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*J Immunol* 2007; 179:1096-1103; doi: 10.4049/jimmunol.179.2.1096

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A Costimulation-Initiated Signaling Pathway Regulates NFATc1 Transcription in T Lymphocytes

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T cell activation and differentiation is accompanied and mediated by transcriptional reprogramming. The NFATc1 transcription factor is strongly induced upon T cell activation and controls numerous genes involved in the T cell effector function. However, its regulation by physiological stimuli in primary T cells has not been well understood. We previously found that ICOS synergizes with TCR and CD28 to greatly enhance NFATc1 expression in primary T cells. In this study, we have examined the signaling mechanisms whereby costimulation regulates NFATc1 expression. We found that CD28 and ICOS regulate sustained PI3K activity in primary T cells, which is required for NFATc1 up-regulation. CD28 and ICOS costimulation, possibly through Itk, a Tec kinase downstream of the PI3K, enhanced phosphorylation of phospholipase Cγ1 and increased and sustained Ca2+ flux in T cells. Costimulation of T cells potentiated transcription of the Nfatc1 gene P1 promoter in a PI3K-dependent manner. This work demonstrates an important role for costimulatory receptors in sustaining T cell activation programs leading to Nfatc1 gene transcription and has implications in our understanding of the immune response and tolerance. The Journal of Immunology, 2007, 179: 1096–1103.

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Received for publication June 14, 2006. Accepted for publication May 11, 2007.

Materials and Methods

Mice

B7h-deficient mice previously analyzed on a (C57BL/6 × 129)F1 background (10) were subsequently backcrossed for more than six generations with C57BL/6 (B6) mice. CD80/CD86 doubly deficient mice on a B6 background were purchased from The Jackson Laboratory and bred with B7h-deficient animals (12). GFP–protein kinase B (PKB)–pleckstrin homology (PH)–transgenic mice (13), provided by Dr. D. Cantrell at University of Dundee (Dundee, U.K.), were bred with OT-II TCR transgenic...
Purified naive CD4+ T cells were conjugated with goat anti-mouse and anti-rat Ig (Polysciences). The using Abs against CD8, MHC class II, and NK1.1 markers and magnetic anti-CD3 Ab in the presence of anti-CD28 and/or anti-ICOS Abs for 24 h microbeads (Miltenyi Biotec). Splenic APC from B6, B7h/B7h T cells were analyzed under each condition.

CD4+ T cells isolated from the lymph nodes and spleens of 6- to 8-wk-old mice were isolated and stimulated as reported previously (9, 11). In brief, the CD4+ T cells were first enriched by immunomagnetic negative selection using Abs against CD8, MHC class II, and NK1.1 markers and magnetic beads conjugated with goat anti-mouse and anti-rat Ig (Polysciences). The CD62L+ naive cells were further purified by an autoMACS sorter (Miltenyi Biotec). Purified naive CD4+ T cells were stimulated with plate-bound anti-CD3 Ab in the presence of anti-CD28 and anti-ICOS Abs for 24 h before analysis of NFATc1 expression.

Measurement of phosphatidylinositol 3,4,5-triphosphate (PIP3) levels in T cells

CD4+ T cells were purified from the spleen and lymph nodes of T cells using anti-CD4-conjugated microbeads (Miltenyi Biotec). Splenic APC from B6, B7+, B7+/−, and B7−/− mice were prepared by complement-mediated lysis of Thy1+ T cells. Purified CD4+ T cells were conjugated with OVA peptide and APC for 1, 3, 5, and 8 h. T cell-APC conjugates and membrane localization of the GFP fusion protein were analyzed by fluorescence microscopy (NIKON Eclipse E400).

