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Relevance of Nck–CD3ε Interaction for T Cell Activation
In Vivo

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On TCR ligation, the adaptor Nck is recruited through its src homology 3.1 domain to a proline-rich sequence (PRS) in CD3ε. We have studied the relevance of this interaction for T cell activation in vitro and in vivo by targeting the interaction sites in both partners. The first approach consisted of studying a knockin (KI) mouse line (KI-PRS) bearing a conservative mutation in the PRS that makes the TCR incompetent to recruit Nck. This deficiency prevents T cell activation by Ag in vitro and inhibited very early TCR signaling events including the tyrosine phosphorylation of CD3ζ. Most important, KI-PRS mice are partly protected against the development of neurological symptoms in an experimental autoimmune encephalitis model, and show a deficient antitumoral response after vaccination. The second approach consisted of using a high-affinity peptide that specifically binds the src homology 3.1 domain and prevents the interaction of Nck with CD3ε. This peptide inhibits T cell proliferation in vitro and in vivo. These data suggest that Nck recruitment to the TCR is fundamental to mount an efficient T cell response in vivo, and that the Nck–CD3ε interaction may represent a target for pharmacological modulation of the immune response. The Journal of Immunology, 2014, 192: 000–000.

In most T cells, the TCR is composed of the sequence-variable TCRα- and TCRβ-chains, which are responsible for pMHC recognition, and the CD3 subunits responsible for signal transduction (CD3γ, CD3β, CD3ε, and CD3ζ [also named as CD247]) (1). The TCR has to be able to interpret small differences in the chemical composition of the peptide Ag bound to MHC as quantitatively and qualitatively different signaling outcomes, although the mechanism underlying this process remains poorly understood. The most prevalent, simplistic model proposes two types of cytoplasmic tyrosine kinases as the sole direct effectors of the TCR: Lck and ZAP70 (2). However, direct recruitment of other proteins to the CD3 subunits of the TCR has also been described (2–6), suggesting that the diversity of signaling outcomes emanating from the TCR may be modulated by the composition of the “TCR signalosome.” Thus, these mechanisms may involve the recruitment and activation of different cytoplasmic and membrane effectors to the TCR.

Nck is an src homology 2 (SH2)/SH3 adaptor protein that plays a universal role in coordinating the signaling networks critical for organizing the actin cytoskeleton, cell movement or axon guidance, connecting transmembrane receptors to multiple intracellular signaling pathways (7, 8). Nck effectors include proteins that have a pivotal role in the nucleation and polymerization of the actin cytoskeleton such as the Scar/Wave proteins and the serine/threonine kinase Pak1. Another important Nck-binding partner in T cells is the cytosolic scaffolding protein SLP76, which interacts with the SH2 domain (9, 10). Typically, Nck is recruited via its SH2 domain to phosphotyrosine residues in the tail of transmembrane receptors, including the BCR (11). On TCR ligation, Nck is, however, recruited to a proline-rich sequence (PRS) in the cytoplasmic tail of CD3ε via its N-terminal SH3 (SH3.1) domain (8, 12, 13).

Bone marrow reconstitution with CD3ε PRS mutants in CD3ε-deficient mice, Nck overexpression in primary T cells, Nck knockout mice, and PRS knockin (KI) mice have all been used to study the role of the PRS and Nck in T cell development and mature T cell activation (14–20). Some experiments have suggested that the PRS is important for thymic maturation, but not for mature T cell activation in vitro (15), although experiments with bone marrow chimeras indicate that the PRS is important to activate mature T cells by weak, but not by strong, agonists in vitro (19). Furthermore, in KI mice bearing an β-aa replacement of the PRS, Nck recruitment to the PRS was shown to regulate TCR levels at the plasma membrane in preselection double-positive CD4+CD8+ thymocytes, but not at later stages, promoting the degradation of the TCR (15, 21). However, a second KI line bearing a more conservative mutation in the PRS did not reveal such TCR degradation defect but a rather partial arrest of thy-
cell differentiation at a double-positive stage with high TCR expression (22).

To evaluate the functional relevance of the Nck–CD3ε interaction in vivo, whereas trying to solve the conflicting data regarding the role of PRS in mature T cell function, we have studied the novel KI mouse line (KI-PRS). Our results demonstrate that Ag-dependent activation of mature T cells is impaired both in vitro and in vivo. The importance of the Nck–CD3ε interaction for T cell activation was also studied using a high-affinity peptide inhibitor of the Nck(SH3.1)–CD3ε interaction. The use of this inhibitor reproduced the effects of the PRS mutation and inhibited T cell activation by Ag in vitro and in vivo. These results suggest that Nck recruitment to the PRS of CD3ε is essential for full T cell activation.

Materials and Methods

Ethics statement

All mice were maintained under specific pathogen-free conditions at the animal facility of the ‘Centro de Biología Molecular Severo Ochoa’ in accordance with current national and European guidelines. All animal procedures were approved by the ethical committee of the ‘Centro de Biología Molecular Severo Ochoa.’

Cells and mice

KI mice bearing the PtxP to AxxA double mutation in the PRS of CD3ε were generated by Genoway (Lyon, France) (22). The human Jurkat T cell lymphoma, the human lymphoblastoid B cell line Raji, and the C57BL/6 mouse melanoma B16-OVA cell line (a generous gift from Dr. I. Melero, Center for Applied Medical Research, Pamplona, Spain) were grown in RPMI 1640 plus 5% FBS. The DCEK fibroblast cell line stably transfected with plasmids encoding I-ε and CD80, and African green monkey COS-7 cells, were grown in DMEM plus 10% FBS. Lymph node T cells were maintained in RPMI 1640 10% FBS supplemented with 20 μM 2-ME and 10 mM sodium pyruvate. KI-PRS mice were crossed with OT-I TCR transgenic (Tg) mice (23) for the AND TCR (MCC specific, I-ε restricted) (24), and for the HY TCR (HY Ag specific, H-2Dβ restricted) (25). The resulting heterozygous mice were crossed again to generate TCR Tg wild type (WT) and KI homozygous mice. All experiments involved the use of littermates homozygous for the WT or the KI alleles.

