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Nck Recruitment to the TCR Required for ZAP70 Activation during Thymic Development

Aldo Borroto,* Irene Arellano,* Elaine P. Dopfer,† Marek Prouza,‡ Miloslav Suchanek,‡ Manuel Fuentes,§ Alberto Orfao,§ Wolfgang W. Schamel,†,* and Balbino Alarcón*  

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The adapter protein Nck is indicatively recruited through its SH3.1 domain to a proline-rich sequence (PRS) in CD3ε after TCR engagement. However, experiments with a knockin mutant bearing an 8-aa replacement of the PRS have indicated that Nck binding to the TCR is constitutive, and that it promotes the degradation of the TCR in preselection double-positive (DP) CD4+CD8+ thymocytes. To clarify these discrepancies, we have generated a new knockin mouse line (KI-PRS) bearing a conservative mutation in the PRS resulting from the replacement of the two central prolines. Thymocytes of KI-PRS mice are partly arrested at each step at which pre-TCR or TCR signaling is required. The mutation prevents the trigger-dependent inductive recruitment of endogenous Nck to the TCR but does not impair TCR degradation. However, KI-PRS preselection DP thymocytes show impaired tyrosine phosphorylation of CD3ζ, as well as impaired recruitment of ZAP70 to the TCR and impaired ZAP70 activation. Our results indicate that Nck is recruited to the TCR in an inducible manner in DP thymocytes, and that this recruitment is required for the activation of early TCR-dependent events. Differences in the extent of PRS mutation could explain the phenotypic differences in both knockin mice.
which the pre-TCR or TCR signaling is required, and that it is necessary for full CD3ε phosphorylation and ZAP70 recruitment to the TCR and activation.

Materials and Methods

Generation of KI-PRS mice

Knockin mice bearing the PxxP to AxxA double mutation in the PRS of CD3ε were generated by Genoway. The BAL2-HR targeting vector was generated that contained a neo cassette flanked by flippase recombination target sequences inserted between exons 4 and 2 and two C to G mutations in exon 5. The mutations were as follows: CCA (CCA > GCA)CTGTT (CCC > GCC)AAC. The construct was electroporated into C57BL/6 ES cells that were selected in G418. Primary screening for 3′ homologous recombination was carried out by PCR, and homologous recombination was verified in 5′ Southern blots followed by 3′ Southern blots. Twelve independent embryonic stem (ES) clones of 547 were positive for homologous recombination and were injected into blastocysts of C57BL/6 mice. Twenty chimeric male mice derived from 8 of the ES clones were crossed with C57BL/6 Flp deleter females, and 14 mice with germline transmission were tested by PCR for excision of the floxed region, which was confirmed in Southern blots. A total of four mice were identified as heterozygous for the knockin allele, of which one male was chosen to cross with C57BL/6 females to generate the KI-PRS colony at the “Centro de Biología Molecular Severo Ochoa.”

Cells and transgenic mice

African green monkey COS7 cells were grown in DMEM plus 10% FBS. Thymocytes were maintained in RPMI 10% FBS supplemented with 20 μM 2-ME and 10 mM sodium pyruvate. KI-PRS mice were crossed with OT-I TCR transgenic mice (OVAp specific, H-2Kb restricted) (17) for the AND 2-ME and 10 mM sodium pyruvate. KI-PRS mice were crossed with C57BL/6 Flp deleter females, and 14 mice with germline transmission were tested by PCR for excision of the floxed region, which was confirmed in Southern blots. A total of four mice were identified as heterozygous for the knockin allele, of which one male was chosen to cross with C57BL/6 females to generate the KI-PRS colony at the “Centro de Biología Molecular Severo Ochoa.”

Flow cytometry

Cells were preincubated with the anti-CD16/32–specific mAb 2.4G2 in PBS, 1% BSA, 0.02% sodium azide before labeling with saturating amounts of the indicated fluorochrome-labeled or biotinylated mAbs and, where applicable, fluorochrome-labeled streptavidin (reagents purchased from BD Pharmingen, eBioscience, Immunotools, Santa Cruz, and Miltenyi). Labeled cells were analyzed on a FACSCalibur or FACSCanto II flow cytometer (Becton-Dickinson), and the data were analyzed with FlowJo software (Tree Star).