GST pull-down, immunoprecipitation, and Western blot analyses

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Luciferase assays

EL-4 cells (2 × 10^5) were cotransfected with 2 μg of NFAT/AP1-Luc or NFAT-P1-Luc plasmid and 0.25 μg of a control reporter plasmid (pPRL-null; Promega). Jurkat cells (2 × 10^5 cells) were cotransfected with 2 μg of NFAT/AP1-Luc or NFAT-P1-Luc reporter plasmid, 1 μg of pCDNA3, pCDNA3-ICOS, or pCDNA3-ICOSmut (Y-F), and 0.25 μg of a control reporter plasmid (pPRL-null; Promega). Cells were pulsed using a Bio-Rad Gene Pulser at 260 volts and 960 farads in 10% FCS. After 16 h, cells were stimulated with plate-bound anti-CD3, anti-CD28, and/or anti-ICOS Abs for 6 h and lysed. Luciferase activity was detected using the Dual luciferase system (Promega). Luciferase units of the experimental vector were normalized at the level of the control vector in each sample.

Calcium flux

EL-4 cells were resuspended at 10^7/ml in medium (RPMI 1640, 10% FBS, 1-glutamine, and penicillin/streptomycin) containing 5 μg of indo-1-acetoxyethyl ester (AM) (Sigma-Aldrich) and incubated for 1 h at 37°C. Light exposure was minimized to prevent photobleaching. Cells were then washed, incubated with the indicated stimulated Abs for 3 h at 4°C, and washed again. Then, 200-μl aliquots containing 5 × 10^6 cells/ml were diluted with 800 μl of complete RPMI 1640 medium prewarmed at 37°C and the sample was applied to a FACSflow fluorescence cytometer (BD Biosciences) for 45 s to measure the basal intracellular Ca2+ concentration. Hamster IgG1 isotype control was added at a final concentration of 10 μg/ml and cytoplasmic Ca2+ levels were measured for 10 min. Changes in intracellular calcium were assessed as the ratio of fluorescence (FL4) to calcium-unbound (FL4) fluorescence. Primary CD4+ T cells were activated with Con A for 48 h. Activated cells were loaded with Fluor-4AM and Fura Red-AM (Invitrogen Life Technologies), activated with polylysine beads coated with a combination of the indicated Abs, and analyzed...
on a FACS Aria flow cytometer (BD Biosciences). Calcium flux data was analyzed using FlowJo (Tree Star) software.

Results

Costimulatory receptors regulate sustained PI3K activity

How NFATc1 expression is regulated by physiological stimuli in primary T cells has not been understood. Previously, we found that during early T cell activation ICOS synergizes with TCR and CD28 in the up-regulation of NFATc1 protein expression (11). We thus seek to elucidate the signaling mechanisms through which CD28 and ICOS regulate NFATc1 expression. Although CD28 signaling has been extensively studied, the mechanism whereby ICOS transduces intracellular signaling is relatively unclear. Like CD28, ICOS contains an evolutionarily conserved YMXM sequence motif in its cytoplasmic region. In CD28 this motif associates with the p85 subunit of PI3K. Several studies indicate that ICOS constitutively associates with the p85 subunit of PI3K and that ICOS ligation led to PI3K activation (14–16). Consistent with these observations, we also found that the association of ICOS with p85 is dependent on the tyrosine residue of the YMXM motif,
Sustained PI3K activity is required for NFATc1 protein expression

We showed previously that CD28 and ICOS synergize in the regulation of NFATc1 protein expression and nuclear translocations (11). To assess the role of PI3K downstream of CD28 and ICOS in NFATc1 induction, we measured the amount of NFATc1 protein in CD4⁺ T cells activated with plate-bound anti-CD3 and anti-CD28 and/or anti-ICOS for 24 h (Fig. 2A). As we showed previously, the expression of NFATc1 is most strongly up-regulated by TCR, CD28, and ICOS agonists (11). NFATc1 up-regulation was completely blocked by LY-294002, a PI3K inhibitor (Fig. 2A). In this culture, cell death remained unaffected by the inhibitor (data not shown). These results suggest that PI3K activity is required for NFATc1 expression upon the activation of T cells.