Abs and other reagents

The following Abs were obtained from BD Pharmingen: FITC-CD4, Alexa Fluor 647–anti-CD4, FITC–anti-CD8α, biotinylated-anti-CD8α, PerCP–anti-CD3 (145-2C11), FITC–anti-CD3 (17A2), FITC–H-57-597 Ab against mouse C57, biotinylated-H57-597, PE–Vio2 Ab against TCRα, FITC–anti-CD69, and anti-hamster IgG (7G7-204). The fluorochrome-conjugated streptavidins used to develop staining with biotinylated Abs and the CD16/32 FeBlock were also purchased from BD Pharmingen. The PerCP–anti-CD25 was from Biologend. The rabbit anti-mouse Alexa Fluor 488 secondary antiserum was from Invitrogen, and the AffiniPure F(ab’)2 fragment Donkey anti-Rabbit Alexa Fluor 488 was from Jackson Immunoresearch. Abs against phosphorylated ZAP70-Y319, Akt-S473, and ERK-T202/T204 were obtained from Cell Signaling. The hybridoma producing the 145-2C11 mAb against mouse CD3 was a generous gift from Dr. Jeffrey Bluestone (University of California San Francisco). The OT I TCR agonist (OVAp, SINFEEKL) and all peptides used in this article were synthesized at the ‘Centro de Biología Molecular Severo Ochoa’ facility by the fmoc method, verified by mass spectrometry, and purified by HPLC to >90% purity.

Confocal microscopy

For confocal microscopy, cells were first adhered to poly-L-lysine-coated coverslips, and they were then fixed and permeabilized as described previously (5), before staining with the appropriate Abs. An inverted Axiovert200M microscope system coupled to a Confocal LSM510 was used with 63× PlanApo oil immersion objective lens (1.4 numerical aperture) and a 100× Plan-Neofluor oil immersion objective lens (1.3 numerical aperture).

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 FACS Calibur or FACSCanto II flow cytometer (Becton-Dickinson), and the data were analyzed with FlowJo software (TreeStar).

In vivo cytotoxicity assay

WT and KI-PRS OT-I mice were sensitized by i.p. inoculation of 1 × 10⁵ PFU modified vaccinia Ankara (MVA)-OVA (26), and 2 d later a mixture of 1 × 10⁵ C57BL/6 spleen cells, labeled with 4, 0.8, 0.016, or 0.0032 μM CFDA-SE (CFSE) and loaded with 0, 100, 10 or 1 nM OVAp, respectively, was injected i.v. into these mice. Twenty-four hours later, lymph node and spleens were isolated and analyzed by flow cytometry. Specific killing was calculated using the formula:

\[ \text{Tumor growth was monitored every 2–3 d. Tumors were measured with a dial-caliper, and areas were determined by multiplying the length and width. Tumors grew quite flat, and measurement of the tumor volume was therefore unreliable.} \]

For vaccination, we used a high-titer MVA-OVA preparation kindly provided by Drs. Astrid Schwantes and Gerd Sutter (Paul-Ehrlich-Institut, Langen, Germany). The preparation was diluted in saline immediately before administration. A total of 200 μl of the viral suspension containing 10⁵ PFU was administered i.p. per mouse a few minutes after injection of the tumor.

Experimental autoimmune encephalomyelitis

Chronic experimental autoimmune encephalitis (EAE) was induced in female C57BL/6 mice (6–8 wk old; 20 g body weight: Janvier), s.c. injecting a total of 150 μg myelin oligodendrocyte glycoprotein peptide (MOG35–55) emulsified in CFA (Sigma-Aldrich) and supplemented with 5 mg/ml Mycobacterium tuberculosis (H37R strain from Difco) into both femoral regions. The mice were immediately i.p. injected with 150 ng pertussis toxin (Sigma-Aldrich) and again 48 h after the immunization. The animals were weighed and inspected for clinical signs of disease on a daily basis by an observer blinded to the genotype. Disease severity of EAE was assessed according to the following scale: 0 = normal; 1 = limp tail or mild hind-limb weakness; 2 = moderate hind-limb weakness or mild ataxia; 3 = moderately severe hind-limb weakness; 4 = severe hind-limb weakness or mild forelimb weakness or moderate ataxia; 5 = paraplegia with no more than moderate forelimb weakness; and 6 = paraplegia with severe forelimb weakness or severe ataxia or moribund condition.

T cell stimulation

The expression of CD69 at the membrane of AND CD4+ cells was analyzed 24 h after stimulation with 5 × 10⁵ DCEK cells preloaded with different concentrations of MCC peptide (ANERADLIALYLVQATK). Cells were incubated with an Ab against CD69 and analyzed in a FACS Calibur (Becton Dickinson) flow cytometer. Likewise, CD25 expression by AND T cell proliferation in response to Ag was measured by CFSE dye dilution after 68 h at 37°C. The proportion of CD4+ T cells was analyzed by gating against M 2-ME and 107 PFU was administered i.p. per mouse a few minutes after injection of the tumor.

Immunoblot analysis of T cell activation

A total of 3 × 10⁵ naïve T cells of each genotype was activated at different times with APCs preloaded with Ag. 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 iodoacetamide, 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 (27) or anti-CD3 mAb (145-2C11) and protein A-Sepharose beads. SDS-PAGE and immunoblotting were performed according to standard protocols, and the membranes were probed with the anti-CD3ε serum and the anti-CD3ε mAb M-20 (Santa Cruz), which were visualized by ECL. Quantification was performed on ECL autoradiography films using ImageJ software.

Flow cytometry analysis of T cell activation

To study protein phosphorylation, we loaded 5 × 10^6 T2k cells overnight with different concentrations of OVAp and discarded the supernatant, and 2 × 10^6 lymph node cells from OT1 mice were at 37°C for 5 min. Stimulation was stopped by adding paraformaldehyde to a final concentration of 2% for 15 min at room temperature, and the fixed cells were then permeabilized for 3 min with 0.1% Nonidet P-40 on ice. Cells were washed and labeled intracellularly overnight with specific phospho-Abs.

