Annexin A5 is essential for PKCθ translocation during T-cell activation

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T-cell activation is a critical part of the adaptive immune system, enabling responses to foreign cells and external stimulus. In this process, T-cell antigen receptor (TCR) activation stimulates translocation of the downstream kinase PKCθ to the membrane, leading to NF-κB activation and thus transcription of relevant genes. However, the details of how PKCθ is recruited to the membrane remain enigmatic. It is known that annexin A5 (ANXA5), a calcium-dependent membrane-binding protein, has been reported to mediate PKCδ activation by interaction with PKCδ, a homologue of PKCθ, which implicates a potential role of ANXA5 involved in PKCθ signaling. Here we demonstrate that ANXA5 does play a critical role in the recruitment of PKCθ to the membrane during T-cell activation. ANXA5 knockout in Jurkat T cells substantially inhibited the membrane translocation of PKCθ upon TCR engagement and blocked the recruitment of CARMA1-BCL10-MALT1 signalosome, which provides a platform for the catalytic activation of IKKs and subsequent activation of canonical NF-κB signaling in activated T cells. As a result, NF-κB activation was impaired in ANXA5-KO T cells. T-cell activation was also suppressed by ANAX5 knockdown in primary T cells. These results demonstrated a novel role of ANXA5 in PKC translocation and PKC signaling during T-cell activation.

T-cell activation is a core part of adaptive immune response, leading to cytokine production and cell proliferation (1). Engagement of TCR-CD3 complex with co-receptor CD28 recruits large signaling complexes to signal transduction cascades. The serine/threonine-specific protein kinase C (PKC) activity is required for TCR/CD3-induced T-cell activation (2). PKCθ, a novel calcium-independent member of the PKC family, is proved to selectively mediate several essential functions in TCR-linked signaling, leading to cell activation (3). PKCθ-deficient T cells displayed defects in TCR-induced proliferation and differentiation (4). PKCθ is usually found in the cytosol when inactive. Upon TCR stimulation, PKCθ rapidly translocates to membrane lipid rafts and activates the downstream signaling, which subsequently results in NF-κB activation (5, 6). PKCθ translocation to lipid rafts plays a pivotal role in T-cell activation, but the molecular basis of PKCθ translocation has not been elucidated.

There have been some investigations on the molecular events of triggering the membrane-bound PKCθ in T-cell activation. PKCθ has no obvious raft-targeting motif, so the lipid raft localization of PKC needs the association with another raft-targeted signaling protein. The cysteine-rich C1 domain of PKCθ can bind membrane-containing second messenger DAG upon stimulation (7). Phosphoinositide-dependent kinase 1 (PDK1) and LCK have been reported to modulate PKC phosphorylation and its translocation (8, 9). Vav and CD28 have also been reported to mediate the recruitment of PKCθ to immunological synapse by interaction with PKCθ (10, 11). Although much is known about PKCθ activation, the process of PKCθ translocation and the proteins that regulate it need further identification.

The annexin superfamily (Anx) is a calcium (Ca^{2+})- and phospholipid-binding protein family. Annexin A5 (ANXA5) belongs to the annexin family, which is well-known for its high affinity to phosphatidylserine (PS) and widely used in apoptosis detection (12), even in molecular imaging for disease diagnosis in clinical applications (13). ANXA5 is involved in various intra- and extracellular processes, including blood coagulation, anti-inflammatory processes, membrane trafficking, and signal transduction (14). However, the biological functions of ANXA5 are believed to depend primarily on its interactions with lipids in membranes. Several annexins have been reported to interact with different PKC isozymes, such as AnxA1, -A2, -A5, and -A6 (15). It has been shown that ANXA5 interacts with PKCδ as an essential step in PKCδ translocation and activation (16). Within the PKC family, PKCδ displays the highest homology with PKCθ. PKCθ is most closely related to PKCδ, because the V1 domains of these two enzymes share 49% homology (17, 18). In addition, the V1 domain of PKCδ is required for interaction with ANXA5, so we hypothesize that ANXA5 might be involved in PKCθ membrane translocation and PKCθ-mediated function by interaction with PKCθ.

In this study, we demonstrate that ANXA5 is involved in T-cell activation by ANXA5-PKCθ interaction. ANXA5 deficiency selectively inhibited PKCθ-mediated NF-κB activation via blocking the recruitment of CARMA1/BCL10/MALT1

