng/ml IL-2, and 10 ng/ml IL-4. Cells were initially stimulated for 3 days then transferred to new plates containing 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/ml with 6 × 10⁵ cells/ml each, IL-2, 10 ng/ml IL-4, and 10 ng/ml IL-4. Cells were initially stimulated for 3 days then transferred to new plates containing anti-OX40L in PBS (0.5 ml), anti-IL-4 (10 μg/ml), anti-IFN-γ (10 μg/ml), anti-IL-2R-α plus-β (5 μg/ml each), L Y (Calbiochem; 3 μM), CsA (Calbiochem; 0.1 μM), VIVIT, or control VEET 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 poly(styrene)-coated 96-well 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 Thy1.1 recipient mice (Thy1.1) were injected i.v. with 5 × 10⁶ naïve Vβ5/Vα2/Thy1.2 CD4 T cells from wild-type or OX40−/− OT-II transgenic donors. One day after cell transfer, mice were im-
muninized s.c. with 10 μg of OVA protein (Sigma) and 2 μg of aluminum hydroxide (InsectAlum; Pierce) in PBS. Inguinal and periaortic draining lymph node cells were harvested 2 and 4 days after immunization. FACS staining with anti-Thy1.2-FITC was used to discriminate 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-TRAviadin, 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-Biotech, Auburn, CA).