Peptide entry assays

FITC-labeled 11Rwt and 11R085 peptides were used to measure the concentration dependence of internalization in Jurkat cells. Cells were attached to poly-L-lysine–coated coverslips and incubated for 30 min with different concentrations of the FITC-labeled peptides before they were examined by confocal microscopy. Alternatively, Jurkat cells were incubated with different concentrations of the FITC-labeled peptides for 30 min and then washed twice for 1 min with acidic medium (50 mM glycine, 150 mM NaCl, 100 mM acetic acid, pH 2.5) to remove all noninternalized peptide. Once washed, the cells were analyzed by flow cytometry.

Surface plasmon resonance

Surface plasmon resonance (SPR) analysis was performed on a Biacore X Instrument (General Electric). Instrument setup was performed according to the manufacturer’s instructions, using CM5 as sensor chip and HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% [v/v] Surfactant P20) as running buffer. The immobilization of the SH3.1 domain (at a final concentration of 30 µg/ml) was performed by the amino coupling strategy using sodium acetate (10 mM) at pH 4.5 and 150 µM solution (v/v) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide, being an optimum immobilization level of approximately 5000 resonance units for both peptides. For interaction experiments, different concentrations of 11R085 and 11Rwt were used onto the same chip. In both cases, the flow rate was 10 µl/s. After each interaction, glycine 2.5 M pH 12 was used to regenerate the surface. In all experiments after the regeneration, the analysis report was constant after repeated injections and within ±10% of the level reached in the first injection. SPR raw data were prepared for global analysis by BIAevaluation 3.2 (General Electric). The corrected binding data were then analyzed by direct curve fitting to a simple bimolecular interaction mechanism (1:1) and mass transport effect. These were fit to the association and dissociation phase sensor curve fitting to a simple bimolecular interaction mechanism (1:1) and mass transport effect. These were fit to the association and dissociation phase sensor data in all the experiments. The error space for each of the parameters was assessed using statistical profiling (Kmax < 0.001). Statistical analysis was performed by MathLab versus7.8 (2009) software considering a normal distribution of each data set (Kes, Kd) per feature and per assay, using median absolute deviation for a robust measure of statistical dispersion of the obtained data. The values (Kes, Kd) outside of the criteria (average ± 3 SD) were removed. Kes was established as Kd/Kes values.

Statistical analysis

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

Results

Inhibition of T cell activation in mice bearing a germline mutation in the PRS

The PRS of CD3ε interacts in an unusual manner with the SH3.1 domain of Nck. The canonical PxxP sequence for PRS–SH3 domain interaction is followed by 2 aa at the position +3 that establish interactions with a third hydrophobic pocket of the SH3.1 domain (pocket magenta in Fig. 1A) (28–30). Thus, the interaction sequence in the PRS of CD3ε is actually defined by the motif PxxPxxDY. This motif overlaps with the N-terminal part of CD3ε’s ITAM and with a consensus binding site (NPxY) for proteins containing PTB domains (Fig. 1A). A first Ki mouse line bearing an 8-aa replacement of the PRS affected both the SH3 and the PTB domain interaction motifs. To fully prevent the interaction with Nck, whereas minimizing possible interferences with other proteins, we generated a second Ki mouse bearing a germline substitution of the two central prolines in the PxxPxxDY motif with alanine. T cell development in these mutant Ki (KI-PRS) mice is partly arrested at each step in which pre-TCR or TCR signaling are required (22). Despite this partial defect during development, peripheral lymphoid organs of Ki-PRS mice, either non-Tg or Tg for two different TCRs, were normally populated with mature T cells expressing normal levels of TCR and the CD4 and CD8 coreceptors (Supplemental Fig. 1). Notwithstanding, recruitment of endogenous Nck to the TCR upon stimulation of lymph node T cells from OT-I TCR Tg (OT-I^Tg) mice with Ag was blocked: a fast and transient recruitment of Nck to the TCR was observed in WT Ag-stimulated OT-I^Tg T cells, but not in KI-PRS cells (Fig. 1B).

To study how blocking the TCR–Nck interaction affected T cell activation in vitro, we first tested the response of KI-PRS T cells to Ag, anti-CD3 plus anti-CD28, stimulation. The proliferative response of KI-PRS T cells was impaired, but not abrogated, when compared with WT T cells (Fig. 1C). Likewise, in a mixed-lymphocyte reaction assay, Ki-PRS T cells were less sensitive to the dose of alloantigen than WT T cells (Fig. 1D). The effect of the PRS mutation on the T cell proliferative response was much stronger when cells were stimulated with Ag-loaded APCs. Thus, the sensitivity of KI-PRS AND TCR Tg (AND^Tg) T cells to Ag stimulation was strongly inhibited (Fig. 1E). In addition to Ag-induced T cell proliferation, Nck recruitment was also important for IFN-γ production by CD8 T cells bearing either a weak (Fig. 1F) or a strong (Fig. 1G) TCR. By contrast, upregulation of CD69 was stronger in AND^Tg Ki-PRS T cells than in WT controls (Fig. 1H). Because CD69 expression is less dependent on the quality or strength of the TCR signal (31), these data suggest that Nck binding to CD3ε is necessary for full T cell activation.

To study how blocking the TCR–Nck interaction affected TCR signaling, we first examined the capacity of T cells to spread on coverslips coated with anti-CD3. TCR triggering promoted the spreading of the WT cells on the coverslip (Fig. 2A) after the rearrangement of the actin cytoskeleton and the formation of an expanding actin ring (32, 33). Cell spreading and actin polymerization were strongly inhibited in KI-PRS T cells (Fig. 2A). When the total F-actin content was measured by flow cytometry after stimulating T cells with soluble anti-CD3, actin polymerization was less evident in KI-PRS than in WT T cells (Fig. 2B). These results indicated that TCR-triggered polymerization of the actin cytoskeleton requires Nck binding to the PRS. As expected, the recruitment of Nck to the immunological synapse (IS) formed by AND^Tg WT cells was strongly inhibited in KI-PRS mice, indicating that the PRS is required for Nck recruitment to the TCR in the IS (Fig. 2C). Interestingly, in AND Ki-PRS T cells, CD3ε accumulation in the IS was clearly inhibited, suggesting that Nck recruitment to the TCR is necessary for the formation or the stabilization of the IS. Furthermore, staining with a mAb specific for the phosphorylated form of the N-terminal tyrosine of the first ITAM of CD3ε (phospho-YN1) or with a polyclonal Ab specific for the N-terminal tyrosine of the ITAM of CD3ε (phospho-eY1) (34) demonstrated deficient phosphorylation of CD3ε and CD3ε at the IS in KI-PRS T cells (Fig. 2C). These data indicate that the PRS is required for the formation of the IS.