PTEN is an antagonistic phosphatase that hydrolyzes PIP3 (17). To further substantiate the role of PI3K in NFATc1 expression, we activated naive CD4⁺ T cells from PTEN⁺/⁻ mice or wild-type mice and assayed the expression of IL-2 and NFATc1 24 h after activation (Fig. 2B). As we showed previously, wild-type cells activated with anti-CD3 plus anti-ICOS Abs produced the same levels of NFATc1 and IL-2 as those stimulated only with anti-CD3; anti-ICOS only had an effect on NFATc1 expression in the presence of anti-CD28, further supporting the notion that CD28 is the predominant costimulatory receptor in CD4⁺ cells (11). Interestingly, there was a strong effect on IL-2 production and NFATc1 up-regulation by anti-ICOS on PTEN⁺/⁻ T cells activated with anti-CD3. Because PTEN⁺/⁺ and PTEN⁺/⁻ cells up-regulated ICOS to a similar extent (data not shown), PTEN appears to be a negative regulator of ICOS signaling; its haploinsufficiency allows T cells to respond strongly to ICOS stimulation.
Because CD28 and ICOS signals resulted in sustained PIP3 production, we next assessed the question of whether sustained PI3K activity is required for NFATc1 expression. LY294002 was added at various time points after CD4/splenic APC. Adding LY294002 0–6 h after T cell activation completely inhibited IL-2 production and NFATc1 expression (Fig. 2C). However, the addition of LY294002 at a later time point (10 h) was less effective, indicating a time window during which PI3K activity was required for the induction of NFATc1 and IL-2.

**Itk is required for PLCγ1 phosphorylation and NFATc1 up-regulation**

PI3K has many important downstream targets. In T cells, PI3P recruits the PH domain-containing Akt/PK-B and the Tec protein kinases to the cell membrane. Interestingly, activated Akt restored T cell proliferation but not the Th2 defect in CD28 effect on T cells (18). In contrast, Itk was shown to be important for full activation of PLCγ1, Ca2+ mobilization, the nuclear localization of NFATc1, and IL-4 expression (19). Because ICOS and CD28 synergize to enhance NFATc1 expression and early IL-4 production (11), we focused our research on the role of Itk in costimulation-mediated NFATc1 induction. Naïve CD4 T cells purified from Itk-deficient mice and their appropriate controls were activated as described above and NFATc1 expression was determined by Western blotting. Itk deficiency led to impaired NFATc1 up-regulation, indicating that Itk is essential for NFATc1 expression mediated by costimulation (Fig. 3A).

Itk, like other Tec kinases, plays an important role in PLCγ1 phosphorylation and Ca2+ mobilization (19). Accordingly, we found that the phosphorylation of PLCγ1, which was enhanced in T cells receiving costimulatory signals, was greatly reduced in the absence of Itk (Fig. 3A). We next examined whether the costimulation of T cells increases Ca2+ mobilization. EL-4 is a thymoma that constitutively expresses TCR/CD3, CD28, and ICOS. To measure Ca2+ mobilization in T cells, EL-4 cells loaded with indo-1 as well as Con A-activated CD4 T cells preloaded with Fluo-4-AM and Fura Red-AM were stimulated with anti-CD3 in the presence of anti-CD28 with or without anti-ICOS. CD28 costimulation increased Ca2+ mobilization in EL4 and primary CD4 T cells. ICOS stimulation, along with TCR and CD28 stimulation, led not only to an increased magnitude but also to a more sustained Ca2+ mobilization (Fig. 3B). These results indicate a crucial role for CD28 and ICOS costimulation in Ca2+ signaling.

**Costimulation regulates Nfatc1 gene transcription**

Our previous and current data have indicated a crucial role of costimulation in the regulation of NFATc1 protein expression (11). The transcriptional initiation of the Nfatc1 gene is controlled by
two different promoters, P1 and P2 (3), which mediate the expression of individual isoforms. Although P1-mediated transcription from exon 1 generates an N-terminal peptide of 42 aa, the P2 promoter initiates transcription from exon 2, which results in the peptide of 29 aa. In addition, alternate RNA splicing events and two poly(A) addition sites, pA1 and pA2, control NFATc1 expression in six individual NFATc1 isoforms, A, B, C, A, B, and C (Refs. 3 and 20 and our unpublished data). To examine which promoter of the Nfatc1 gene is the target of costimulation-dependent regulation, we hybridized RNA from EL-4 or primary CD4 cells activated under various conditions with RNA probes specific for the detection of mRNA transcripts initiated at the P1 or P2 promoter. We found that the Nfatc1 P1 promoter activity was minimal upon anti-CD3 stimulation alone but was enhanced by CD28 costimulation (Fig. 4A). Anti-ICOS treatment did not affect NFATc1 P1 activity mediated in anti-CD3-treated T cells. The combination of anti-CD3, anti-CD28, and anti-ICOS Abs strongly enhanced the transcription of the Nfatc1 P1 promoter (Fig. 4A), correlating with NFATc1 protein expression. In contrast, P2 activity was not found to be different in these cells (data not shown). These data suggest that costimulation may regulate the transcription of the P1 promoter.

