Antigen-independent signalosome of CARMA1, PKCθ, and TNF receptor-associated factor 2 (TRAF2) determines NF-κB signaling in T cells

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NF-κB activation is essential for T-cell responses, and costimulatory molecules in the TNF receptor (TNFR) superfamily are viewed as a major source of this signal. Although the TNFR family recruits TNFR-associated factor (TRAF) molecules leading to IKKα/β/γ activation, it is not clear whether simple binding of TRAFs explains why they are such strong activators of NF-κB and so important for T-cell immunity. We now show that one TNFR family member, OX40 (CD134), after ligation by OX40L, assembles a unique complex that not only contains TRAF2, RIP, and IKKα/β/γ but also CARMA1, MALT1, BCL10, and PKCθ, molecules previously shown to regulate NF-κB activation through the T-cell receptor (TCR). The OX40 signalosome is formed in membrane microdomains irrespective of TCR engagement, and strongly promotes NF-κB activation only if CARMA1 and PKCθ are recruited. This NF-κB signal allows effector/memory T cells to survive when antigen is no longer available. Thus, by recruiting TCR-related intracellular molecules into the TRAF2 complex, OX40 provides the T cell with a high level of NF-κB activity needed for longevity.

T lymphocytes require costimulatory or second signals for their response, provided by receptor ligands within the CD28-B7 family as well as by molecules such as OX40 (CD134), CD27, 4-1BB (CD137), CD30, GITR, DR3, and HVEM, which are members of the TNF receptor (TNFR) superfamily (1, 2). The best-characterized costimulatory receptor, CD28, which is constitutively expressed, promotes initial responses. However, for differentiation and longevity of T cells, the receptors in the TNFR family can play dominant roles. Clonal expansion and accumulation of effector and memory CD4 and CD8 T cells are controlled by several TNFR family interactions (1, 2), typified by the ligand-receptor pair OX40-OX40L (3).

One defining feature of the TNFR family is that they are strong activators of NF-κB (4, 5). Data on molecules such as OX40 have shown this NF-κB signal is essential for a major component of their function in T cells (6). Studies primarily of TNFR1 have suggested that NF-κB activity induced by TNFR family molecules is mediated by recruitment of adaptors called TNF receptor-associated factors (TRAFs) that link a serine/threonine kinase, RIP, to activation of IKKβ and degradation of IκB (7, 8). In accordance with this, the T-cell costimulatory members OX40, 4-1BB, CD30, CD27, HVEM, and GITR have been found in transient transfection systems to recruit various TRAF molecules, resulting in NF-κB activity (9–14). Interestingly, the T-cell receptor (TCR) and CD28 are capable of synergizing together and activating NF-κB, but in this case through the adapter protein CARMA1 (15, 16). PKCθ is recruited after ligating the TCR and CD28 (17), and phosphorylation of CARMA1 by PKCθ induces a CARMA1–BCL10–MALT1 (CBM) complex that activates IKKβ (18, 19). This raises the question of how much cross-talk there might be between the TCR and costimulatory receptors in the TNFR family. One study of 4-1BB showed that cross-linking this molecule resulted in localization of PKCθ at the T-cell synapse (20), although a caveat here is that 4-1BB is atypical within the TNFR superfamily and can bind Lck and might directly cooperate with the TCR. However, this suggested that members of the TNFR family might have the ability to recruit signaling molecules normally associated with the TCR, and that this could then allow a level of NF-κB activation that could not be achieved otherwise.

Here we show that engagement of OX40 on activated/effector T cells by OX40L resulted in not only the recruitment of the canonical TRAF-RIP-IKKα/β/γ complex but additionally recruited PKCθ and the CARMA1-MALT1-BCL10 complex. This signalosome directly controlled NF-κB activation without antigen/TCR engagement, but was dependent on OX40 being aggregated by its ligand and moving into detergent-insoluble membrane lipid microdomains (DIMs). Without recruitment of CARMA1 or PKCθ, OX40 only induced NF-κB activation at a low level, approximating that promoted through the TCR when recognizing peptide/MHC, and this prevented OX40 from imparting a survival signal to effector T cells.

Results

OX40–OX40L Interactions Augment Antigen-Independent NF-κB Signaling. To address the signaling complex formed by OX40, we established a moth cytochrome *c* (MCC) peptide-specific T-cell hybridoma derived from activated/effector T cells obtained from OX40-deficient AND Vα11/Vβ3 TCR transgenic mice. These were transfected with cMyc-OX40. Both control vector and cMyc-OX40 T cells had equivalent Vα11, Vβ3, CD3ε, and CD28 (Fig. S1*A*). These T cells were stimulated with MCC presented on $CD80^+OX40L^+$ antigen-presenting cells (APCs) (21) (Fig. S1*B*).