Likewise, the defective phosphorylation of CD3ε at the IS was paralleled by defective phosphorylation of CD3ε immunoprecipitated with anti-CD3 from KI-PRS T cells (Fig. 3A). The inhibi-
Regulation of TCR signaling by Nck

Mutation of the two central proline residues in CD3ε abolishes Nck recruitment to the TCR. (A) Surface model of the SH3.1 domain of Nck1 (PDB 2JW4) and amino acid sequence of the cytoplasmic tail of CD3ε. The three shallow hydrophobic pockets of Nck(SH3.1) that bind CD3ε are shown in cyan, green, and magenta. The two prolines of the PxxPxxy Dy SH3.1-binding motif of CD3ε are shown in cyan and green, and the Asp-Tyr residues in magenta. Boxes indicate amino acids comprising the PRS, the PTB, and the ITAM motifs. (B) OT-I transgenic WT and KI-PRS lymph node T cells were stimulated with T2-Kb APCs loaded with OVAp. TCR–Nck copurification was assessed in anti-Nck immunoblots after immunoprecipitation with anti-CD3. Reprobing with anti-CD3ζ served as a loading control. Data are representative of three experiments. (C) Proliferation of non-Tg T cells 96 h after stimulation with plastic-bound anti-CD3 and 1 μg/ml soluble anti-CD28. Data represent the mean ± SD of triplicates and are representative of four experiments. (D) MLR of 5 × 10^5 spleen WT and KI-PRS T cells in response to different doses (1.2 × 10^3 to 5 × 10^3) of irradiated BALB/c spleen cells. Data represent the mean ± SD of triplicates and are representative of three experiments. (E) Proliferation of AND Tg T cells 68 h after stimulation with MCC peptide Ag-loaded DCEK APCs. Data represent the mean ± SD of triplicates and are representative of four experiments. (F) Intracellular IFN-γ expression by T cells from female HY^Tg mice after stimulation for 48 h with HY peptide Ag-loaded irradiated spleen cells from female C57BL/6 mice. Data represent the mean ± SD of triplicates and are representative of four experiments. (G) Intracellular IFN-γ expression by OT-I^Tg T cells after stimulation for 48 h with OVAp-loaded T2-Kb APCs. Data represent the mean ± SD of triplicates and are representative of four experiments. (H) CD69 induction in AND Tg T cells measured 24 h after stimulation with DCEK APCs loaded with MCC peptide Ag. Data represent the mean ± SD of triplicates and are representative of four experiments. *p < 0.05, **p < 0.005.

bition in total CD3ζ tyrosine phosphorylation was accompanied by a general reduction of tyrosine phosphorylated proteins of 37, 50, 75, and 100 kDa that copurified with the TCR (Fig. 3A). Immunoblotting with anti–phospho-ζY1 showed that at least part of the defective phosphorylation of CD3ζ in KI-PRS T cells is due to a deficient phosphorylation of the first tyrosine of the membrane-proximal ITAM (Fig. 3B). Likewise, immunoblotting with an Ab specific for ZAP70 phosphorylated on residue Tyr^319 showed less active ZAP70 copurified with the TCR in KI-PRS than in WT control T cells (Fig. 3C). The reduction in CD3ζ and ZAP70 phosphorylation was paralleled by an inhibition, downstream, of ERK phosphorylation (Fig. 3C). The results generated by immunoblotting from Ab-stimulated T cells were confirmed by flow cytometry after intracellular staining of OT-I

FIGURE 1. Mutation of the two central proline residues in CD3ε abolishes Nck recruitment to the TCR. (A) Surface model of the SH3.1 domain of Nck1 (PDB 2jw4) and amino acid sequence of the cytoplasmic tail of CD3ε. The three shallow hydrophobic pockets of Nck(SH3.1) that bind CD3ε are shown in cyan, green, and magenta. The two prolines of the PxxPxxy Dy SH3.1-binding motif of CD3ε are shown in cyan and green, and the Asp-Tyr residues in magenta. Boxes indicate amino acids comprising the PRS, the PTB, and the ITAM motifs. (B) OT-I^Tg WT and KI-PRS lymph node T cells were stimulated with T2-Kb APCs loaded with OVAp. TCR–Nck copurification was assessed in anti-Nck immunoblots after immunoprecipitation with anti-CD3. Reprobing with anti-CD3ζ served as a loading control. Data are representative of three experiments. (C) Proliferation of non-Tg T cells 96 h after stimulation with plastic-bound anti-CD3 and 1 μg/ml soluble anti-CD28. Data represent the mean ± SD of triplicates and are representative of four experiments. (D) MLR of 5 × 10^5 spleen WT and KI-PRS T cells in response to different doses (1.2 × 10^3 to 5 × 10^3) of irradiated BALB/c spleen cells. Data represent the mean ± SD of triplicates and are representative of three experiments. (E) Proliferation of AND Tg T cells 68 h after stimulation with MCC peptide Ag-loaded DCEK APCs. Data represent the mean ± SD of triplicates and are representative of four experiments. (F) Intracellular IFN-γ expression by T cells from female HY^Tg mice after stimulation for 48 h with HY peptide Ag-loaded irradiated spleen cells from female C57BL/6 mice. Data represent the mean ± SD of triplicates and are representative of three experiments. (G) Intracellular IFN-γ expression by OT-I^Tg T cells after stimulation for 48 h with OVAp-loaded T2-Kb APCs. Data represent the mean ± SD of triplicates and are representative of four experiments. (H) CD69 induction in AND Tg T cells measured 24 h after stimulation with DCEK APCs loaded with MCC peptide Ag. Data represent the mean ± SD of triplicates and are representative of four experiments. *p < 0.05, **p < 0.005.
TCR Tg T cells stimulated with Ag. Compared with WT controls, KI-PRS cells responded with weaker phosphorylation of ZAP70, ERK, and Akt (Fig. 3D), indicating that mutation of the PRS inhibited, but not blocked, immediate TCR-proximal and down-stream activation events.