To substantiate these results, we performed RT-PCR analyses for the detection of individual isoforms generated from either the P1 or the P2 promoter. Transcripts generated from the P1 promoter encoding α peptides with short (A) or long (B plus C) C termini were induced after T cell activation (Fig. 4B). Expression of the αA isoform and, to a less extent, the αB plus C isoforms, is highly responsive to CD28 and ICOS costimulation, consistent with a role for NFATc1 α proteins in T cell proliferation (3). In contrast, the synthesis of short and long β isoforms appeared not to be stimulated upon T cell activation.

We further examined costimulation regulation of P1 promoter using a P1-driven luciferase reporter gene. In EL-4 cells, the P1 promoter was only weakly activated by stimulation with anti-CD3 in the absence or presence of anti-ICOS. However, anti-CD3, anti-CD28 and anti-ICOS synergized to induce significant P1 activation (Fig. 5A). Therefore, from our above three experiments it appears that P1 is a target of costimulation-dependent regulation.

The Nfatc1 P1 promoter has been previously reported to be autoregulated by NFATc1 itself (3). When we transfected an NFAT-AP1 reporter construct carrying multiples of the distal NFAT/AP-1 site from the IL-2 promoter into EL4 cells, we found that it was induced in a similar fashion as that of NFATc1 protein or the P1 transcript by TCR and costimulation (Fig. 5A). Furthermore, the induction of the NFATc1 protein (11) or P1 transcript (Fig. 4A) was strongly inhibited by cyclosporin A. Therefore, costimulation by CD28 and ICOS resulted in NFATc1 nuclear translocation and possibly autoregulation of the NFATc1 P1 promoter.

FIGURE 5. Regulation of NFAT activity by ICOS. A, NFAT/AP1-Luc or Nfatc1 P1-Luc constructs and a pPRL-null construct were cotransfected into EL-4 cells by electroporation. After 16 h cells were treated with the indicated combination of anti-CD3, anti-CD28, and anti-ICOS for 6 h and lysed. Luciferase activity was detected by using a Dual luciferase system (Promega). Results from one of four independent experiments were shown as relative luciferase activity (RLA). N/A, Nonactivated. B, Jurkat cells were cotransfected with an NFAT/AP1-Luc reporter plasmid and various expression vectors (pCDNA3, pCDNA3-ICOS, or pCDNA3-ICOSmut (Y-F)) and a control reporter plasmid, pPRL-null. After 16 h, cells were treated with the indicated combination of anti-CD3, anti-CD28, and anti-ICOS for 6 h and luciferase activity was detected by a Dual luciferase assay. A representative of three independent experiments with similar results is shown.
**PI3K activity is required for NFAT activation**

Because CD28 and ICOS function to regulate PI3K activity, we next examined the importance of PI3K activity in transcription of the *Nfatc1* gene. We first treated EL-4 cells with LY294002 and found that the transcription of the *Nfatc1* P1 promoter that was induced by TCR and costimulation was inhibited with this treatment (Fig. 4A). Second, we used human Jurkat cells that do not express ICOS for transfections with constructs expressing wild-type mouse ICOS or ICOS harboring a Y181F mutation. These two forms of ICOS were expressed at similar levels as determined by flow cytometry analysis (data not shown). Anti-CD3 activated a NFAT-AP1 reporter, which was enhanced by anti-CD28 (Fig. 5B). Because Jurkat cells are deficient in PTEN, in cells transfected with wild-type ICOS, anti-ICOS enhanced the NFAT-AP1 activity activated by anti-CD3 with or without CD28 costimulation (Fig. 5B), which correlates with our previous data on PTEN+/− cells (Fig. 2B). Cells transfected with mutant ICOS/Y181F did not respond to anti-ICOS in enhancing NFAT reporter activity. Therefore, PI3K activated by ICOS appears crucial in the regulation of NFAT activity.

