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Fine Tuning of the Threshold of T Cell Selection by the Nck Adapters

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Thymic selection shapes the T cell repertoire to ensure maximal antigenic coverage against pathogens while preventing autoimmunity. Recognition of self-peptides in the context of peptide-MHC complexes by the TCR is central to this process, which remains partially understood at the molecular level. In this study we provide genetic evidence that the Nck adapter proteins are essential for thymic selection. In vivo Nck deletion resulted in a reduction of the thymic cellularity, defective positive selection of low-avidity T cells, and impaired deletion of thymocytes engaged by low-potency stimuli. Nck-deficient thymocytes were characterized by reduced ERK activation, particularly pronounced in mature single positive thymocytes. Taken together, our findings identify a crucial role for the Nck adapters in enhancing TCR signal strength, thereby fine-tuning the threshold of thymocyte selection and shaping the preimmune T cell repertoire. *The Journal of Immunology, 2010, 185: 000–000.

Thymic ontogeny leads to the generation of functionally competent T cells through somatic rearrangement of the TCRs and TCRβ loci and selective maturation of the thymocytes, whose TCR interacts with self peptide-MHC (pMHC) complexes within a defined range of avidity. Thymic development proceeds through discrete stages, which can be differentiated based on the configuration of the TCRα and TCRβ loci and of the expression of the CD4 and CD8 coreceptors. First, the productive rearrangement of the TCRβ locus in CD4+ CD8+ (double negative; DN) thymocytes leads to expression of the rearranged TCRβ-chain with the invariant pre-β-chain. The concerted actions of the CD3 signalosome and the Src family and Syk/ZAP-70 tyrosine kinases (1) are then required for progression to the CD4+ CD8+ (double positive; DP) stage and expansion through a series of rapid cell divisions. During the DP stage, rearrangement of the TCRα-chain results in the expression of the mature TCRαβ complex. The TCR ability to discriminate between small amino acid variations in antigenic peptides controls repertoire selection and induction of self-tolerance (2). Only TCR–pMHC interactions within a relatively narrow range of functional avidity lead to thymocyte differentiation into mature T cells. In contrast, weak TCR–pMHC binding causes “death by neglect,” while high-avidity interactions result in clonal deletion, anergy, clonal diversion, or receptor editing, a series of events collectively responsible for the induction of central tolerance.

The role of the Nck adapters in T cell development has been inferred from their interaction with key players in TCR signaling, such as SLP-76 and CD3ε, but has not been genetically demonstrated. The Nck adapter family (3) comprises two members, Nck1 and Nck2, which are highly homologous and structurally characterized by one Src homology (SH)2 domain and three SH3 domains. Their wide tissue distribution includes developing and mature T cells. In a variety of cell types, the Nck adapters act as a molecular link between phosphotyrosine signals and regulation of the actin cytoskeleton (4–6), such as the Wiskott-Aldrich syndrome protein (WASP), VAV1, and the p21 activated kinase (7). In T cells, biochemical (7) and live colocalization (8) analyses in transfected Jurkat T cells support the hypothesis that Nck recruitment to the immune synapse is phosphorylation dependent and is mediated by binding of its SH2 domain to phospho–SLP-76. Upon formation of a trimolecular complex, Nck–SLP-76–Vav1, Nck would recruit WASP, thereby promoting actin cytoskeletal rearrangement (9). Pull-down experiments in resting and activated Jurkat T cells suggest that the first SH3 domain of Nck binds to the proline rich sequence (PRS) of CD3ε upon TCR engagement. In this context, a “closed” conformation of CD3ε, masking the PRS and preventing its interaction with Nck (10), would account for the inability of Nck to bind to CD3ε from resting T cells. Biochemical and structural studies have shown that a noncanonical PxxDY170 region juxtaposed to the PRS may also be crucial for Nck–CD3ε interaction and functionally relevant as Nck binding prevented Fyn-mediated Y170 phosphorylation, and Y170 phosphorylation resulted in Nck displacement (11). Notably,
the characterization of mice deprived of the CD3ε.PRS region has shown no effects on the development and function of the polyclonal T cell repertoire (12), whereas alterations in the development (13) and responsiveness (14) of CD3ε.PRS mutant T cells have been reported in CD3ε.PRS mutant mice expressing different transgenic TCRs. The Nck adapters are essential to enhance mature T cell sensitivity and reactivity to TCR-mediated stimulation (15). However, their role in T cell development is unknown. To address this issue, we used a conditional genetic system (5, 16, 17) in combination with two TCR transgenic models of different avidity for endogenous ligands. Our findings indicate that the Nck adapters are essential for positive and negative selection upon TCR engagement by low-avidity ligands but are not required for selection of high-avidity T cells. Nck-deficient thymocytes exhibited enhanced CD3ε phosphorylation and impaired ERK activation. Taken together, our findings provide genetic evidence that the Nck adapters regulate the threshold of thymic selection, thereby contributing to shape the preimmune T cell repertoire.