These results suggest that Nck recruitment to the TCR in mature T cells requires an intact CD3ζ PRS, and that disruption of this interaction results in decreased tyrosine phosphorylation of CD3ζ, impaired formation of the IS, and the failure to trigger TCR-dependent actin cytoskeleton remodeling. Furthermore, the effect of the KI-PRS mutant on CD3ζ tyrosine phosphorylation suggests that Nck recruitment to the PRS of CD3ζ affects either the recruitment of priming tyrosine kinases or the accessibility of CD3ζ tyrosine residues.

**FIGURE 2.** Blocking Nck–CD3ζ interaction impairs TCR-triggered actin polymerization and IS formation. (A) Cell spreading and polymerization of the actin cytoskeleton of lymph node WT and KI-PRS cells incubated for 15 min on coverslips coated with either anti-TCRβ (H57) or anti-CD3 (2C11) Abs, or uncoated (polylysine). Photographs were taken at ×25 and ×63 magnification of cells stained with Texas Red–conjugated phalloidin. Scale bars: 50 µm (original magnification ×25), 20 µm (original magnification ×63). Quantification of cell spreading was performed with cells incubated on anti-TCRβ–coated coverslips by counting cells with a diameter >30 µm in the major axis in 10 photographs per cell type each containing ~40 cells. Data represent the mean ± SD of triplicates and are representative of three experiments. (B) Flow cytometry analysis of F-actin content in CD4 and CD8 T cells stimulated for 5 min with anti-CD3 (filled bars) or unstimulated (open bars). Values represent the mean fluorescence intensity of fluorescein-conjugated phalloidin staining after permeabilization. Data represent the mean ± SD of triplicates and are representative of three experiments. (C) Formation of the IS by AND Tg T cells stimulated for 15 min with MCC-loaded DCEK APCs was evaluated in fixed cells after staining with anti-CD3ζ. Nck recruitment to the IS, and phospho-CD3ζ(Y1) and phospho-CD3ζε(Y1) localization were visualized with specific Abs; actin polymerization with Texas Red–conjugated phalloidin. Arrows indicate the presence of polymerized actin in the IS of WT cells. Staining with anti–phospho-e was carried out separately. Scale bars, 5 µm. Quantification of labeled ISs was performed by counting 5 photographs per cell type and condition, each containing ~20 cells. Data represent the mean ± SD and are representative of three experiments. *p < 0.05, **p < 0.005.
Impairment of T cell–dependent tumor rejection in KI-PRS mice

Given the impaired response to Ag observed in vitro, we studied whether KI-PRS mice could mount an efficient immune response in vivo. To this end, we analyzed s.c. growth of the B16 mouse melanoma cell line expressing OVA as a model Ag, and the antitumor response elicited after vaccination (Fig. 4A). After vaccination with a recombinant nonreplicative vaccinia virus encoding the OVA protein (MVA-OVA) (26), tumors progressed much slower in WT mice. However, KI-PRS mice responded 

![Image](http://www.jimmunol.org/)  

**FIGURE 3.** Blocking Nck–CD3ε interaction impairs early activation events in mature T cells. (A) Tyrosine phosphorylation of the TCR and TCR-associated proteins was analyzed after T cell stimulation with anti-CD3 and probing with anti-phosphotyrosine Ab 4G10 after immunoprecipitation with anti-CD3. The position of tyrosine phosphorylated CD3ζ in its different m.w. forms is indicated. Data are representative of four experiments. (B) Tyrosine phosphorylation of CD3ζ was analyzed as in (A) after probing with anti-phospho(Y1). Reprobing with anti-CD3ζ served as loading control. Data are representative of three experiments. (C) ZAP70 phosphorylation was analyzed after T cell stimulation with anti-CD3 and probing with anti–phospho-ZAP70 (Y319) Ab after immunoprecipitation with anti-CD3. Anti-ERK phosphorylation triggered after stimulation with anti-CD3 evaluated by immunoblotting with anti–phospho-ERK. Data are representative of three experiments. (D) Defective ZAP70 and ERK, and Akt phosphorylation, in CD8+ T cells from lymph nodes of OT-I Tg mice stimulated with OVAp-loaded APCs as analyzed by flow cytometry. Data represent the mean ± SD mean fluorescence intensity (n = 3 mice/group). *p < 0.05.

**Impairment of T cell–dependent tumor rejection in KI-PRS mice**

Given the impaired response to Ag observed in vitro, we studied whether KI-PRS mice could mount an efficient immune response in vivo. To this end, we analyzed s.c. growth of the B16 mouse melanoma cell line expressing OVA as a model Ag, and the antitumor response elicited after vaccination (Fig. 4A). After vaccination with a recombinant nonreplicative vaccinia virus encoding the OVA protein (MVA-OVA) (26), tumors progressed much slower in WT mice. However, KI-PRS mice responded
FIGURE 4. Blocking Nck–CD3ε interaction abrogates T cell–mediated immunity in vivo. (A) Protective response after vaccination with MVA-OVA (10^7 PFU, i.p.) against OVA-expressing B16 melanoma cells (5 × 10^6) injected s.c. just after vaccination (day 0). Data represent the mean ± SD for n = 4 mice/group and are representative of three experiments. (B) Protective response after adoptive transfer on day 0 of OT-I Tg T cells (10^3, i.v.) from either WT or KI-PRS mice to non-Tg WT recipient mice that were subsequently immunized and challenged with B16-OVA cells as described in (A). Data represent the mean ± SD for n = 5 mice/group and are representative of three experiments. (C) Proliferative response to MVA-OVA vaccination of CFSE-labeled OT-I T cells (10^7) inoculated i.v. into C57BL/6 mice immunized with MVA-OVA. Sixty hours after transfer, mice were sacrificed and CFSE fluorescence intensity within the CD8^+ Vα2^+ (OT-I TCR) population in spleen cells was analyzed by flow cytometry. The proliferation index was calculated as indicated in Materials and Methods. Data represent the mean ± SD for n = 4 mice/group and are representative of two experiments. (D) In vivo cytotoxicity. Spleen cells from C57BL/6 mice were labeled with CFSE at four different concentrations. Each CFSE-labeled population was preincubated with a dose of OVA p and then mixed in approximately equal proportions before i.v. injection into OT-I WT or OT-I KI-PRS mice. Recipient mice were sacrificed 24 h later, and the percentage of CFSE-labeled cells corresponding to each intensity peak was calculated after flow cytometry analysis. To the left are examples of cells analyzed according to CFSE intensity and size (forward scatter [FS]). OVA p dose-dependent killing was calculated according to the ratio of cells under each CFSE peak divided by the percentage of cells nonloaded with OVA p. Data represent the mean ± SD for n = 5 mice/group and are representative of two experiments. *p < 0.05, ***p < 0.0005, ****p < 0.00005. n.i., WT mice injected with B16-OVA melanoma cells but not immunized with MVA-OVA; N.T., WT vaccinated mice that did not receive OT-I T cells; WT n.i., proliferation of OT-I WT CD8 T cells in non-immunized C57BL/6 recipient mice.
poorly to vaccination and developed large tumors (Fig. 4A). These results indicate that KI-PRS mice were unable to mount an efficient adaptive immune response to a model tumor Ag after vaccination.