When assessed in total lysates from cultures, phosphorylation of IκBα and RelA was strongly promoted by engagement of OX40 (cMyc-OX40 T cells) with $OX40L^+$ APCs compared with that induced by peptide in OX40 negative T cells (control vector) (Fig. 1 *A* and *B*). In accordance, OX40–OX40L interactions drove strong accumulation of nuclear NF-κB (p50 and RelA) (Fig. 1*B*). Interestingly, phosphorylation of IκBα and nuclear p50 and RelA was promoted by $OX40L^+$ APCs independently of antigen recognition (Fig. 1*B*). Showing this reflected an OX40 signal to the T cells, cMyc-OX40 T cells displayed significantly less nuclear p50/RelA when stimulated with OX40L negative APCs in the absence of antigen (Fig. S1*C*). Furthermore, OX40L protein on an immobilized surface induced comparable NF-κB activation to that observed in cocultures of T cells with APCs (Fig. S1*D*). Nuclear NF-κB in cocultures was primarily detected in T cells, again showing it was largely derived from OX40 signaling (Fig. S1*E*). Phosphorylation of Erk was unaffected by OX40–OX40L interactions, demonstrating some specificity (Fig. 1*A*). CD80 blockade had little or no effect on NF-κB activation (Fig. 1*B* and Fig. S1*C*). Showing OX40 ligation did not cross-link the TCR in the absence of peptide, phosphorylation of Zap70

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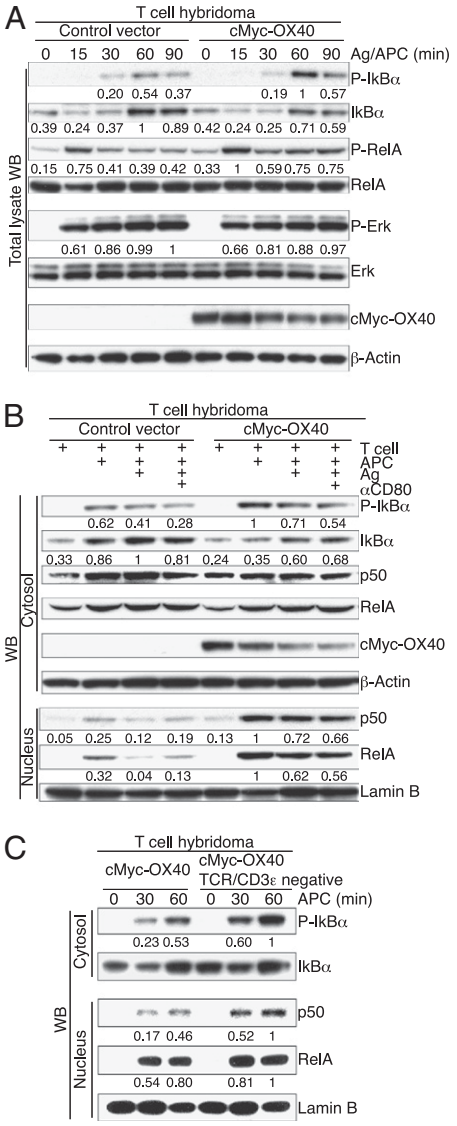

Fig. 1. OX40 promotes antigen-independent NF-κB activation. Control vector (OX40$^{-/-}$) and cMyc-OX40 transduced T-cell hybridomas were stimulated in the presence or absence of MCC peptide and B7^{+}OX40L^{+} DCEK APCs, and anti-CD80/B7 blocking antibody. (*A*) Protein expression levels were evaluated by immunoblotting of total lysates from cells cultured for 0–90 min. (*B*) Cytosolic and nuclear protein levels were evaluated after 60 min in lysates from cultures as in *A*. (*C*) NF-κB activation in a cMyc-OX40 T hybridoma defective in expression of Vα11, Vβ3, and CD3ε, evaluated in the absence of antigen (Ag) as in *B*. Densitometry was performed with 1 as a reference for the highest level normalized to actin or lamin expression. All results are representative of at least two experiments. WB, Western blot.

was not observed (Fig. S1*F*). OX40 signals augmented antigen-dependent IL-2 production by the T-cell hybridoma and this was suppressed by dominant-negative (DN) IκBα (Fig. S1*G*), showing the OX40-induced NF-κB signal was functional. OX40 signals in isolation did not induce IL-2, as TCR signaling is required for Ca^{2+} entry and NFAT activation is essential for IL-2 secretion. Antigen signals did not significantly augment P-IκBα and nuclear p50/RelA over that induced by OX40, and depending on the time examined resulted in less NF-κB activation (Fig. 1*B* and Fig. S1*H*), suggesting that too strong stimulation caused down-regulation of this pathway. Last, OX40–OX40L interactions still strongly promoted NF-κB activation in a TCR/CD3 negative T-cell hybridoma (Fig. 1C and Fig. S2 *A* and *B*). These results show that OX40 is a dominant antigen/TCR-independent stimulus for promoting NF-κB activity in effector-like T cells.