Materials and Methods

Mice

Nck1<sup>1<sup>Cre</sup>/Cre</sup> (16) and Nck2<sup>flx/flx</sup> (5) mice were crossed to Lck-Cre mice (17) (a generous gift of Dr. J. Marth, University of California at San Diego, La Jolla, CA). HY-TCR (18), P14-TCR (19), and RAG2<sup>2<sup>−/−</sup></sup> mice were kindly provided by Drs. B. Rocha (INSERM), A. Freitass (Institut Pasteur, Paris, France), and S. Eznine (INSERM), respectively. Nck1<sup>1<sup>Cre</sup>/Cre</sup>Nck2<sup>flx/flx</sup>Lck-Cre (Nck.T<sup>−/−</sup>) mice were back-crossed to C57BL/6 mice three to four times and crossed to RAG2<sup>2<sup>−/−</sup></sup> HY-TCR or P14-TCR mice to obtain HY.Nck.T<sup>−/−</sup> and P14-Nck.T<sup>−/−</sup> mutant mice. Given the high number of mutations (3-5) and the difficulty to obtain mutants and wild-type mice in the same litter, an initial set of experiments was conducted to establish the equivalence of Lck-Cre<sup>Nck1</sup>/Cre<sup>Nck2</sup>/Nck2<sup>flx/flx</sup>/Nck1<sup>−/−</sup>/Cre<sup>Nck2</sup> littermates, obtained from Lck-Cre<sup>Nck1</sup>/Cre<sup>Nck2</sup>/Nck2<sup>flx/flx</sup> intercrossing, and Lck-Cre<sup>Nck2</sup>/Nck2<sup>−/−</sup> mice, obtained from Lck-Cre<sup>Nck</sup>+/Cre<sup>Nck2</sup>+/Cre<sup>Nck2</sup> intercrossing (Supplemental Fig. 1). Notably, Nck.T<sup>−/−</sup> and Nck.T<sup>−/−</sup> mice on the C57BL/6.N10 background retained their phenotypic differences.

Flow cytometry

The Abs used are listed in Supplemental Table I. Surface staining was carried out according to standard procedures. DN thymocytes were defined as CD45.2<sup>−/−</sup>CD3<sup>−/−</sup>CD4<sup>−/−</sup>CD8<sup>−/−</sup>CD25<sup>−/−</sup>CD44<sup>−/−</sup>CD19<sup>−/−</sup> (NK1.1<sup>−/−</sup> CD11b<sup>−/−</sup> CD11c<sup>−/−</sup> CD19<sup>−/−</sup> Ter119<sup>−/−</sup>) thymocytes. For CD3ε intraacellular staining, Cytofix/Cytoperm and Perm/Wash Buffer (BD Biosciences, Franklin Lakes, NJ) were used according to the manufacturer’s instructions. Four-color FACS samples were acquired on a FACSCalibur (BD Biosciences); 8-color FACS samples were acquired on a BD LSRII (BD Biosciences) using the BD FACS Diva acquisition software. Data analysis was performed using the FlowJo software (Tree Star, Ashland, OR).