Control of the B16-OVA melanoma requires an efficient CD8 T cell cytotoxic response (35). To determine whether the antitumor immunity conferred by CD8 T cells was defective in the absence of a functional PRS, purified OVA-responsive CD8 T cells from either WT or KI-PRS OT-I Tg mice were adoptively transferred to WT mice on the day of immunization (Fig. 4B). Compared with WT mice just vaccinated with MVA-OVA, tumor progression was significantly reduced when mice received OT-I WT CD8 T cells. Conversely, no protection other than that afforded by vaccination was observed when immunized WT mice received OT-I CD8 T cells from KI-PRS mice (Fig. 4B). Overall, these data indicate that Nck recruitment to the TCR is required to elicit a protective adaptive immune response to tumor Ags in vivo.

To understand the inability of KI-PRS mice to mount an efficient antitumor response after vaccination, we first tested the proliferative response of OT-I T cells to MVA-OVA in vivo. WT and KI-PRS OT-I T CD8 T cells were CFSE-labeled and adoptively transferred into MVA-OVA–vaccinated WT mice. The proliferation of OT-I cells was calculated 60 h after inoculation according to CFSE dilution. KI-PRS OT-I Tg mice proliferated poorly in response to MVA-OVA vaccination, compared with WT cells (Fig. 4C), suggesting that Nck binding to the PRS of CD3ε is required for an efficient proliferative response of CD8 T cells to the MVA-OVA vaccine. In addition, we tested the in vivo cytotoxic response of KI-PRS T cells by inoculating WT and KI-PRS OT-I Tg mice with CFSE-labeled target spleen cells preloaded with different amounts of OVAp. Twenty-four hours after inoculation, target cells preloaded with the lowest concentration of OVAp (1 nM) had been efficiently killed in WT mice, whereas KI-PRS mice required target cells to be preloaded with a 100-fold higher concentration of OVAp to achieve a similar response (Fig. 4D). These results indicated that the PRS is required for an efficient cytotoxic T cell response in vivo. Therefore, the inefficient proliferative response to the vaccine and the inefficient cytotoxic response of T cells bearing the PRS mutation could be behind their inability to reject a model tumor in vivo.

Delayed onset of neurological symptoms in an EAE model in the absence of a functional PRS

EAE is the most commonly used experimental model for multiple sclerosis, which is frequently induced by immunization with myelin-derived Ags in adjuvant, apparently reproducing the key pathological features of multiple sclerosis: inflammation, demyelination, axonal loss, and gliosis (36). To assess the effect of the PRS mutation on the development of EAE symptoms, WT and KI-PRS mice were immunized with MOG35–55 and scored according to the acquisition of neurological symptoms and weight loss. Both symptoms were significantly delayed in KI-PRS compared with WT controls (Fig. 5), suggesting that in the absence of a functional PRS, T cell response against an autoantigen is impaired.

A high-affinity peptide for the SH3.1 domain of Nck inhibits T cell activation in vitro and in vivo

The effect of the KI-PRS mutation indicated that the PRS sequence responsible for Nck binding is important both for a functional immune response of mature T cells in vitro and in vivo. To prevent the CD3ε PRS–Nck interaction by blocking the SH3.1 domain of Nck, that is, without affecting the other domains of Nck, we used a synthetic peptide derived from the CD3ε PRS, identified after two sequential rounds of PEPSscan to ensure high-affinity binding to the SH3.1 domain of Nck1 (29). This peptide (peptide 085) was synthesized with an N-terminal polyarginine sequence that favored cell entry (37) (Fig. 6A). For comparison, we synthesized the corresponding peptide derived from the WT sequence (11Rwt) and another with the same amino acid composition as peptide 085 but with a scrambled sequence (11Rscr). Using a pull-down assay with GST fused to a truncated Nck2 construct containing the three SH3 domains, binding of anti-CD3–triggered TCR to GST-Nck was completely abolished by the 11Rwt peptide at 100 μM, but only slightly affected at a dose of 30 μM (Fig. 6B). Peptide 11R085 was more potent, blocking TCR binding at 30 μM and with only residual binding observed at 10 μM. Peptide 085 was selected by its high affinity for the SH3.1 domain and unaffected binding to the SH3.2 and SH3.3 domains (29). Therefore, it was not surprising that neither 11R085 nor 11Rwt affected the binding of Pak1 to the SH3.2 domain of Nck (Fig. 6B), suggesting that 11R085 is selective for the SH3.1 domain.

Because the pull-down assay is not very sensitive for inhibition studies because of the large amounts of GST-Nck fusion protein required, we analyzed the inhibitory capacity of these peptides in vitro using a more sensitive immunoprecipitation measured by flow cytometry method, as previously described (38). Peptide 11R085 inhibited TCR binding to the SH3.1 domain of Nck1 with an IC50 of 0.3 nM, whereas peptide 11Rwt was 300-fold less potent (Fig. 6C). These data are consistent with the affinity of 11R085 and 11Rwt for SH3.1 of Nck1 observed by SPR (0.28 and 580 nM, respectively; Fig. 6D).