Thymocyte proliferation

A total of 1.5 × 10⁵ thymocytes of each genotype were stimulated at different times with 3 × 10⁵ irradiated spleen cells from CD3ε™ mice, as APCs, preloaded with OVAp (SIINFEKL), with Q4R7 (SIIQFERL), or with G4 (SIIGFEKL). Forty-eight hours later, a 12-h pulse with 1 μCi/well ['H]thymidine (Perkin Elmer) was given and radioactivity collected in a glass fiber filter (Perkin-Elmer), and counted in a 1450 microbeta Wallac Trilux liquid scintillation counter.

Immunoblot analysis of T cell activation

A total of 3 × 10⁶ thymocytes of each genotype were activated at different times with T2K² APCs preloaded with OVAp peptide (SIINFEKL). After different incubation times, the cells were lysed in 1 ml BriJ96 lysis buffer containing protease and phosphatase inhibitors (0.3% BriJ96, 140 mM NaCl, 20 mM Tris-HCl [pH 7.8], 10 mM iodacetamide, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, and 20 mM sodium fluoride). Immunoprecipitation was performed with anti-CD3ε serum 448 (20) or anti-CD3 mAb (145-2C11) and protein A Sepharose beads. SDS-PAGE and immunoblotting was performed according to standard protocols, and the membranes were probed with anti-CD3ε serum or different anti-phospho-specific Abs, and were visualized by ECL. Quantification was performed on ECL autoradiography films using ImageJ software.

Pull-down assay

The construct pGEX-4T1-GST-Nckβtrn (GST-Nckβ[ASH2]) was made by PCR of human Nckβ. The pGEX-4T1 derivative GST-SH3.1α was kindly provided by Dr. R. Geha (Children’s Hospital, Harvard Medical School, Boston, MA). Pull-down assays were performed as described previously (21).

Generation of anti-Nck Abs

BALB/c mice were biweekly immunized s.c. with 30 μg and intraperitoneally with 10 μg GST fused to the second SH3 domain (SH3.2) of human Nck1. The first s.c. immunization was performed with CFA, and it was followed by two injections inIFA. Intrasplenic immunization was done in IFA. Ten days after the second and third immunization, the animals were tail-bleed, and the immune response to Ag was measured by ELISA. The mouse selected for generation of mAbs was boosted i.v. with 5 μg Ag in saline. Four days later, the spleen was harvested and used for cell fusion with myeloma cells. A total of 350 × 10⁶ spleen cells were fused to 60 × 10⁶ Sp/20 myeloma cells using polyethylene glycol 1500 (STEMCELL Technologies) according to the manufacturer’s recommendations. The fused cells were initially seeded in tissue culture plates containing semisolid Clontech HY Selection and Cloning Medium D (STEMCELL Technologies). About 700 hybridoma clones were picked from semisolid medium after 14 d of growth. Primary screening to test positive clones for the production of anti-Nck Abs was performed using an ELISA assay, selecting those reactive with GST-SH3.2 but not with GST. All animal experiments were performed according to Czech Central Commission for Animal Welfare guidelines.

Statistical analysis

Quantitative data are shown as the mean ± SD. A nonparametric two-tailed Student t test was used to assess the confidence intervals.

Abs and other materials

Abs and other materials are described in Table I.

Results

Nck is inducibly recruited to the TCR in DP thymocytes

The cytoplasmic tail of CD3ε contains multiple confirmed and potential sequences for protein interactions (Fig. 1A). The PRS of CD3ε casts an elongated footprint on the SH3.1 domain of Nck, interacting with three shallow hydrophobic pockets (22–24). The canonical PxxP sequence for PRS-SH3 domain interaction is followed by 2 aa at the position +3 that establish interactions with the third hydrophobic pocket of the SH3.1 domain (Supplemental Fig. 1). Thus, the interaction sequence in the PRS of CD3ε is actually defined by the motif PxxPxxDY. To minimize possible interference with other proteins and to fully prevent the interaction with Nck, we generated a knockin mouse bearing a germline substitution of the two central prolines in the PxxPaxxDY motif with alanine (Fig. 1A). This mutation is more conservative than others previously used to study the PRS (Fig. 1A).