This article contains supporting information.
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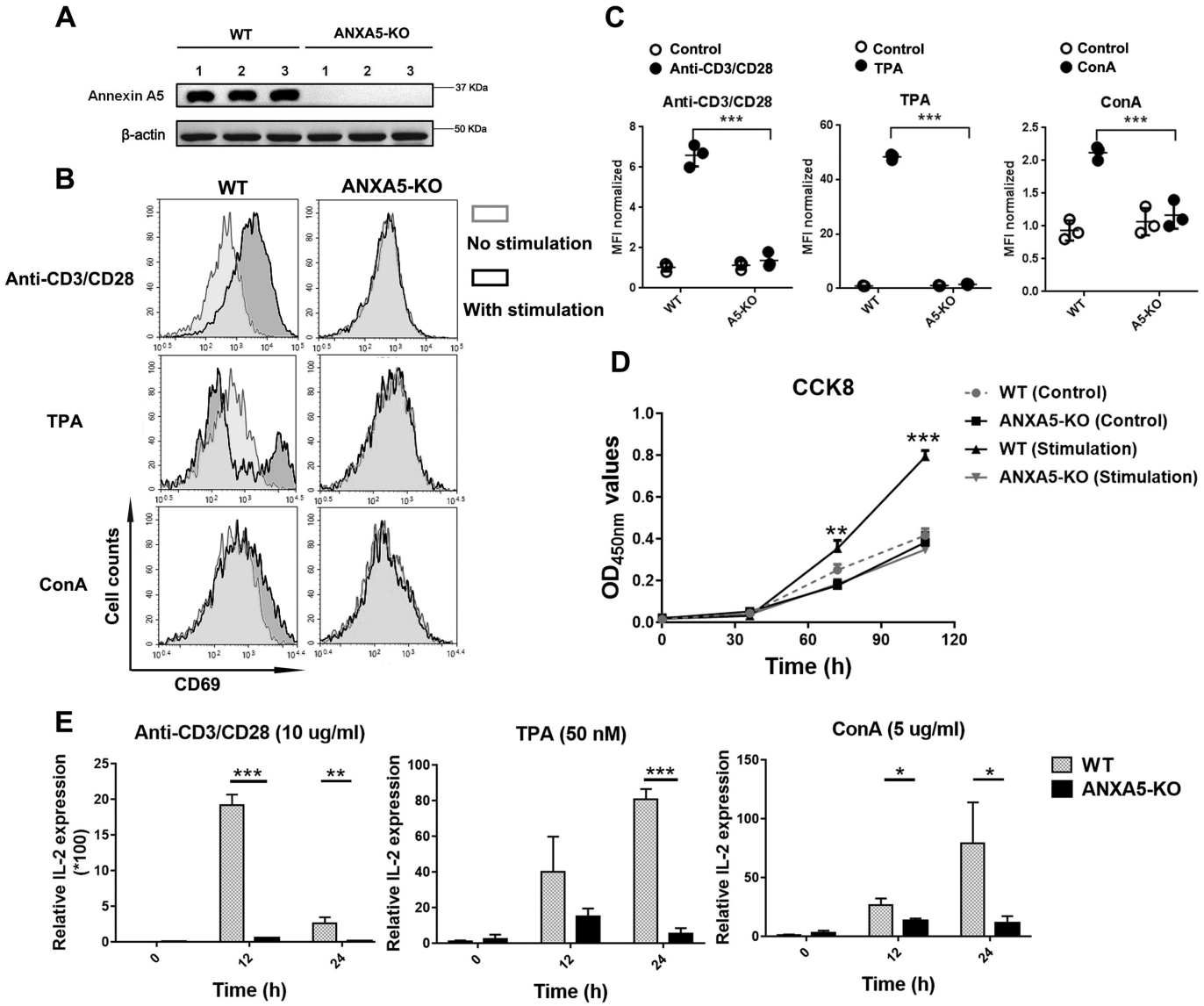

Figure 1. Knockout of ANXA5 inhibits T-cell activation. *A*, ANXA5 expression was analyzed by Western blotting in ANXA5-KO cells and parent Jurkat T cells. *B*, cells were stimulated with anti-CD3/CD28 (10 μg/ml), TPA (50 nM), or ConA (5 μg/ml), respectively. After a 24-h treatment, T cells were collected for anti-CD69 APC staining. Representative FACS analysis of $CD69^+$ T cells is shown in response to stimulations. *C*, mean fluorescence intensity (*MFI*) of flow cytometric measurements of $CD69^+$ T cells for quantitative analysis. Values were normalized by the mean fluorescence intensity of controls with no stimulation. *D*, T cells were treated with anti-CD3/CD28 co-stimulation for the indicated times. Cell proliferation was measured by Cell Counting Kit-8. *E*, after T cells were stimulated by activator as indicated for 12 or 24 h, the expression of IL-2 was detected by quantitative RT-PCR. *Error bars*, S.D. ($n = 3$/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

complex. Our results present a novel role of ANXA5 on PKCθ signaling in T-cell activation.

Results

Knockout of ANXA5 inhibits T-cell activation

To investigate the role of ANXA5 in T-cell activation, we used CRISPR/Cas9 technology to generate ANXA5-KO Jurkat T cells (Fig. 1*A*). There are three main types of stimulus for T-cell activation: anti-CD3/CD28 co-stimulation, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and concanavalin A (ConA) (1, 19). With the treatment of each stimulus, T-cell activation was examined in both ANXA5-KO and WT Jurkat T cells. CD69 as a T-cell activation marker is always used to evaluate the degree of T-cell activation by FACS analysis. After 24 h of stimulation, the induction of CD69 expression was readily apparent in WT Jurkat T cells, but not in ANXA5-KO Jurkat T cells (Fig. 1, *B* and *C*). By CCK8 assay, ANXA5-KO Jurkat T cells were also shown to be defective in cell proliferation upon anti-CD3/CD28 co-stimulation (Fig. 1*D*). During T-cell activation, TCR-CD3 engagement leads to increased production of interleukin-2 (IL-2) (20). Consistently, we found that the production of IL-2 was effectively up-regulated in Jurkat T cells responding to various stimulus, but little change was detected in ANXA5-KO cells (Fig. 1*E*). Collectively, our data suggested that ANXA5 plays an important role in T-cell activation.