In spite of the presence of the poly-basic amino acid stretch, the permeability of T cells to these peptides was low, and peptide entry was barely detectable at concentrations <1 μM (Fig. 6E, 6F). Peptide 11R085 was nevertheless useful to test the effect of inhibition of Nck recruitment to the TCR on T cell activation. Stimulation of Jurkat T cells with superantigen-loaded APCs produced a fast and transient recruitment of Nck to the TCR (Fig. 6G), such as it had been observed for OT-I WT T cells (Fig. 1B). The recruitment of endogenous Nck was prevented in
FIGURE 6. A high-affinity peptide inhibitor of the Nck–CD3ε interaction. (A) Sequence of the polybasic cell-permeable peptides used for inhibition. The three peptides have an N-terminal polyarginine stretch of 11 residues followed by a 3-glycine residue spacer, and the specific sequence was either derived directly from the PRS and surrounding residues (peptide 11Rwt) or from a PEPSCAN selection (11R085). A control for 11R085 was generated by randomizing the specific amino acid sequence (11Rscr). A D-lysine residue present in peptides 11R085 and 11Rscr is indicated in bold in small caps. (B) The inhibitory capacity of the 11Rwt and 11R085 peptides was analyzed in the pull-down assay with GST-Nck2(DSH2) using lysates of anti-CD3–stimulated Jurkat T cells followed by immunoblotting with anti-CD3ε. The membrane was reprobed with anti-Pak1 to study the effect of the peptides on the SH3.2–Pak1 interaction. NS, pull-down from unstimulated Jurkat cells. Data are representative of three experiments. (C) Inhibitory effect of 11Rwt and 11R085 peptides in a quantitative pull-down assay performed by immunoprecipitation measured by flow cytometry (IP-FCM) with beads coated with GST-SH3.1. Coated beads were incubated with lysates of anti-CD3–stimulated Jurkat and the amount of TCR bound to the beads was quantified by staining with PE-labeled anti-TCR (Vβ8) Ab. Data represent the mean ± SD of triplicates. (D) Label-free bimolecular interaction analysis of peptides 11R085 and 11Rwt with domain SH3.1 of Nck1 immobilized onto the surface of a CM5 sensor chip. The corrected binding data for three peptide concentrations (cyan, green, and magenta lines) is represented together with the fit association and dissociation phase sensor data (black lines). The binding affinities were calculated dividing the koff by the kon values. Data are representative of two experiments. (E) Internalization of fluorescein-labeled 11R085 by Jurkat cells incubated with 10 μM peptide for 15 min. After incubation, some cells have concentrated the peptide and appear brighter than the extracellular medium (arrows), other cells are starting to internalize the peptide in vesicles (arrowheads), and others have not yet internalized the peptide and appear black. Original magnification ×63. (F) Uptake of the indicated peptides in Jurkat cells by flow cytometry. Cells were incubated for 30...
min with fluorescein-labeled 11R085 or 11Rwt peptides and intake calculated according to the mean fluorescence intensity of the cells after washing out the bound, but not internalized peptide, with acidic buffer. Autofluorescence of cells is represented by a black line, green fluorescence of cells incubated with 10 nM peptide by a red line, with 1 μM peptide by a blue line, and with 20 μM peptide by a green line. The panel on the bottom shows a plot of mean fluorescence intensity data for all concentrations of peptides 11R085-FITC and 11Rwt-FITC. Data represent the mean ± SD of triplicates and are representative of three experiments. (**G**) Recruitment of endogenous Nck to the TCR was assessed by precipitation with anti-Nck of lysates from Jurkat cells stimulated with SEE-loaded Raji APCs for the times indicated. Immunoblots were subsequently probed with anti-CD3ζ. Jurkat cells were either incubated with 20 μM 11R085 or mock treated. The membrane was reprobed with anti-Nck as a loading control. Data are representative of two experiments.

**FIGURE 7.** The inhibitor of the Nck–CD3ζ interaction blocks T cell activation in vitro and in vivo. (A) Proliferation of T cells from lymph nodes of OT-I TCR Tg WT mice was examined 72 h after stimulation with T2-Kb APCs loaded with 1 nM OVAp in the presence of the indicated concentrations of the inhibitory peptides. Data represent the mean ± SD of triplicates and are representative of five experiments. ***p < 0.0005. (B) Proliferation of human blood CD8+ T cells in response to anti-CD3 plus anti-CD28 for 96 h in the presence of the concentrations of peptide inhibitors indicated. The effect of the peptide inhibitors on IL-2–dependent T cell proliferation was analyzed on T lymphoblasts stimulated for 72 h with IL-2. Data represent the mean ± SD of triplicates and are representative of five experiments. (C) Intracellular IFN-γ expression by lymph node T cells from OT-I TCR Tg WT and KI-PRS mice after stimulation for 48 h with T2-Kb APCs loaded with OVAp in the presence of the indicated concentrations of peptides 11R085 or 11Rscr. Data represent the mean ± SD of triplicates and are representative of three experiments. *p < 0.05. (D) Proliferation of T cells from lymph nodes of AND TCR Tg WT and KI-PRS mice was examined 68 h after stimulation with MCC peptide Ag-loaded DCEK APCs. Data represent the mean ± SD of triplicates and are representative of three experiments. ***p < 0.0005. (E) Stability of intracellular 11R085 in T cells in vivo. Lymph node cells from OT-I WT mice were preincubated ex vivo with 10 μM FITC-labeled 11R085 for 1 h. Treated cells were subsequently inoculated (10⁷, i.v.) into C57BL/6 mice previously immunized with MVA-OVA. Two mice were killed at each indicated time point after inoculation and FITC-11R085 fluorescence intensity within the CD8+ Vα2+(OT-I TCR) population in lymph node cells was analyzed by flow cytometry. Data represent the mean ± SD for n = 2 mice per group analyzed in triplicates. Mean fluorescence values (MFI) were normalized to those of the cells before inoculation. (F) Effect of the Nck–CD3ζ interaction inhibitor on the T cell proliferative response to MVA-OVA vaccination. Lymph node cells from OT-I WT mice were labeled with CFSE and preincubated ex vivo with the indicated concentrations of peptides 11R085 or 11Rscr for 1 h. Treated cells were subsequently inoculated (10⁷, i.v.) into C57BL/6 mice previously immunized with MVA-OVA. Sixty hours after transfer, mice were killed and the CFSE fluorescence intensity within the CD8+ Vα2+(OT-I TCR) population in lymph node and spleen cells was analyzed by flow cytometry. The proliferation index was calculated as indicated in Materials and Methods. Data represent the mean ± SD for n = 4 mice/group and are representative of two experiments. *p < 0.05. n.i., Proliferation of OT-I WT CD8 T cells in nonimmunized C57BL/6 recipient mice; n.s., not significant, p > 0.05.
the presence of the 11R085 peptide (Fig. 6G), demonstrating that the peptide is capable of entering cells and inhibiting the Nck–CD3ε interaction.