In vitro thymocyte stimulation

Thymocytes from Nck.T<sup>−/−</sup> and Nck.T<sup>−/−</sup> mice (4 × 10<sup>6</sup>/well) were cocultured with irradiated (30 Gy) T cell-depleted spleen cells from C57BL/6 mice (4 × 10<sup>6</sup>/well) in the presence of graded doses of the Staphylococcus aureus enterotoxin A (SEA). Alternatively, thymocytes (10<sup>6</sup>/well) were cultured in the presence of graded doses of plate-bound εCD3ε and αCD28 Abs. After 18 h of culture in a humidified 37°C, 5% CO<sub>2</sub> incubator, cells were harvested, stained for extracellular markers, and subsequently incubated with annexin V/propidium iodide, according to standard procedures. The amount of specific apoptosis was calculated by the following equation: (CD3/CD28-induced apoptosis − spontaneous apoptosis)/100 − spontaneous apoptosis)%.

RT-PCR analysis

Total RNA was extracted with the RNaseasy kit (Qiagen, Hilden, Germany) and reverse transcribed with the ImProm-II Reverse Transcription System kit (Promega, Madison, WI) according to the manufacturer’s instructions. GAPDH amplification of graded doses of each cDNA sample was used to quantify and “normalize” their levels, using the ImageJ software. After normalization, equal cDNA amounts were amplified using Nck1- and Nck2-specific primers.

Bone marrow chimeras

Bone marrow (BM) cells were obtained by flushing femurs and tibias of donor mice. After magnetic T cell depletion, 12 × 10<sup>6</sup> to 13 × 10<sup>6</sup> BM cells were injected i.v. into irradiated (4.5 Gy) RAG2<sup>2<sup>−/−</sup></sup> recipients. Mice were treated with antibiotic for 8 d, starting one day before BM transfer. BM chimeras were analyzed 6–8 wk after transfer.

Immunoprecipitation and Western blot analysis

Freshly isolated thymocytes were incubated with biotinylated α-CD3ε and α-CD8α (10 μg/ml) for 15 min on ice. After washing with cold medium, cells were prewarmed for 1 min at 37°C and cross-linked with prewarmed streptavidin (20 μg/ml). Lysis was performed in cold buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate freshly supplemented with protease inhibitors (Roche, Mannheim, Germany), 1 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, and 1 mM okadaic acid. The cytosolic fraction was assessed for protein content (BCA protein assay kit; Pierce, Rockford, IL). Equal protein amounts were immunoprecipitated with the indicated Abs. For subsequent Western blotting, immunoprecipitates were washed three times with lysis buffer. Immune complexes were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore, Billerica, MA), and probed with biotinylated primary Abs, followed by streptavidin-HRP (Millipore). Whole-cell lysates resolved by SDS-PAGE were immunoblotted with Abs, including anti-phospho-linker of activated T cells (LAT) (Tyr191), and anti–phospho-ERK (Thr202/Tyr204) antibodies (E10; Cell Signaling, Danvers, MA). Detection was carried out by chemiluminescence (Pierce, Rockford, IL). Membranes were stripped and reprobed for β-actin (AC-15; Sigma, St. Louis, MO) or ERK1/2 (rabbit polyclonal Ab; Cell Signaling).

Statistics

All two-group comparisons were performed with the exact Wilcoxon rank sum test using SAS version 9.1 (SAS Institute, Cary, NC). In the graphs of Figs. 1, 2, and 3, *p < 0.05 (significant) and **p < 0.001 (highly significant).

Results

Targeted deletion of the Nck adapters in developing and mature thymocytes

Deletion of both Nck adapters in the T cell compartment was obtained using a conditional genetic approach. Nck1<sup>1<sup>Cre</sup>/Cre</sup> (16) and Nck2<sup>flx/flx</sup> (5) mice were crossed to Lck-Cre transgenic mice (17) to obtain Lck.Cre × Nck1<sup>−/−</sup>/Nck2<sup>flx/flx</sup> mice, which will be referred to as Nck.T<sup>−/−</sup> throughout the paper, and Lck.Cre × Nck1<sup>+/+</sup>Nck2<sup>−/−</sup> controls, which will be referred to as Nck.T<sup>+/−</sup> mice (Supplemental Fig. 1). The expression of the Cre recombinase (Supplemental Fig. 2) and the deletion efficiency of the floxed Nck2 alleles (Supplemental Fig. 3) were analyzed at different stages of T cell development. Cre expression was low in immature thymocytes (DN1/DN2) and increased throughout thymic ontogeny to reach virtually 100% in mature thymocytes. At the mRNA level, expression of Nck2 was readily identified at the DN stage, detectable at low levels in DP thymocytes, and absent in mature single positive (SP) thymocytes (Supplemental Fig. 3A). At the protein level, the Nck proteins were undetectable in the thymic thymocytes of Nck.T<sup>−/−</sup> mice (Supplemental Fig. 3B), indicating that the residual expression of Nck2 was minimal and/or restricted to a minute thymocyte fraction.