ANXA5 knockout inhibits NF-κB signaling in T-cell activation

To explore the signal transduction pathway of ANXA5 in T-cell activation, three major signaling pathways ERK, p38

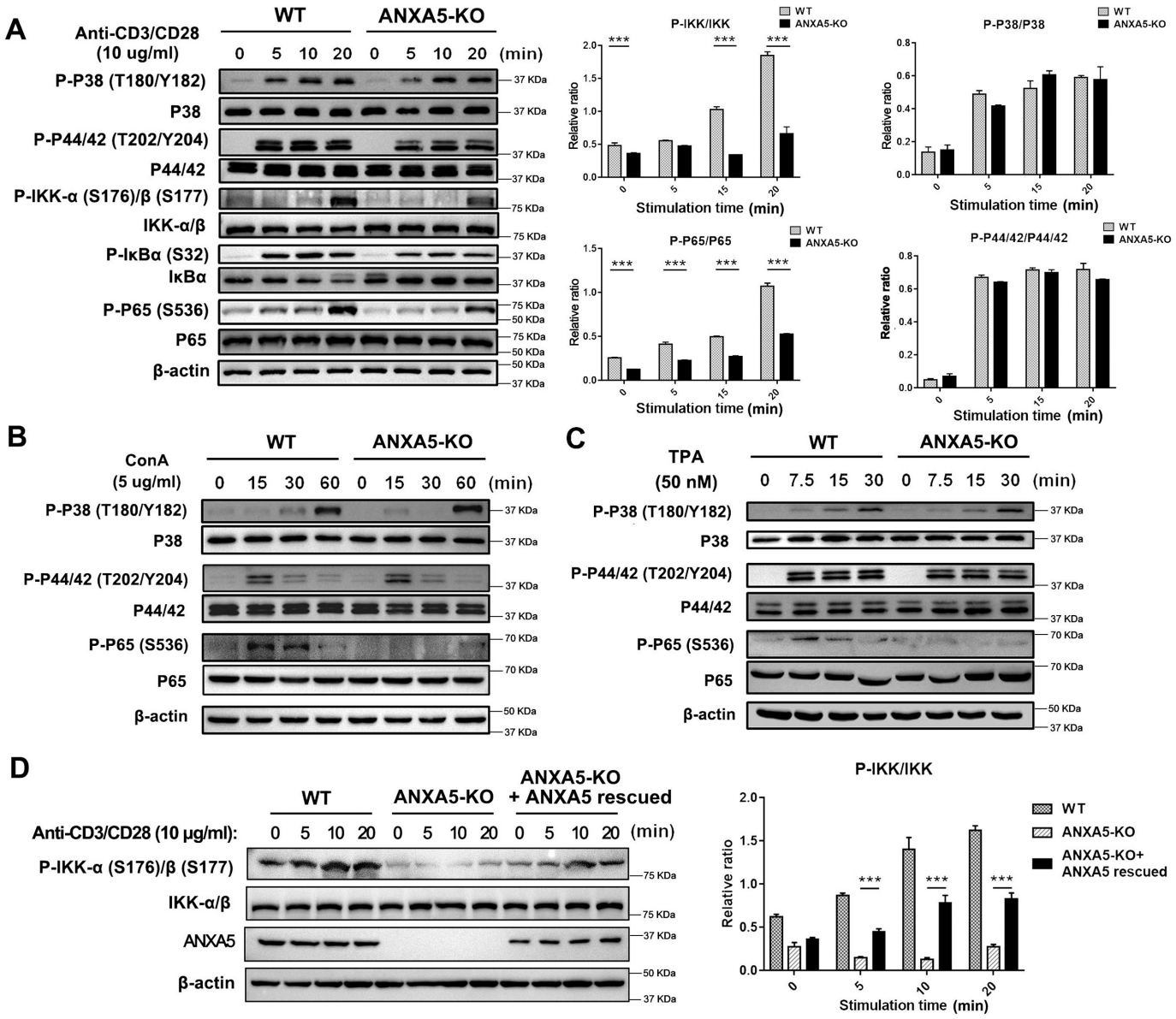

Figure 2. ANXA5 knockout inhibits NF-κB signaling in T-cell activation. *A*, T cells were treated with anti-CD3/28 antibody (10 μg/ml) for various times. The cell lysates were analyzed by Western blotting with the indicated antibodies. Relative ratios of the phosphorylated protein *versus* the corresponding total protein based on *grayscale* are shown as mean ± S.D. (*error bars*) (n = 3/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, T cells were stimulated by ConA (5 μg/ml) for Western blotting analysis. *C*, T cells were stimulated by TPA (50 nM) for Western blotting analysis. *D*, ANXA5-KO T cells were transfected with ANXA5 expression vector by lentiviral infection. After treatment with anti-CD3/CD28 co-stimulation for various times, these cells were harvested for Western blotting analysis with the indicated antibodies. Based on *grayscale* values, relative ratios of the phosphorylated IKK normalized to total IKK are shown as mean ± S.D. (n = 3/group). ***, $p < 0.001$.

MAPK, and NF-κB, were examined. In response to anti-CD3/CD28 co-stimulation, the activations of ERK and MAPK pathways were intact in ANXA5-KO Jurkat T cells, but NF-κB activation was impaired (Fig. 2*A*). The defects on the NF-κB pathway were reconfirmed in two other clones of ANXA5-KO Jurkat T cells (Fig. S1). Similarly, with the treatment of ConA or TPA, ANXA5-KO Jurkat T cells also showed impaired NF-κB activation but normal ERK and p38 MAPK pathways (Fig. 2, *B* and *C*). When the expression of ANXA5 was recovered by transfection in ANXA5-KO Jurkat T cells, partial rescue of the IKK activation was observed (Fig. 2*D*). Together, these results suggested that ANXA5 modulated T-cell activation via the NF-κB signaling pathway.