OX40 Forms a Functional Signalosome with TRAF2, the IKK Complex, and CARMA1. OX40 was then immunoprecipitated via cMyc. TRAF2 can associate with OX40 in transient transfection systems (9, 10). After stimulation with OX40L-expressing APCs, but not before, OX40 associated with endogenously expressed TRAF2 (Fig. 2). Moreover, IKKα, IKKβ, IKKγ, and RIP were found within this signalosome (Fig. 2 *A* and *B*), similar to the complex immunoprecipitated with TNFR1 after TNF engagement (7, 8). Most significantly, we visualized CARMA1, the adapter linking the TCR to NF-κB (15, 16, 18). Again, this was not dependent on TCR engagement (Fig. 2*B*) or expression of TCR/CD3 (Fig. 2*C*). Zap70, Lck, SLP-76, CD28, and CD3 were not found in the immunoprecipitates (Fig. S3). Thus, OX40 binding to OX40L promotes a unique complex separate from the TCR and CD28 signalosomes.

T cells expressing a TRAF2 dominant-negative transgene were unreceptive to OX40 signals in vivo (22). We knocked down TRAF2 with shRNA (Fig. S4*A*), which did not affect OX40 expression (Fig. S4*B*) but impaired activation of NF-κB following OX40L interaction (Fig. 3*A*). Correspondingly, the association of OX40 with IKKα/β was severely reduced, but interestingly not the association with RIP and CARMA1 (Fig. 3*B*).

CARMA1 was also reduced with shRNA (Fig. S5 *A*–*C*). Importantly, activation of NF-κB driven by OX40L^{+} APCs was severely impaired (Fig. 3*C*) and reduced to approximately the level provided by recognition of antigen in the context of CD28–B7 interactions (Fig. 1). Association of OX40 with RIP and IKKα/β was inhibited but not association with TRAF2 (Fig. 3*D*). Thus, CARMA1 together with TRAF2 promotes strong NF-κB activation through OX40 and is also required for full assembly of the OX40 signalosome.

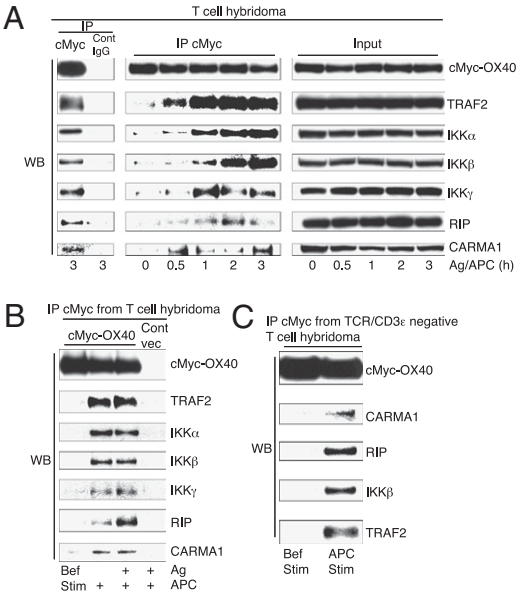

Fig. 2. Antigen-independent induction of a unique CARMA1-containing OX40 signalosome. (*A* and *B*) cMyc-OX40 or control vector (OX40$^{-/-}$) T-hybridoma cells were stimulated with APCs as in Fig. 1 in the presence (*A* and *B*) or absence (*B*) of antigen for 0–3 h. Cells were lysed in RIPA buffer containing Nonidet P-40 and OX40 was immunoprecipitated. Samples were analyzed for the indicated proteins. (*C*) OX40 signalosome formation in TCR/CD3 negative T cells, evaluated at 1 h after APC stimulation in the absence of antigen as in *B*. Bef Stim indicates OX40 immunoprecipitated from unstimulated T cells. All results are representative of at least two experiments. IP, immunoprecipitation.

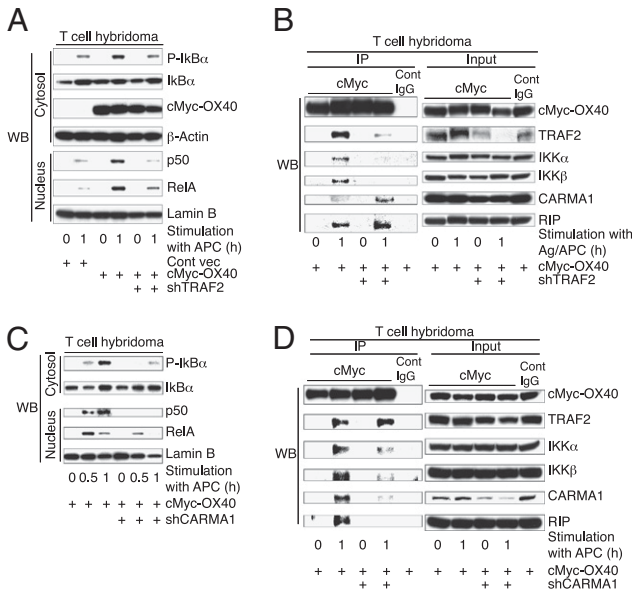

Fig. 3. TRAF2 and CARMA1 act as critical OX40 signalosome organizers. (*A* and *C*) NF-κB activation was evaluated in shTRAF2 (*A*) and shCARMA1 (*C*) transduced T-cell hybridomas as in Fig. 1*B* before stimulation or after stimulation with APCs in the absence of antigen. (*B* and *D*) OX40 was immunoprecipitated via the cMyc tag as in Fig. 2 from shTRAF2 (*B*) or shCARMA1 (*D*) transduced T cells that were unstimulated (0 h) or stimulated with APCs for 1 h. Accompanying proteins were visualized by immunoblotting. Results are representative of at least two experiments.