Initially, we verified that no interaction occurred between the double mutant and the SH3.1 domain in a pull-down assay performed in transfected COS cells (Fig. 1B). This analysis was repeated with thymocyte lysates from knockin (KI-PRS) mice, revealing that TCR does not bind to the SH3.1, irrespective of anti-CD3 stimulation (Fig. 1C). Conversely, stimulation of WT thymocytes with anti-CD3 clearly provoked TCR binding to SH3.1, in contrast with previous results (10), suggesting that TCR engagement was necessary for the CD3ε PRS to bind SH3.1, probably by inducing the TCR to adopt an active conformation (8). Indeed, the TCR adopted the active conformation in WT thymocytes upon triggering as it did in mature T cells (Fig. 1C, spleen). Because total thymocytes were used in the pull-down assay, the induction of TCR binding to SH3.1 (Fig. 1C) may originate through single-positive (SP) and not through DP thymocytes. To determine whether the CD3ε PRS of preselection DP
thymocytes was induced or constitutively exposed, we depleted the TCR\textsuperscript{high} thymocytes, thereby obtaining a 94% pure population of preselection DP thymocytes with low levels of TCR (Fig. 1D). Binding of the TCR to SH3.1 in the preselection DP population above background levels was induced upon TCR engagement (Fig. 1D), in agreement with a recent report (25).

The pull-down assay determines whether the TCR can bind the SH3.1 domain but not whether Nck is indeed recruited to the TCR. Indeed, defective ubiquitylation of the CD3 ε chain was thought to play an important role in the degradation of the TCR in DP thymocytes, perhaps by promoting the recruitment of the SLAP adaptor protein to the TCR. Indeed, defective ubiquitylation of the CD3 ε chain has been described (16) in those knockin mice (10), further suggesting that the PRS, and perhaps Nck recruitment, favors the polyubiquitylation and degradation of the TCR in DP thymocytes. Therefore, we analyzed how the TCR in thymocytes of our KI-PRS mice became ubiquitylated and degraded in comparison with WT thymocytes. After immunoprecipitation with anti-CD3 ε and immunoblotting with anti-ubiquitin, we detected two major bands, one corresponding to bis-ubiquitylated CD3 ε homodimer in which each subunit is modified with one ubiquitin molecule and a larger one corresponding to bis-ubiquitylated CD3 ε polyubiquitylated TCR in DP thymocytes.

Table I. Abs and other materials

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BD, Becton Dickinson; CBMso, Centro de Biología Molecular Severo Ochoa; FC, flow cytometry; IF, immunofluorescence; IP, immunoprecipitation; UCSF, University of California, San Francisco; WB, Western blot.
FIGURE 1. Mutation of the two central prolines in CD3ε abolishes Nck recruitment to the TCR. (A) Cartoon of the cytoplasmic tail of CD3ε illustrating different sequence motifs. A cartoon indicating the mutations in the PRS previously described (in boldface) is shown (right panel). (B) Pull-down (pd) of lysates of COS cells transfected with either Flag-tagged WT or CD3ε double proline mutant (fεwt or fεmut) with GST-SH3.1 analyzed in immunoblots (IB) probed with anti-Flag. Relative densitometric values of three samples run in parallel are shown (right panel). Data are representative of two experiments. (C) Thymocytes and splenocytes isolated from either WT or KI-PRS mice were left unstimulated or stimulated with anti-CD3 for 5 min before lysis and GST-SH3.1 pd. Relative densitometric values of three samples run in parallel are shown (right panels). Data are representative of three experiments. (D) Preselection DP thymocytes from WT mice were enriched by panning on anti-TCRβ–coated plates at 0°C. The double-color plot shows that 94% of the nonattached thymocytes were DP for CD4 and CD8, whereas the single-color histogram demonstrates that all TCR high and TCR int were removed. The purified preselection DP WT thymocytes were stimulated with anti-CD3 for the times indicated and lysed. Subsequently, a GST-SH3.1 pd and anti-CD3ε IB were performed. Relative densitometric values of three samples run in parallel are shown (right panel). Data are representative of two experiments. (E) Nontransgenic WT and KI-PRS total thymocytes were stimulated with anti-CD3, and the lysates precipitated with anti-CD3 (Ip) were analyzed in IB probed with anti-Nck (top). Anti-CD3ε served as a loading control. Likewise, total cell lysates were immunoblotted with anti-Nck to reveal equal levels of expression. Relative densitometric values of three samples run in parallel are shown (right panel). (F) A similar experiment was carried out with thymocytes from OT-I TCR transgenic mice that were stimulated with T2-Kb APCs loaded with OVA for the times indicated. Relative densitometric values of three samples run in parallel are shown (right panel). Data are representative of three experiments. BRS, basic amino acid–rich sequence; EC, extracellular domain; ER-RS, endoplasmic reticulum retention sequence; IC, intracellular domain; PTB, phosphotyrosine-binding motif; PxxP, consensus SH3 domain–binding motif; TM, transmembrane domain.
degradation (Fig. 2B). Therefore, the increased TCR expression detected in DP thymocytes of our KI-PRS mice could not be explained by decreased rates of TCR ubiquitylation and degradation. These observations raised the question why DP thymocytes exhibit higher levels of TCR expression in our KI-PRS mice if the PRS mutation and blockage of Nck recruitment accelerates TCR degradation.