ANXA5 is required for PKCθ membrane translocation

A number of studies have indicated that PKC isozymes play a critical role in mature T-cell activation. We examined the kinase activity of PKC isozymes in ANXA5-KO Jurkat T cells. Upon TPA stimulation, PKC activity was weaker in ANXA5-KO Jurkat T cells compared with the parent Jurkat T cells (Fig. 3*A*), suggesting that ANXA5 deletion partially suppressed PKC activation. By examination of various PKC isoforms, we found that ANXA5 knockout selectively inhibited PKCθ activation, whereas it had no impact on PKCα and PKCμ activations (Fig. 3*B*). PKCθ is mainly expressed in T cells and involved in TCR-induced proliferation, cytokine production, and differentiation (21). Differing from other PKCs in T cells, PKCθ is unique in its

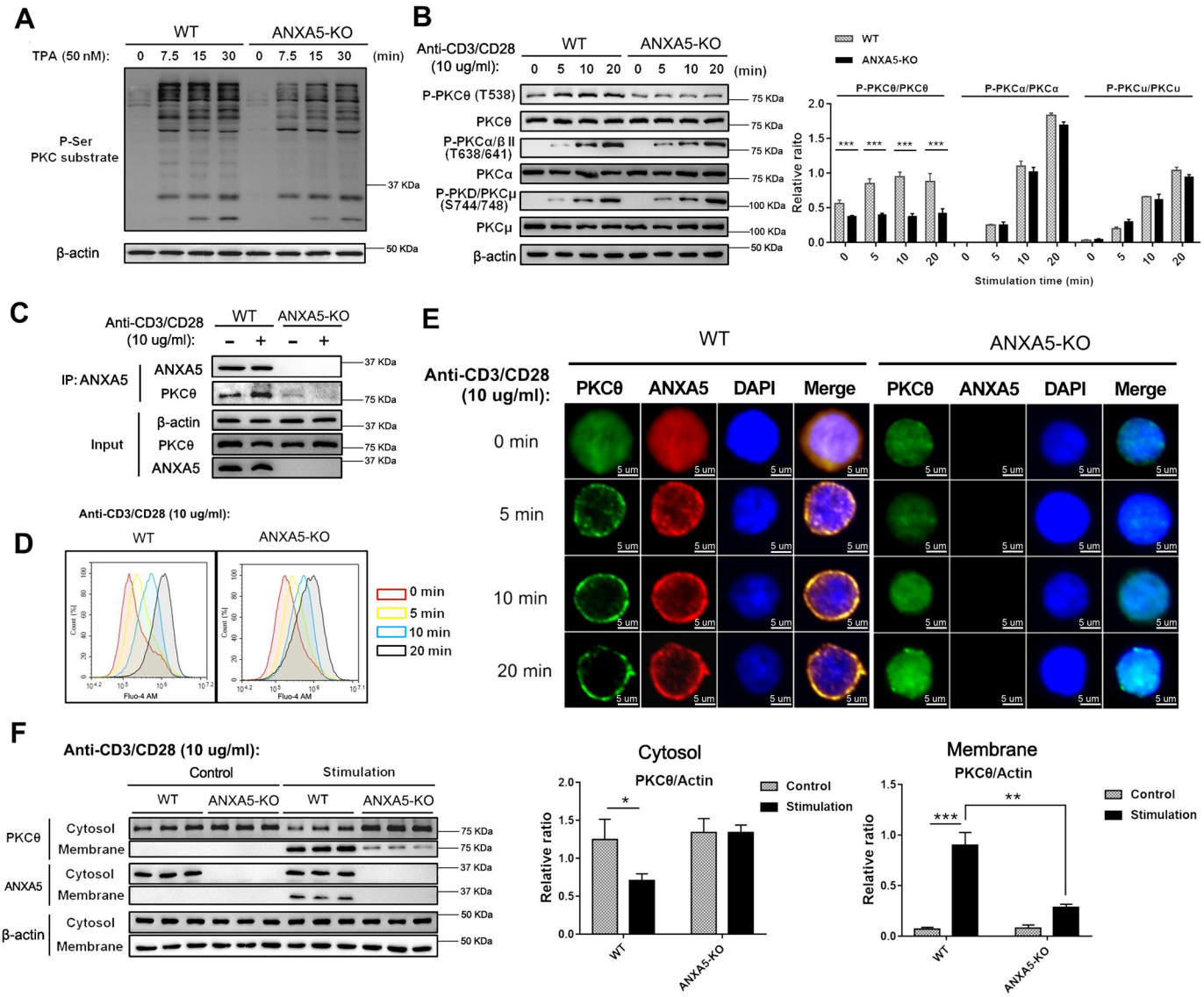

Figure 3. Association of ANXA5-PKCθ is required for PKCθ membrane translocation. *A*, ANXA5-KO cells and parent T cells were treated with TPA for different times and collected for Western blotting analysis with the antibody against the phosphorylation of PKC substrate. *B*, T cells were stimulated with anti-CD3/CD28 antibodies for various times and then analyzed by Western blotting with the indicated antibodies. Relative ratios of protein level based on *grayscale* are shown as mean ± S.D. (*error bars*) ($n = 3$/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *C*, T cells were treated or untreated with anti-CD3/CD28 antibodies for 20 min. Then cell lysates were immunoprecipitated (*IP*) with anti-ANXA5 and probed with anti-PKCθ antibody. 10% of the lysate was used as input control. A co-immunoprecipitation (*co-IP*) assay indicated that PKCθ interacted with ANXA5. *D*, T cells were treated with anti-CD3/CD28 (10 μg/ml) for various times and then detected for cellular Ca^{2+} elevation with Fluo-4 AM (calcium fluorescent probe) by FACS. *E*, after anti-CD3/CD28 co-stimulation, T cells were subjected to an immunofluorescence assay with antibodies against PKCθ and ANXA5. ANXA5 co-localized with PKCθ in the membrane of activated T cells, not in ANXA5-KO cells. *Scale bars*, 5 μm. *F*, after stimulation for 20 min, T cells were fractionated and analyzed by Western blotting. Actin was used as an internal control, and the relative ratio of PKCθ or ANXA5 normalized to actin is shown on the *right* as mean ± S.D. ($n = 3$/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

translocation to the site of the immunological synapse on the plasma membrane (3). Membrane translocation of cytosolic PKC is the hallmark of PKC activation (22). Based on the above finding, we speculated a possibility that ANXA5 acts as a PKCθ membrane target. To test this hypothesis, we performed a co-immunoprecipitation assay in Jurkat T cells. There was ANXA5-PKCθ interaction in normal T cells, and their interaction was significantly enhanced in activated T cells (Fig. 3*C*), suggesting that ANXA5 is involved in T-cell activation by association with PKCθ.