CARMA1/TRAF/RIP Signalosome Is Formed in Detergent-Insoluble Microdomains. Detergent-insoluble microdomains (DIMs or lipid rafts) might be important for efficient intracellular signaling (23). The majority of OX40 resided outside the DIMs before stimulation (Fig. 4*A*), but activation induced strong OX40 translocation accompanied by TRAF2, IKKα, IKKβ, IKKγ, and RIP (Fig. 4*A*). The DIM fractions of T cells stimulated with OX40L-expressing APCs were additionally enriched in ubiquitinated RIP and phosphorylated IKKα/β. This was without antigen recognition. Antigen in the absence of OX40 resulted in only moderate accumulation of phosphorylated IKKα/β, and no additive effect was seen with antigen and OX40L (Fig. 4 *B* and *C*).

CARMA1 can be expressed in several compartments, including cytosol, and lipid raft and nonraft membrane fractions in unstimulated T cells (15). A proportion of CARMA1 preexisted in the DIMs of T-cell hybridomas before stimulation, and TCR/OX40L stimulation induced a marginal increase (Fig. 4*D*). Total and phospho-IKKα/β in the DIMs was dramatically reduced when TRAF2 was knocked down, but expression of CARMA1 and ubiquitination of RIP were unaffected (Fig. 4*D*).

The cholesterol/DIM-depleting drug methyl-β-cyclodextrin (MβCD) (Fig. S6*A*) at 10 mM completely suppressed phosphorylation of IκBα and nuclear p50 and RelA driven by OX40L$^+$ APCs (Fig. 5 *A* and *B*). Suppression of sphingolipid and cholesterol synthesis by myriocin (M) and zaragozic acid (Z), respectively, produced the same results (Fig. 5*C* and Fig. S6*B*). Importantly, OX40 association with TRAF2 and IKKα/β/γ was not altered, whereas that with RIP and CARMA1 was abolished, and phosphorylation of IKKα/β in the complex did not occur (Fig. 5*D*). OX40L$^+$ APC stimulation resulted in ubiquitination of OX40 (Fig. S7*A*), which was also sensitive to MβCD (Fig. 5*D*), suggesting this might have a functional role in OX40 association with CARMA1 and RIP. Together with data in Fig. 3, this implies that OX40 recruits the IKK complex through TRAF2 outside the DIMs. CARMA1 and RIP are then recruited after translocation of OX40 into the DIMs, and CARMA1 allows IKK to remain associated and be strongly phosphorylated.

PKCθ and the CBM Complex Associate with OX40 and Play a Role in OX40-Driven NF-κB. We then investigated whether the other components of the TCR-driven NF-κB pathway, namely PKCθ, MALT1, and BCL10, were recruited to OX40. We did not find these in the stringent immunoprecipitation conditions using RIPA/Nonidet P-40 (Figs. 2, 3, and 5), but when n-dodecyl-β-maltoside was used, a detergent that preserves membrane protein structure but solubilizes DIMs, we found that PKCθ, MALT1, and BCL10 were precipitated after OX40 was ligated using OX40L-expressing APCs, along with TRAF2, CARMA1, RIP, and IKK (Fig. 6*A*). Again, this was independent of TCR engagement (Fig. 6*A*), and was also induced with an agonist antibody to OX40 in the absence of APCs (Fig. S7 *B* and *C*). After knockdown of TRAF2, total PKCθ levels were unaffected (Fig. S4*C*) but association of PKCθ with OX40 was impaired (Fig. 6*B*), strongly contrasting with CARMA1 (Figs. 3*B* and 6*B*) and RIP (Fig. 3*B*).

To verify that the CARMA1–BCL10–MALT1–PKCθ interactions were relevant to the ability of OX40 to signal in primary effector CD4 T cells, we attempted to immunoprecipitate OX40, but a technical limitation prevented efficient pull-down with anti-OX40 reagents. However, precipitation of RIP, after stimulation with OX40L$^+$ APCs in the absence of antigen, revealed both OX40 and the PKCθ-CBM complex (Fig. 6*C*). We also assessed NF-κB activation in primary effector populations derived from PKCθ$^{-/-}$ and CARMA1$^{-/-}$ mice. Because OX40 is transiently inducible in primary T cells, it was expressed at variable levels on wild-type effector cells, depending on the experiment, but ap-