Blocking Nck recruitment to the TCR impairs DP thymocyte differentiation at each of the TCR-dependent selection stages

The OT-I TCR transgenic mice provided a clue to resolve the above question, as anti-CD4 and anti-CD8 staining revealed an abnormal distribution of thymic subpopulations in KI-PRS mice. Preselection DP thymocytes with high levels of both CD4 and CD8 were strongly diminished, with thymocytes accumulating at intermediate stages of differentiation from DP to CD8SP (i.e., CD4<sup>+</sup>CD8<sup>lo</sup> and CD4<sup>lo</sup>CD8<sup>lo</sup>; Fig. 3A) (26). This result indicated that the PRS mutation provoked a partial arrest beyond the positive selection checkpoint at the DP stage. The DP thymocyte population can be subdivided in three different stages (DP1-DP3), classified according to the expression of the TCR and CD5 (27). Accordingly, OT-I DP thymocytes from KI-PRS mice accumulate at the DP3 stage, visualized both in percentages and in the absolute number of thymocytes (Fig. 3B). Therefore, the accumulation of thymocytes at the DP3 stage and not a reduction in TCR degradation apparently accounts for the potential increase in TCR expression in DP thymocytes induced by the PRS mutation.

Positive selection is accompanied by downregulation of CD24 and upregulation of CD69. Analysis of the DP3 subpopulation revealed an increase and decrease in the expression of these markers, respectively, in the OT-I KI-PRS versus WT OT-I thymocytes (Fig. 3B), indicating defective positive selection in KI-PRS OT-I thymocytes. Defects in positive and negative selection were also detected in female and male HY TCR transgenic mice, respectively, on a mutant KI-PRS background (Supplemental Fig. 3A, 3B). OT-I and HY are models for CD8 T cell selection. Defects in positive selection were also manifested in the AND TCR transgenic model for CD4 T cell selection. In this model, the formation of CD4SP thymocytes and the upregulation of TCR transgene (V<sub>B</sub>3) expression were reduced in KI-PRS mice (Supplemental Fig. 3C, 3D).

Although the PRS mutation reduced the absolute number of total thymocytes in OT-I TCR transgenic mice (Fig. 3A), we detected no differences in the distribution (Fig. 2A) or absolute numbers (Fig. 3C) of nontransgenic thymocytes between mutant and WT genotypes. However, a more careful analysis of the DP population indicated an accumulation of the DP2 subpopulation of thymocytes (Fig. 3C). Furthermore, analysis of total thymocytes based on their expression of CD24 and TCR revealed an accumulation of thymocytes at a DP stage with partially upregulated TCR levels and high CD24 expression (population b in Fig. 3D), which corresponded to the DP2 subpopulation. Thymocytes also accumulated at transitional stages in KI-PRS mice, as defined by high levels of TCR and CD24 expression (population c in Fig. 3D).
These results confirm that high TCR expression in the DP population of KI-PRS mice occurs because of an arrest of thymic maturation at an intermediate stage, and not because of decreased TCR degradation.

The accumulation of thymocytes at the DP2 stage in KI-PRS mice suggests positive selection is impaired. In contrast, the reduction in DP1 cells compared with WT mice indicates that the input of DP1 precursors from the DN stages is also impaired in KI-PRS mice. Indeed, the analysis of the DN populations in OT-I TCR transgenic, as well as nontransgenic mice, of the WT and mutant genotypes revealed that the PRS mutation caused a partial arrest at the DN3 stage, suggesting that pre-TCR signaling is deficient (Fig. 4). An accumulation of DN thymocytes at the DN3 stage was also observed in mice expressing the AND and HY TCR transgenes (Supplemental Fig. 3E, 3F). Overall, these results indicate that the PRS of CD3ε is necessary for full pre-TCR and TCR signaling at each step during thymic maturation, that is, β-selection, positive and negative selection, and maturation to the CD4 and CD8 T cell lineages.