The Ca^{2+} increase is an early signaling following the engagement of TCR (2). ANXA5 can rapidly translocate from the cytosol to the plasma membrane upon Ca^{2+} elevation (the elevation of calcium ion concentration) (15). There was no obvious difference in the increase of Ca^{2+} initiated by anti-CD3/CD28 co-stimulation between ANXA5-KO and WT Jurkat T cells (Fig. 3*D*). Next, we tested whether ANXA5 is required for PKCθ translocation. By immunofluorescence observation, ANXA5 and PKCθ were distributed in the cytoplasm in resting Jurkat T cells and rapidly translocated and co-localized in the plasma membrane upon anti-CD3/CD28 co-stimulation (Fig. 3*E*). However, in ANXA5-KO Jurkat T cells, PKCθ was predominately located in the cytosol, and there was almost no localization on the membrane in response to TCR stimulus,

supporting ANXA5 as a binding target for PKCθ translocation in the process of T-cell activation (Fig. 3*E*). Further confirmation was performed using cellular fractionation for Western blotting analysis. Consistent with the immunofluorescence observation, PKCθ was not detected in the membrane fraction, indicating that PKCθ translocation was lost in ANXA5-KO Jurkat T cells. In contrast, the presence of ANXA5 in Jurkat T cells led to membrane-bound PKCθ following anti-CD3/CD28 co-stimulation (Fig. 3*F*). Together, our data suggested that ANXA5 was a PKCθ-binding protein required for PKCθ translocation on the membrane.

ANXA5 knockout inhibits PKCθ-mediated assembly of CARMA1-Bcl10-MALT1 complex

Recent reports have shown that PKCθ cooperates with CARMA1-Bcl10-MALT1 complex to activate the NF-κB pathway. To validate the importance of ANXA5-PKCθ association for NF-κB activation, we generated PKCθ knockout cells on the basis of Jurkat T cells for investigation (Fig. S2*A*). As expected, NF-κB activation was inhibited, but ERK and p38 activations were unaffected in PKCθ-KO Jurkat T cells (Fig. 4*A* and Fig. S4), just like the same phenotype in ANXA5-KO Jurkat T cells. Next, we examined the effect of ANXA5 on PKCθ-mediated function. The PKCθ-mediated CARMA1 phosphorylation is crucial for the assembly of CARMA1-Bcl10-MALT1 (CBM) signaling complex in T cells (23). Consistent with this report, the phosphorylation of CARMA1 was actually inhibited in PKCθ-KO Jurkat T cells (Fig. 4*B*), which was reconfirmed by another clone of PKCθ-KO cells (Fig. S2*B*). Similar to PKCθ-KO Jurkat T cells, ANXA5-KO Jurkat T cells also showed the inhibition on the phosphorylation of CARMA1 in response to anti-CD3/CD28 co-stimulation (Fig. 4*C*) or TPA (Fig. 4*D*) or ConA treatment (Fig. 4*E*). Phosphorylated CARMA1 acts as a seed for CBM complex assembly in TCR-mediated cell activation. Consistently, we found that there were normal translocations of CARMA1, Bcl10, and MALT1 from the soluble to the cell membrane fraction in WT Jurkat T cells, but little in ANXA5-KO Jurkat T cells (Fig. 4*F*). Furthermore, we validated the involvement of ANXA5 in the CBM complex formation by a co-immunoprecipitation assay. The result showed that the formation of the CBM complex was weakened in the absence of ANXA5 but strengthened in the presence of ANXA5, especially in activated T cells (Fig. 4*G*).