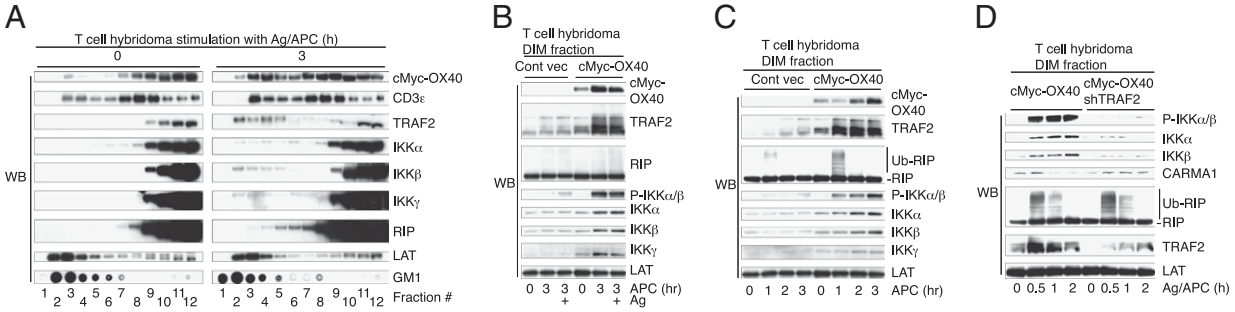

Fig. 4. OX40 translocation into DIMs is associated with activation of IKK. (*A*) cMyc-OX40 T cells were stimulated with APCs in the presence of antigen as in Figs. 1 and 2. Fractions from a sucrose gradient separating lysates of unstimulated (0 h) and stimulated (3 h) T cells were immunoblotted for the indicated proteins. LAT and GM1 are markers of the DIMs. (*B* and *C*) Control vector (OX40$^{-/-}$) and cMyc-OX40 T cells were stimulated with APCs in the presence or absence of antigen for the indicated times as in *A*. The DIM fractions were obtained by a two-step separation method and protein levels were evaluated by immunoblotting. (*D*) DIM fractions were obtained and visualized by immunoblotting from cMyc-OX40 T cells transduced with shTRAF2 and stimulated for 0–2 h with antigen/APCs. Results are representative of at least two experiments.

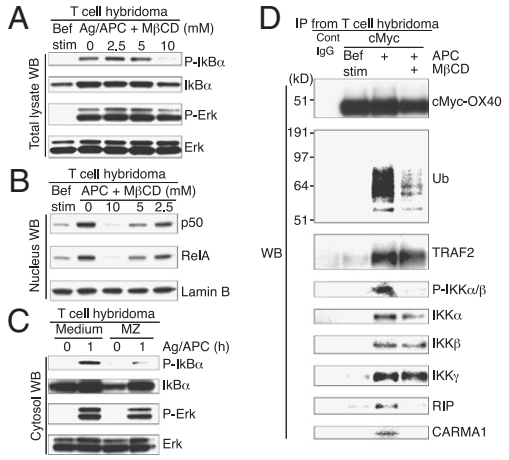

Fig. 5. Disruption of DIMs partially blocks formation of the OX40 signalosome and inhibits NF-κB signaling. (*A* and *B*) cMyc-OX40 T cells were cultured with various concentrations of MβCD. Total and nuclear protein levels of the indicated molecules were evaluated at 1 h by immunoblotting after stimulation with APCs. Bef stim, unstimulated T cells. (*C*) cMyc-OX40 T cells treated with myriocin (M) and zaragozic acid (Z) were cultured with APCs for 1 h and the cytosolic protein levels were evaluated by immunoblotting. (*D*) cMyc-OX40 T cells were cultured with APCs in the presence or absence of 10 mM MβCD for 1 h as in *A* and *B*. OX40 was immunoprecipitated from total cell lysates as in Fig. 2 and associated proteins were analyzed as well as ubiquitination (Ub). Bef stim, OX40 precipitated from unstimulated T cells. Results are representative of at least two experiments.

proximating that seen on the cMyc-OX40 transduced hybridoma (Fig. S8*A Upper* to *Lower*). OX40 was also expressed on the majority of primary effector T cells induced from PKCθ$^{-/-}$ and CARMA1$^{-/-}$ mice (Fig. S8*A*). OX40L-expressing APCs induced phosphorylation of IKKα/β and IκBα and nuclear RelA and p50 in WT T cells, whereas these were strongly impaired in both knockout T cells (Fig. 6*D* and Fig. S8*B*). The defect in NF-κB activation was also evident when PKCθ was knocked down (Fig. S8 *C–E*). Neither CARMA1 nor PKCθ was prelocalized with OX40 in primary effector T cells before OX40 was ligated, but both became tightly focused with OX40 in the contact region after engagement of OX40L expressed on an interacting cell. This was in the absence of antigen, and when MHC class II was blocked (Fig. 6*E*) and showing specificity and further confirming TCR independence, TCRβ did not cocluster with OX40 (Fig. S8 *F–H*). Thus, PKCθ and CARMA1 form a signalosome with OX40 in the immune synapse and play a major role in promoting maximum and prolonged NF-κB activity mediated by OX40.