**Discussion**

Costimulation critically determines T cell activation and tolerance. In this study, we demonstrated a signaling pathway activated by CD28 and ICOS leading to activation of the *Nfatc1* P1 promoter. ICOS is a novel costimulatory receptor important in Th2 cytokine production (9, 21, 22). However, the signal transduction mechanisms of ICOS have not been well characterized. Several studies have previously shown that ICOS, like CD28, could associate with p85 and activate PI3K (14). In the current study, we have characterized the function of the YMXM motif in the ICOS cytoplasmic region. We found that the Y181 residue in this motif is required for association with p85 (Fig. 1A) and for ICOS-dependent NFAT activation (Fig. 5B). Thus, the YMXM motif, commonly found in costimulatory receptors in leukocytes, confers at least one important aspect of ICOS signaling. Interestingly, PTEN phosphatase limits the response by CD4+ T cells to ICOS, so that ICOS can only enhance the signals induced by TCR and CD28. The loss of one allele of PTEN resulted in a hyperresponsiveness of T cells to ICOS costimulation. It has been previously reported that PTEN+/− mice developed fatal autoimmune disease (23). It is not clear whether this results from increased response to ICOS. It is noteworthy that the disruption of an ICOS repressor, roquin (Rc3h1) E3 ubiquitin ligase, also resulted in autoimmune diseases in mice. Thus, maintaining the signaling strength of ICOS is an important regulation on immune tolerance.

Costimulation is critical in regulating T cell activation and tolerance. Recently, we found that absence of both CD28 and ICOS costimulation resulted in T cell clonal tolerance (12, 24). In vitro, T cells activated in the absence of both CD28 and ICOS costimulation were able to up-regulate activation markers and undergo 2–3 rounds of division but were unable to differentiate into effector cells (11). In the current study, we found that in the absence of costimulation T cells were able to produce PIP3 within 1 h of activation, which was rapidly reduced to background within 3–5 h of T cell activation (Fig. 1B). Thus, we propose that costimulation is not required for the initial T cell activation events but rather to enhance and sustain this activation, at least in part, via signaling through the PI3K pathway. Sustained T cell activation mediated by costimulation is needed for productive T cell activation, because the expression of IL-2, an important T cell growth factor, and the induction of the NFATc1 transcription factor required at least 6 h of PI3K activity (Fig. 2C).

NFATc1 expression is induced after naive T cell activation; the physiological signals that regulate NFATc1 induction has not been understood. Previously, we showed that T cell activation led to a strong induction of the P1 promoter while the synthesis of other NFATc1 isoforms driven by the P2 promoter and of NFATc2 remained unaffected (3, 20). The autoregulation of the P1 promoter by NFAT factors results in strongly increased NFATc1 protein expression. In our current study, we found that signals from CD28 and ICOS costimulatory receptors synergize with TCR, resulting in the activation of the *Nfatc1* P1 promoter activity. This work illustrates a physiological context of *Nfatc1* transcriptional regulation. NFATc1 is important in T cell proliferation and Th2 differentiation (1, 2). In addition to CD28 and ICOS, OX40 (25) and IL-25 (26) have been shown to enhance NFATc1 nuclear translocation in activated T cells. This effect is associated with potentiation of cytokine-independent early IL-4 production and Th2 differentiation. It is not clear whether the NFATc1 P1 promoter is regulated by these factors. NFATc1 likely functions where the multiple signaling pathway is integrated. The mechanisms illustrated in the current study may be applied to other systems, which will further elucidate the molecular mechanisms governing T cell activation and functional differentiation.

**Acknowledgments**

We thank Drs. Doreen Cantrell for providing the GFP-Akt-PH transgenic mice and Dan Littman for permission to use 5lk-deficient mice, Dr. Jonathan Cooper for TKX1 bacteria, Drs. Yang Wang and Qing Ma for help on microscopy, Dr. Doreen Cantrell for critical reading of the manuscript, and the entire Dong laboratory for their help and discussion.

**Disclosures**

The authors have no financial conflict of interest.

**References**