Finally, we verified the role of ANXA5 in primary T-cell activation. T lymphocytes isolated from lymph nodes were electrotransfected with ANXA5 siRNAs to knock down endogenous ANXA5 level. With about 16–20% transfection efficiency, the reduced endogenous ANXA5 was detected by Western blotting (Fig. S3). Then these ANXA5 siRNA–treated T cells were activated with anti-CD3/CD28 co-stimulation and analyzed for CD69 expression by FACS. In both $CD4^+$ and $CD8^+$ T cells, the increased CD69 expression induced by TCR stimulation was clearly inhibited by ANXA5 knockdown and, importantly, was rescued by the recovery expression of ANXA5 (Fig. 4*H*). These results demonstrate that ANXA5 is an important regulator linked to T-cell activation.

Discussion

T-cell activation is accompanied by the clustering of lipid rafts to the site of T-cell engagement and the recruitment of different intracellular signaling proteins into these rafts. Lipid raft recruitment is required for PKCθ to activate NF-κB (5). The details of PKCθ translocation are not completely defined in T-cell activation. DAG weakly recruited PKCθ to the membrane, and the PS binding of PKCθ phosphorylation was reported to enhance its binding to DAG, resulting in PKCθ activation (21). ANXA5 has high PS-binding ability and translocates to membranes, dependent on the increased intracellular Ca^{2+} levels (14, 24). In this study, we reveal an important role of ANXA5 in T-cell activation and provide evidence that ANXA5 may act as an early sensor in PKCθ translocation and activation.

As we all know, TCR-stimulated PLCγ1 activity stimulates Ca^{2+}-permeable ion channel receptors (IP3R) on the endoplasmic reticulum membrane, leading to the release of endoplasmic reticulum Ca^{2+} stores into the cytoplasm. We found that ANXA5 translocated to the membrane along with Ca^{2+} elevation during T-cell activation, which was essential for the lipid raft recruitment of PKCθ. ANXA5 was previously reported to play a prominent scaffolding role for PKCδ in various signal transduction pathways relevant in cancer (16). Here we demonstrate that ANXA5 interacts with PKCθ and contributes to the membrane translocation of PKCθ in Jurkat T cells (Fig. 3).

Our studies indicate that the ANXA5-PKCθ interaction precedes PKCθ translocation and is an essential step in the function of PKCθ. ANXA5 knockout inhibited TCR-induced PKCθ activity. Like the downstream signal transduction of PKCθ, the assembly of CARMA1-Bcl10-MALT1 complex and NF-κB activation were both inhibited by ANXA5 knockout in T-cell activation (Fig. 4). Consistently, the formation of CARMA1-Bcl10-MALT1 complex in the membrane-bound state was undetected in ANXA5-KO Jurkat T cells. Furthermore, there were the same phenotypes between ANXA5-KO and PKCθ-KO T cells, such as the defective NF-κB activation and intact ERK and p38 MAPK pathways, suggesting a functional link between ANXA5 and PKCθ on NF-κB signaling in T cell activation.

Recent studies have highlighted the essential role of PKCθ in activating the NF-κB signaling pathway in T cells. PKCθ, but not other PKCs, mediates the activation of the NF-κB complex induced by TCR/CD28 co-stimulation via selective activation of IκB kinase β (IKKβ) (25). Thus, NF-κB activation represents the most critical target of PKCθ in the TCR signal leading to production of IL-2, a major T-cell growth factor. In ANXA5-KO T cells, IL-2 production was significantly inhibited, supporting the involvement of ANXA5 in TCR-induced PKCθ activity. Notably, the phosphorylation of PKCθ, not PKCα and PKCμ, was inhibited in the absence of ANXA5, suggesting the selective regulation of ANXA5 on PKCθ function. Studies on PKCθ-deficient mice showed apparently relieved symptoms of multiple sclerosis, inflammatory bowel disease, arthritis, and asthma (26–30). Future research on ANXA5 in modulating PKCθ activation might serve as a new target for the selective regulation of PKC signaling in health and disease.