We then examined the effect of the 11R085 peptide on T cell activation. This peptide blocked Ag-triggered proliferation of OT-I WT T cells at a concentration of 10 μM, whereas peptide 11Rwt was less potent (Fig. 7A). The scrambled peptide did not affect OT-I T cell proliferation even at 60 μM, demonstrating the specificity of the inhibitory effect. To determine whether the inhibitory effect of the 11R085 peptide was restricted to TCR-dependent stimuli, its effect on cell proliferation was studied in freshly isolated human T cells stimulated with anti-CD3 and in human T lymphoblasts stimulated with exogenous IL-2. The peptide only inhibited TCR-dependent proliferation, indicating that no nonspecific toxic effects on non–TCR-dependent pathways occurred (Fig. 7B). Further proof of the specificity of 11R085 peptide inhibition came from its dependence on an intact PRS. Thus, both IFN-γ production (Fig. 7C) and Ag-triggered proliferation (Fig. 7D) of WT OT-I T cells were inhibited by peptide 11R085. However, this peptide did not inhibit Ag-triggered activation of KI-PRS OT-I T cells (Fig. 7C, 7D), indicating that it does not have targets other than the Nck–CD3ε interaction during TCR-triggered T cell activation.

Because of the presence of the polyarginine stretch, both the 11R085 and the control 11Rscr peptides were too toxic when directly administered to mice as to perform experiments in vivo (data not shown). Notwithstanding, to test the inhibitory capacity of the 11R085 on T cell activation in vivo, we performed an experiment similar to this of Fig. 4C. To this aim, we first tested the intracellular stability of the 11R085 peptide in vivo. OT-I CD8 WT T cells were preincubated with FITC-labeled peptide 11R085 ex vivo and were adoptively transferred into MVA-OVA–vaccinated WT mice. Forty and twenty-five percent of the initial fluorescence intensity was still detected in OT-I T cells taken from draining lymph nodes 4 and 16 h after inoculation, respectively (Fig. 7E). These data suggested that after preincubation ex vivo, peptide 11R085 may be sufficiently stable as to have an impact on initial TCR triggering events in vivo. To study the effect of peptide 11R085 on T cell proliferation in vivo, CD8 T cells from OT-I WT mice were labeled with CFSE and preincubated ex vivo with peptides 11R085 or 11Rscr. Sixty hours after adoptive transfer into MVA-OVA–vaccinated WT mice, T cell proliferation was calculated according to CFSE dilution on CD8 OT-I T cells removed from draining lymph nodes and spleen. A pretreatment with 11R085 inhibited OT-I T cell proliferation in response to MVA-OVA vaccination, compared with 11Rscr-treated cells (Fig. 7F). These results suggest that the Nck–CD3ε interaction could be an interesting target for immunosuppressive or immunomodulatory drugs.

**Discussion**

In this study, we used a dual approach to study the relevance of the Nck–CD3ε interaction in T cell responses to Ag in vivo. We generated a genetically modified mouse line bearing alanine replacements for the two central prolines of the canonical PxxP motif of CD3ε. This mutation severely impaired T cell response to Ag in vitro in all the TCR systems used. More importantly, the capacity of mutant T cells to kill target cells in vivo and of mutant mice to acquire immunity to a model tumor Ag after vaccination was compromised. This incapacity to respond in vivo was probably caused by deficient T cell proliferation in response to the viral vaccine and by inefficient cytotoxicity. The second approach was to inhibit the Nck–CD3ε interaction by specifically targeting the SH3.1 domain of Nck. This approach confirmed the importance of this interaction for T cell activation. Accordingly, we generated a peptide inhibitor with an extremely high affinity for the SH3.1 domain in cell-free systems (Kd < 1 nM), although with considerably reduced potency in intact cells, probably because of poor entry.

An important role for Nck during mature T cell function has been demonstrated in double knockout mice lacking Nck1 in all tissues and conditionally lacking Nck2 in T cells only (16, 17). 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 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 (16, 17), this article highlights the importance of Nck in T cell activation by binding to CD3ε, and therefore being directly recruited to the TCR. Furthermore, our data with the 11R085 peptide pinpoints the SH3.1 domain of Nck, the one binding CD3ε, as a fundamental domain for T cell activation.

In summary, our results demonstrate that the Nck–CD3ε interaction plays an important role in TCR signaling, rearrangement of the actin cytoskeleton, and T cell activation. Disruption of Nck recruitment to the TCR results in an impaired T cell Ag response in vitro and prevents the T cell response to a model tumor Ag in vivo. Furthermore, pretreatment with a peptide that prevents Nck–CD3ε interaction inhibits T cell activation in vitro and in vivo, suggesting that this interaction might be an interesting target for the development of immunosuppressive or immunomodulatory drugs. Indeed, KI-PRS mice present a delayed onset of neurological symptoms after vaccination with a self MOG peptide. Of note, the PRS mutation and the peptide inhibitor 11R085 inhibited but did not block T cell responses to Ag, suggesting that the Nck–CD3ε interaction modulates the TCR signal rather than being absolutely required. It will be interesting to determine whether such a modulating role could make it more attractive to selectively inhibit some, but not all, T cell responses in models of autoimmunity.

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**Disclosures**

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**References**

REGULATION OF TCR SIGNALING BY Nck