TCR-Independent NF-κB Signals from OX40 Control Effector T-Cell Survival. OX40–OX40L interactions regulate the expansion/survival of effector T cells due to promoting or maintaining expression of molecules such as Bcl-xL, Bcl-2, and survivin, which are responsible for proliferation and survival (24–29). As OX40 is expressed shortly before and during the peak phase of effector T-cell expansion, our data suggested that it might contribute to maximizing clonal expansion by forming the PKCθ/CBM/TRAF signalosome, enabling a late but strong NF-κB–dependent survival signal after antigen is cleared. To evaluate this, WT CD45.1$^+$ effector T cells were adoptively transferred into congenic mice that were injected with adjuvants that promote OX40L expression, but in the absence of antigen. Significantly, blocking OX40–OX40L interaction in vivo reduced donor T-cell survival by more than 50% (Fig. 7*A*). WT effector T cells were also transduced with a GFP retroviral vector containing DN IκBα or control. Equal numbers of GFP$^+$OX40$^+$ T cells and GFP$^-$OX40$^+$ T cells were transferred to the congenic hosts. The GFP$^-$ T cells from cultures transduced with control or DN IκB vectors survived equivalently as an internal control (Fig. 7*B Lower*), but those GFP$^+$ T cells expressing DN IκBα did not survive well (Fig. 7*B Upper*), replicating the 50% reduction revealed by blocking OX40L. Furthermore, effector T cells in which PKCθ was knocked down by shRNA also displayed a similar defect (Fig. 7*C*). These results support the contention that in vivo survival of effector T cells can be dependent on OX40 promoting PKCθ/CBM-driven NF-κB activity during late-phase T-cell responses when antigen is cleared.

Discussion

We have identified signaling mechanisms of OX40 that are shared by both the TNFR family and the TCR. TNF binding with TNFR1, the best-characterized TNFR family member, recruits TRADD, TRAF2, and RIP, and induces ubiquitination of RIP to form a signaling complex. RIP's ubiquitination is mediated by TRAF2 but requires TRADD (30–32), and the polyubiquitinated RIP recruits IKKγ (33) and TRAF2 binds IKKα and IKKβ (7). Therefore, TRADD works primarily as an adaptor protein. OX40 triggering with OX40L also recruited TRAF2 and RIP, and resulted in ubiquitination of RIP. However, OX40 does not have a death domain to recruit TRADD. The absence of TRAF2 resulted in a lack of association of OX40 with PKCθ, IKKα, and IKKβ, which contributed to a defect in NF-κB activation, but RIP and CARMA1 association were not affected. In contrast, CARMA1 was required for RIP recruitment to OX40, and also strongly participated in allowing association and phosphorylation of the IKK complex, drawing some comparisons with the action of TRADD. We therefore conclude that both TRAF2 and CARMA1 serve as keystones for formation of the full OX40 signalosome and for allowing NF-κB activation by OX40 in T cells.

The important role of the CBM complex has been well-characterized for antigen receptors, but other receptor systems also use this or similar types of signalosome for activation of NF-κB, including CD28 (34). Although we ruled out a role for the TCR in formation of the OX40 signalosome, it is presently not clear whether OX40 collaborates with other receptors to recruit the PKCθ-CBM complex. Blocking CD80 on the OX40L$^+$ APC did not prevent OX40 activity, and the complex was also formed by engagement of OX40 with anti-OX40 in the absence of APCs. However, CD28 engagement alone can recruit PKCθ (35), and a possibility remains that CD28 or other receptors might cooperate with OX40 in that T cells themselves can express CD86, or some other ligands, that possibly could have ligated their receptors. This needs to be investigated in the future. In the TNFR superfamily, our finding that CARMA1, PKCθ, BCL10, and MALT1 are components of the OX40 signalosome is presently a unique observation, although other results imply that this signalosome could be broadly used. In CD8 T cells, PKCθ was also found to colocalize with 4-1BB in immune synapses (20), and a role for MALT1 in B-cell activation induced by another family member, BAFF, was shown (36). Several studies have reported that soluble TNF binding to the TNFR1 can recruit PKCδ in non-T cells and that this can contribute to NF-κB activity (37), perhaps suggesting that PKC use by TNFR family members might also be widespread. In contrast, soluble TNF induced NF-κB equivalently in CARMA1-deficient Jurkat T cells (15, 16), either implying TNFR1/2 cannot form the same signalosome as OX40, or that soluble TNF does not provide a sufficient level of aggregation of the receptor to induce recruitment of the CBM complex and/or PKCθ.