Nck recruitment to the TCR is required for the most immediate TCR signaling events in the thymus

The impairment of thymic differentiation in KI-PRS mice suggested that the recruitment of Nck to the PRS is required for TCR...
FIGURE 4. Partial arrest of thymocyte differentiation at the pre-TCR stage in nontransgenic and OT-I TCR transgenic KI-PRS mice. (A) Flow cytometry of thymocytes from nontransgenic WT and KI-PRS mice showing thymocyte distribution in the four major DN subpopulations. Thymocytes were gated in the DN quadrant according to CD4 and CD8 expression, and the selected cells were reanalyzed for the expression of CD25 and CD44 markers. The DN3 and DN4 subpopulations correspond to CD44^−CD25^− and CD44^CD25^ DN thymocytes, respectively. Absolute cell number in each thymic subpopulation was counted in four mice per group (right panel). Data are representative of six experiments. (B) The analysis described in (A) was also performed on thymocytes from OT-I TCR transgenic WT and KI-PRS mice. Absolute cell number in each thymic subpopulation was counted in three mice per group (right panel). Data are representative of six experiments.

signaling. Furthermore, because both positive and negative selection are impaired by the PRS mutation (Fig. 3, Supplemental Fig. 3), we studied whether the proliferative response of OT-I thymocytes to a panel of positive- or negative-selecting peptide Ags was affected. The response to the two negatively selecting peptides (OVAp and Q4R7) was inhibited in cells expressing the mutant PRS, whereas the proliferative response to the two positively selecting peptides (Q4H7 and G4) was too weak even in WT thymocytes to detect significant differences (Supplemental Fig. 4).

To evaluate how the PRS mutation affected TCR-proximal signals that could explain its effects on thymic differentiation, we carried out a number of flow cytometry experiments with ZAP70 and phospho-ZAP70–specific Abs. DP thymocyte differentiation from the DP1-DP3 stages is accompanied by the upregulation of ZAP70 (27). Although ZAP70 expression was higher in KI-PRS versus WT DP thymocytes when the total population was examined (Fig. 5A), there were no differences in ZAP70 levels when the DP1, DP2, and DP3 subpopulations of WT and KI-PRS thymocytes were analyzed, suggesting that the PRS mutation affects DP maturation, but not the upregulation of ZAP70. However, a similar analysis using an anti–phospho-Tyr319-ZAP70 Ab, which detects active ZAP70, revealed deficient ZAP70 activation at all stages of DP maturation in KI-PRS mice (Fig. 5B). Defective phosphorylation of ZAP70 in Tyr319 was also detected in all post-DP stages in KI-PRS thymocytes when compared with WT counterparts (Fig. 5B). Similar findings were observed with another phospho-ZAP70 Ab specific for Tyr292 (Fig. 5C). Hence, Nck binding to CD3ε appears to be required for efficient activation of ZAP70 during thymic differentiation.

Because phosphorylation of ZAP70 requires recruitment to the CD3 phospho-ITAMs (2), we investigated whether deficient phosphorylation of the CD3 ITAMs could underlie the observed defects in ZAP70 phosphorylation. When total thymocytes from OT-I transgenic mice were stimulated with T-2K0 APCs loaded with OVAp, CD3ε was rapidly phosphorylated in WT OT-I thymocytes, as detected by anti-CD3 immunoprecipitation and immunoblotting with a pan-phosphotyrosine Ab, peaking 30 s after stimulation (Fig. 5D, upper row). By contrast, induction of CD3ε tyrosine phosphorylation in KI-PRS OT-I transgenic mice was both delayed and weaker. When the membranes were reprobed with a mAb specific for the phosphorylated form of the N-terminal tyrosine of the first ITAM of CD3ε (phospho-εY1) (29), defective tyrosine phosphorylation of CD3ε in thymocytes bearing the mutation in the PRS was confirmed (Fig. 5D, second row). Finally, the coimmunoprecipitation of ZAP70 with the TCR was impaired by the PRS mutation (Fig. 5D, third row). Together, these results indicate that Nck binding to the PRS sequence of CD3ε is required for full tyrosine phosphorylation of the CD3ε ITAMs and for ZAP70 recruitment and activation in thymocytes.