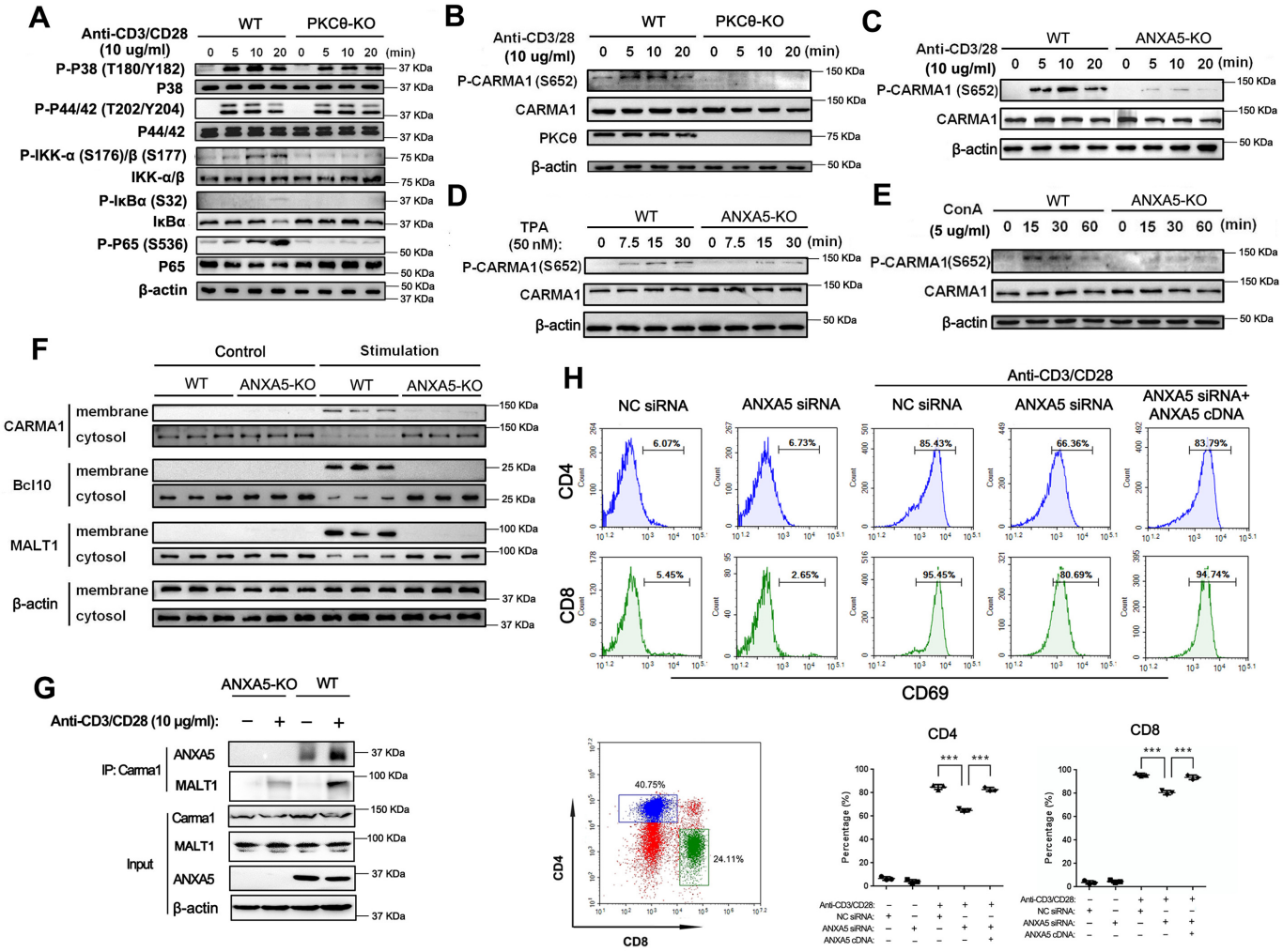

Figure 4. Loss of ANXA5 inhibits PKCθ-mediated assembly of CARMA1-Bcl10-MALT1 complex. *A*, T cells were treated with anti-CD3/CD28 antibodies for various times and analyzed by Western blotting with the indicated antibodies. *B*, the changes of phospho-CARMA1 were analyzed by Western blotting in PKCθ-KO and WT Jurkat T cells. ANXA5-KO cells were stimulated with anti-CD3/CD28 antibodies (*C*), TPA (*D*), or ConA (*E*) and analyzed for phospho-CARMA1 by Western blotting. *F*, T cells were fractionated to detect the localization of CARMA1, Bcl10, and MALT1 in T cells activated by anti-CD3/CD28 stimulation. *G*, Jurkat T cells were treated or untreated with anti-CD3/CD28 antibodies for 20 min. Then cell lysates were immunoprecipitated (*IP*) with anti-CARMA1 and probed with antibodies against ANXA5 and MALT1. 10% of the lysate was used as input control. *H*, using a mouse T cell Nucleofector™ kit, primary T cells were transfected with ANXA5 siRNAs or ANXA5 expression vector as indicated and then induced by anti-CD3/CD28 stimulation for 12 h. $CD4^+$ or $CD8^+$ T cells were gated, respectively, for the detection of CD69 expression. Representative FACS and quantitative analysis of $CD69^+$ cells are shown. Statistical analysis of the percentage of $CD69^+$-positive cells is shown as mean ± S.D. (*error bars*) ($n = 3$/group). ***, $p < 0.001$.

Several annexins have been reported in immunological process, such as ANXA1, ANXA2, and ANXA6 (31–33). However, little attention has been paid to the immunological role of ANXA5. Our study fills a gap in this knowledge about an important role of ANXA5 in T-cell activation. ANXA5 as a PKCθ association partner will provide new clues to the complicated molecular mechanism of PKCθ function during T-cell activation.

Experimental procedures

Cells culture

The human acute T-cell leukemia cell line Jurkat (ATCC TIB-152™) was cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 1% (v/v) penicillin–streptomycin (Gibco). Primary T cells were prepared from lymph node of mice. Cells were grown at 37 °C in 5% CO_2. All experiments were approved by the State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University.

CRISPR/Cas9 system for gene knockout

Gene knockout was conducted with a CRISPR/Cas9 system as described previously (34). Single guide RNAs targeting the ANXA5 (5′-AGGGTACTACCAGCGGATGT-3′) and PKCθ (5′-GCCGCCATGTTTACCGACAC-3′) genes were designed using an online CRISPR design platform (https://zlab.bio/guide-design-resources), and each was cloned into pLenti-CRISPRv2. Briefly, HEK-293T cells were co-transfected with constructed pLentiCRISPRv2 plasmid and lentiviral envelope plasmids (PL3, PL4, and PL5). The viruses were harvested by ultracentrifugation 3 days after transfection. Viruses were then added into the cultures of Jurkat T cells, followed by selection with puromycin (2 μg/ml). Finally, single-cell clones were

separated by serial dilutions in a 96-well plate and then transferred to 6-well plates. The knockout cell clones were identified by Western blotting.