Our functional studies here and elsewhere provide strong clues as to where the OX40 signalosome is relevant to T-cell immunity (6, 26, 38). Although it has been implied that OX40L will be provided on an activated dendritic cell or B-cell presenting antigen during the early phase of clonal expansion of T cells, fulfilling a conventional costimulus role (25, 39–41), Ishii and colleagues found that OX40L could be expressed on the responding T cells themselves (42), and that OX40L interactions could help survival of effector memory CD4 T cells in the apparent absence of antigen (29). Furthermore, lymphoid tissue-inducer cells (LTi), which express OX40L, can promote CD4 T-

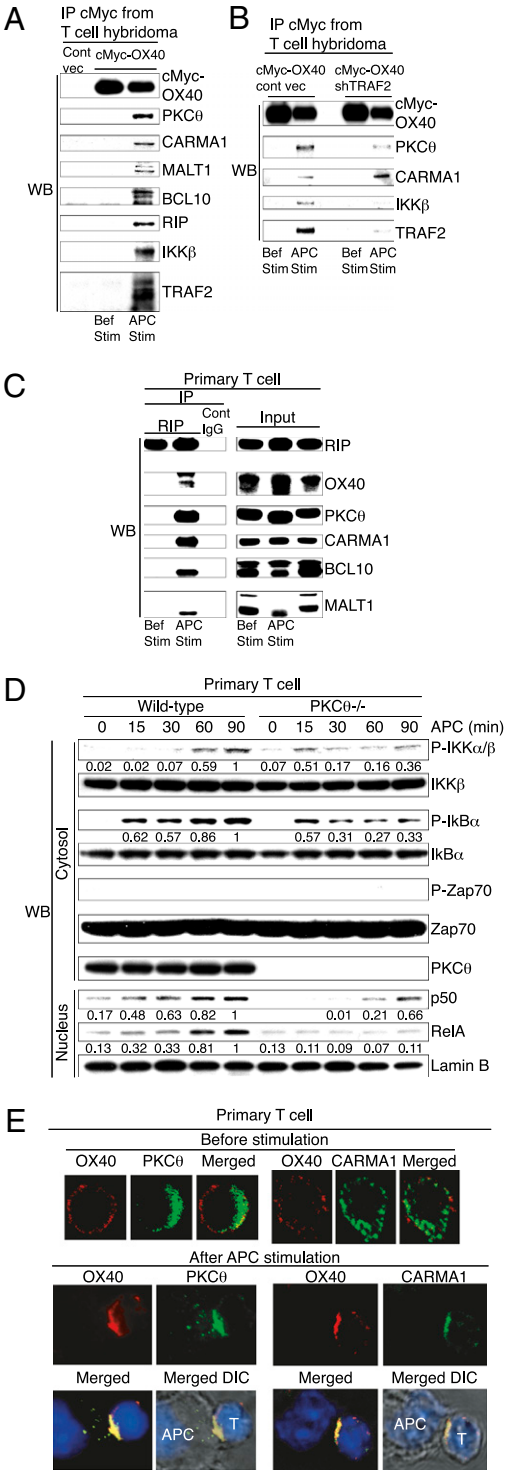

Fig. 6. PKCθ is required for activation of NF-κB mediated by OX40. (*A* and *B*) cMyc-OX40, cMyc-OX40-shTRAF2, or control T-cell hybridomas were left unstimulated (Bef Stim) or stimulated with APCs in the absence of antigen for 15 min. Cells were lysed in 1% n-dodecyl-β-maltoside and cMyc-OX40 was immunoprecipitated. Indicated proteins were visualized. (*C*) WT primary effector T cells were left unstimulated, or stimulated with APCs in the presence of anti-MHC class II for 2 h. Cells were lysed in 1% n-dodecyl-β-maltoside and RIP was immunoprecipitated with associated proteins as indicated. (*D*) NF-κB activation was evaluated by immunoblot of cytosol and nuclear lysates from WT and PKCθ$^{-/-}$ primary effector CD4 T cells that were cultured for 0–90 min with APCs in the presence of anti-MHC class II. (*E*) WT primary effector CD4 T cells were allowed to form conjugates with APCs in the presence of anti-MHC class II. T cells were stained before culture (*Upper*) or after culture (*Lower*) for OX40 (red, Alexa Fluor 555), PKCθ (green, Alexa Fluor 488), CARMA1 (green, Alexa Fluor 488), and nuclei (blue, DAPI), and visualized by microscopy. Representative images are shown. Results are representative of at least two experiments.

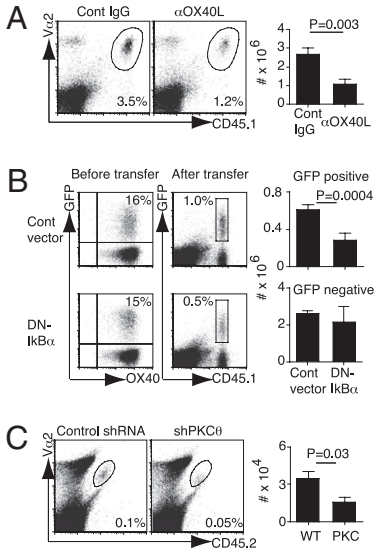

Fig. 7. OX40-PKCθ-NF-κB axis controls antigen-independent survival in primary effector T cells. (*A* and *B*) WT primary effector OT-II T cells (CD45.1$^+$) were adoptively transferred into congenic recipients and cell survival was tracked in the absence of antigen over 7 d. In *A*, anti-OX40L was injected in vivo at the time of transfer. In *B*, effector T cells were transduced with control or DN IκBα GFP vectors and equal numbers of GFP$^+$OX40$^+$ or GFP$^-$OX40$^+$ cells were transferred. Transduction efficiency and expression of OX40 with GFP is shown before adoptive transfer. Data show percentage and total numbers of donor GFP$^+$ and GFP$^-$ CD4 T cells recovered from the spleen. (*C*) WT primary effector OT-II T cells (CD45.2$^+$) were transduced with control shRNA and shPKCθ and transferred into congenic recipients. T-cell survival was evaluated at day 14. All data are means with SEM and *P* values in unpaired Student's *t* test for four mice per group. All results are representative of at least two experiments.