Discussion
In this study, we have generated a genetically modified mouse line bearing alanine replacements for the two central prolines of the canonical PxxP motif of CD3ε. This mutation impaired T cell development at each individual step at which the pre-TCR or TCR are required: pre-TCR signaling at the DN3-DN4 transition, positive and negative selection at the DP stage, and maturation into CD4SP and CD8SP thymocytes. Unlike in a previously described genetically modified mouse line bearing an 8-aa replacement in the PRS of CD3ε (10, 16), we did not find that PRS mutation resulted in slower degradation of the TCR. Furthermore, we found that endogenous Nck is not constitutively bound to the TCR, but inducibly recruited to the TCR in WT thymocytes upon TCR triggering. How can we explain the discrepancy in the phenotypes of our KI-PRS with the previous KI? We suggest that the discrepancy is explained by the different extent of the PRS mutations used. Although in the previous knockin mutant, 8 aa of CD3ε containing the PRS were replaced by the sequence cont-
FIGURE 5. PRS mutation impairs early activation events in thymocytes. (A) Intracellular flow cytometry analysis of ZAP70 expression in total DP and DP1-DP3 subpopulations. Histograms corresponding to WT are shadowed, and those corresponding to KI-PRS are indicated by solid lines. Mean fluorescence intensity (MFI) values for WT and KI-PRS mice are shown in gray and black type, respectively. Data are representative of three experiments. (B) Intracellular flow cytometry analysis of phospho-Y319 ZAP70 in total DP, in DP1-DP3 subpopulations, and in transitional (CD4^hiCD8^- and CD4^-CD8^hi) and mature (CD8SP) thymocytes. Data are representative of three experiments. (C) Intracellular flow cytometry analysis of phospho-Y292 ZAP70 of thymocyte populations as in (B). Data are representative of three experiments. (D) Phospho-CD3 was analyzed after stimulation of OT-I thymocytes from WT and KI-PRS mice with T2-K^b APCs loaded with OVAp, probing anti-CD3 precipitates in immunoblots with anti-phosphotyrosine. The membrane was reprobed with an Ab specific for the phosphorylated form of the N-terminal tyrosine of the first ITAM of CD3ξ (EM-26) and with an Ab directed against total ZAP70. Data are representative of three experiments.
tained in a similar position of the γ-chain of the FcεRI (10), our knockin mice bear a more conservative mutation: the two central proline residues of the canonical SH3-binding PxxP motif were replaced by alanine residues. The 8-aa replacement affects the PRS, as well as a potential phospho-tyrosine-binding site that conforms to the canonical NPXY sequence contained within the PRS. Thus, although our PxxP-to-AxxA mutation exclusively abrogates Nck recruitment, the 8-aa replacement might prevent the recruitment of a second protein to the NPXY sequence. A defective recruitment of the second protein might explain the milder phenotype of the 8-aa replacement mutant. Studies aimed at identifying this second protein are currently under way. The differences between PRS mutations could also explain other observations in transgenic and retrogenic mice (9, 13, 14).

An important role for Nck during thymic maturation and mature T cell function has been demonstrated in double-knockout mice lacking Nck1 in all tissues, and conditionally lacking Nck2 in T cells only (11, 12). The phenotype of mice lacking Nck in T cells resembles that of our KI-PRS mice, although in some aspects the phenotype of these mice is stronger or milder than ours. This may be explained by the participation of Nck in T cell activation in both TCR recruitment-dependent and -independent pathways, and by the incomplete elimination of Nck2 through the conditional approach. Therefore, unlike previous articles on Nck-deficient mice (11, 12), this article highlights the importance of Nck in T cell activation by binding to CD3ε.

We investigated the effect of inhibiting the Nck-CD3ε interaction on early TCR signaling in KI-PRS TCR transgenic mice. ZAP70 recruitment to the TCR and ZAP70 activation, revealed by its phosphorylation at tyrosine residues 292 and 319, were inhibited in KI-PRS T cells. This is probably due to an unexpected effect of the PRS mutation on CD3ε tyrosine phosphorylation. The analysis of thymocytes from KI-PRS mice shows impaired CD3ε tyrosine phosphorylation, suggesting that Nck binding to CD3ε is required for full tyrosine phosphorylation of CD3ε. This effect of Nck binding to CD3ε could be explained by Nck mediating the recruitment of priming tyrosine kinase, perhaps Lck, to the TCR or by the stabilization of the active conformation of the TCR after Nck binding to the PRS. Because it has recently been shown that Lck activity remains unaltered after TCR triggering (30), regulating the TCR triggering-dependent phosphorylation of the CD3 ITAMs may, in fact, be the result of ITAM accessibility rather than of kinase activity. The conformational change of the TCR may be the mechanism responsible for placing the cytoplasmic tails of the CD3 subunits in an appropriate position for phosphorylation, and Nck binding to the PRS a stabilizing factor. It remains to be determined what is the importance of Nck recruitment to the TCR for mature T cell activation in our KI-PRS mice compared with the 8-aa replacement mutant.

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Disclosures
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References