For the recovery expression in ANXA5-KO Jurkat T cells, human ANXA5 cDNA was cloned into the plenti6/v5-D-Topo expression vector (Invitrogen). Then ANXA5 expression plasmid and lentiviral envelope plasmids (PL3, PL4, and PL5) were co-transfected into HEK-293T cells. The viruses were harvested on day 3. Then ANXA5-KO cells were treated with lentiviral transduction. 24 h after transduction, 10 μg/ml blasticidin was added into the medium for selection. Finally, the expression of ANAX5 was detected by Western blotting.

Immunofluorescence

Cells were cytospun, fixed with 3.75% formaldehyde/PBS, and permeabilized with 0.1% (v/v) Triton X-100. After blocking with 10% goat serum, sections were incubated with anti-human PKCθ and ANXA5 primary antibodies (1:50; Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After washing, slides were incubated with appropriate fluorochrome-conjugated secondary antibody (1:1000; Invitrogen) for 1 h at room temperature in the dark. Slides were counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen). All images were visualized and captured by a fluorescence microscope (Zeiss AX10, Carl Zeiss AG, Jena, Germany).

Cell activation and proliferation assay

Cells were seeded at a density of 1000 cells/plate and were maintained in culture for 0, 48, 72, and 108 h. Cell proliferation was detected by a CCK8 cell proliferation kit (Beyotime, Shanghai, China).

Cell activation was measured by a flow cytometer (FACSCalibur, BD Biosciences, Mississauga, Canada) equipped with Cell Quest software (BD Biosciences). Antibodies against human CD69 or mouse CD69 were purchased from BD Pharmingen (San Diego, CA).

Nuclear and cytoplasmic extracts

Cells were fractionated using a membrane protein extraction kit (Beyotime). Briefly, cells were lysed using lysis buffer A provided by the kit and then homogenized in ice. The lysates were centrifuged at 700 × *g* for 10 min at 4 °C, and the supernatant was collected and spun at 14,000 × *g* for 30 min at 4 °C. The pellets were suspended using extraction buffer B and incubated for 20 min. After centrifugation at 14,000 × *g* for 5 min at 4 °C, the supernatant was used as the membranous fraction. The samples were then analyzed by Western blotting.

Quantitative RT-PCR

Total RNA was extracted using TRIzol (Invitrogen Life Technologies). Reverse transcription was accomplished with a PrimeScript RT reagent kit (Takara). Quantitative PCR was performed with SYBR Green PCR Master Mix according to the manufacturer's instructions (Vazyme) on a StepOne/StepOne Plus™ real-time PCR system (Applied Biosystems). Sequence-specific primers for human IL-2 (forward primer, 5′-TACAAGAATCCCAAACTCACCAG-3′; reverse primer, 5′-GGCACAAAAAGAATCATAAAAGA-3′) and human actin (forward primer, 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′; reverse primer, 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′) were used.

Primary murine T-cell transfection

6–12-week-old mice were purchased from the Model Animal Research Center of Nanjing University. All the animal experiments were approved by the Nanjing University Animal Care and Use Committee. The mice were sacrificed and sterilized with 75% ethanol. Lymph nodes were isolated and prepared for single-cell suspension. Primary murine T cells were maintained in RPMI 1640 medium (Gibco) supplemented with 100 mg/ml streptomycin, 100 units/ml penicillin, and 10% fetal calf serum.

For primary murine T-cell transfection, the mouse T Cell Nucleofector™ kit (Lonza) was used for electrotransfection with the Amaxa transfection device Nucleofector™ II according to the manufacturer's instructions. In brief, 1×10^7 cells were resuspended in 100 μl of room temperature Nucleofector® solution (Lonza) and electroporated with 40 pmol of negative control siRNAs, 40 pmol of ANXA5 siRNA, or 40 pmol of ANXA5 siRNAs together with 2 μg of plasmid pCMV-ANXA5, respectively. CD69 expression was examined by flow cytometer 24 h after electrotransfection. The transfection of 2 μg of pmax-GFP (Lonza) was used as a positive control for indicating the transfection efficiency measured by flow cytometer. Negative control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) and mouse ANXA5 siRNA (5′-AUGCUCCGAAUAGACUUCACGTT-3′) were purchased from GenePharma. The plasmid of pCMV-ANXA5 was constructed and stored by our laboratory.

Statistics and reproducibility

All experiments were conducted at least three times. The experimental data were processed by GraphPad Prism 7.00 and are presented as mean ± S.D. A p value of <0.05 shows that there is a statistically significant difference, marked with an *asterisk*. p values of <0.01 and <0.001 are marked with *two* and *three asterisks*, respectively.

Data availability

All data are included in the article.

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X. Z. project administration; X. Z. software; X. Z. writing-original draft; X. Z. and J. Z. writing-review and editing; Z. Hua funding acquisition.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: TCR, T-cell receptor; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ConA, concanavalin A; IL, interleukin; ERK, extracellular signal–regulated kinase; MAPK, mitogen-activated protein kinase; CBM, CARMA1-Bcl10-MALT1; KO, knockout.

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