cell survival in an OX40-dependent manner (27), even though these cells are thought not to express MHC class II. Together, this implies that after clearance of antigen, OX40 signals allow effector T cells to be maintained and active, ensuring efficient late primary responses as well as development of high-frequency memory T-cell pools. Although the location and timing of late cellular interactions with T cells in vivo have not been characterized, T–LTi- and T–T-type cell interactions might support antigen-independent but OX40-OX40L-dependent late activity by forming the OX40 signalosome that productively activates NF-κB. Functional studies of 4-1BB also support this general concept, showing 4-1BB signals can regulate late survival of CD8 T cells in the absence of antigen (43).

In summary, our experiments demonstrate a previously unrecognized facet of TNFR family costimulatory receptor action on T cells whereby the level of NF-κB activation that is essential for T-cell fate is determined by an antigen-independent signalosome that incorporates intracellular molecules known to be key to both the TCR and the TNFR family. The data provide a possible explanation as to why such late-acting signaling from this family of costimulatory receptors is so important for sustaining T-cell function.

Materials and Methods

Mice. OX40-deficient AND [Tg(TcrAND)53Hed] on a B10.BR background and WT OT-II TCR-transgenic mice on a B6 or B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (The Jackson

Laboratory) background were bred in-house (6, 26, 38). Wild-type or PKCθ$^{-/-}$ (17) mice on a B6 background were also bred in-house. B6 CARMA1$^{-/-}$ (44) mice were bred by Michael Farrar at the University of Minnesota and, with permission from Dan Littman (New York University), spleens were transferred to the La Jolla Institute for Allergy and Immunology.

Cells. CD4 T cells from OX40-deficient AND mice (Vβ3/Vα11) were activated with MCC peptide ($MCC_{88–103}$), and primary effector cells were then fused with BW5147 using polyethylene glycol. A representative IL-2-producing clone was established. N-terminal cMyc-tagged mouse OX40 was subcloned into the pEF1 vector (Invitrogen). T-cell hybridomas were transfected with control or cMyc-OX40 vector and selected with G418 (Invitrogen). A fibroblast (DCEK) cell line expressing endogenous CD80 was transfected with I-E^k and OX40L as described (21). T cells (5×10^5 cells per mL) were stimulated with DCEK cells (5×10^3 to 1×10^5 cells per mL) in the presence or absence of 10 μM T102S, a superagonist MCC peptide.

For generation of primary effector T cells, naive CD4 T cells from wild-type OT-II TCR transgenic mice (Vβ5/Vα2) or wild-type, PKCθ$^{-/-}$ (17), or CARMA1$^{-/-}$ (44) B6 mice were stimulated with 5 μg/mL plate-bound anti-CD3, 5 μg/mL soluble anti-CD28, 10 μg/mL anti–IFN-γ, 10 ng/mL IL-2, and 10 ng/mL IL-4. T cells were harvested at days 5–7 and recultured with OX40L-expressing DCEK cells in the absence of antigen and anti-IE^k blocking antibody (10 μg/mL).

DIM Isolation, Immunoprecipitation, and Western Blotting. For discontinuous sucrose-gradient ultracentrifugation, cells were lysed in 1 mL of 1% Brij-58 lysis buffer. Lysates were mixed with 1 mL of 80% wt/vol sucrose in the same lysis buffer and overlaid with 2 mL of 30% sucrose followed by 1 mL of 5% sucrose. Samples were ultracentrifuged in a Beckman SW50Ti rotor at 200,000 × *g* for 16 h at 4 °C. Alternatively, the DIM fractions were prepared by a two-step separation method. Postnuclear lysates in 1% Brij-58 lysis buffer were ultracentrifuged at 100,000 × *g* for 50 min at 4 °C and DIM proteins in the cell pellets were extracted with 1% SDS containing ice-cold RIPA buffer. For immunoprecipitation, cells were lysed in ice-cold RIPA or 1% n-dodecyl-β-maltoside. Lysates were immunoprecipitated with primary Abs, cMyc, and protein G beads. Samples were loaded onto NuPage bis-Tris precasting gels, transferred onto PVDF membrane (Invitrogen), and immunoblotted.

Immunofluorescence. Immunofluorescence of effector CD4 T cells with OX40L-expressing DCEK cells was analyzed in the presence of anti-IE^k under a Zeiss Axiovert 200M microscope.

Detailed procedures are described in *SI Materials and Methods*.

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