Reduced thymic cellularity in Nck.T<sup>−/−</sup> mice

The phenotypic analysis of Nck.T<sup>−/−</sup> mice revealed a 3- to 4-fold reduction of the thymic cellularity (Fig. 1A) compared with that of age-matched Nck.T<sup>+/+</sup> mice. The thymic architecture and the levels of in vivo apoptotic cells remained unaffected by Nck deletion, as assessed by histological analysis and TUNEL assay (Supplemental Fig. 4). The decreased thymic cellularity in Nck.T<sup>−/−</sup> mice was mainly accounted for by a reduction in DP cells (3- to 4-fold) in the absence of significant alterations of the CD4/CD8 profile (Fig. 1C). Notably, DN thymocytes were less severely reduced in Nck.T<sup>−/−</sup> mice compared with their Nck.T<sup>+/−</sup> counterparts. Notably, Nck.T<sup>−/−</sup> mice remain almost normal in splenic cellularity (Supplemental Fig. 5).
Reduced thymic cellularity and partial block at the β-checkpoint in Nck.T−/− mice. A, The thymic cellularity was reduced in 4- to 5-wk-old Nck.T−/− mice (△, n = 13) compared with that of Nck.T+/+ controls (○, n = 14; p < 0.00001), CD4+CD8− DP (△, p < 0.00001) and CD4−CD8+ (SP4; p < 0.001) thymocytes were strongly reduced in Nck.T−/− mice compared with those of Nck.T+/+ mice. *p < 0.05; **p < 0.001. B, The number of CD8low TCRlow ISP thymocytes was significantly reduced in Nck.T−/− mice compared with that of wild-type controls (p = 0.0001). **p < 0.001. C, The CD4/CD8 profile remained unaltered in the absence of Nck. D and E, The percentage of Lin−CD45.2+CD4−CD25+CD44+ (DN1), CD25−CD44+ (DN2), CD25+CD44+ (DN3), and CD25−CD44− (DN4), which correspond with the phases of TCRβ gene rearrangement (20). CD27 is upregulated after TCRβ selection, in the transition from the DN3a to DN3b stage (21). In Nck.T−/− mice, we observed an increase in Lin−CD45.2+CD25+CD44− (DN3) thymocytes (Fig. 1D) associated with a reduction in post-β selection CD27high cells (Fig. 1E). As maturation to the DP stage is associated with CD8 upregulation and thymocyte proliferation, we next investigated the characteristics of CD8low TCRlow immature single-positive (ISP) thymocytes (Supplemental Fig. 6). Total CD8low thymocytes (Supplemental Fig. 6C) and ISP thymocytes (Fig. 1B) were significantly reduced in Nck.T−/− mice. However, the intrinsic proliferative capacity of ISP thymocytes, similar to that of other thymic subsets, was not affected by Nck deletion (Supplemental Fig. 7). Thus, our findings are compatible with the hypothesis that the Nck adapters influence TCRβ selection and the progression to the DP stage.

Nck deletion results in TCR overexpression on DP thymocytes

The Nck adapters have been implicated in the regulation of the surface levels of the TCR (11, 22, 23). The levels of TCR expression are finely regulated at the DP stage (24), as both positive and negative selection depend on the strength of TCR signaling (25). Notably, we found that the surface levels of TCRβ (Fig. 1F, top) and CD3ε (Fig. 1G, top) expression were increased in DP cells from Nck.T−/− mice compared with those of wild-type controls. However, loss of Nck function did not alter the size of the total CD3ε pool in DP thymocytes, as assessed by intracellular staining (Fig. 1G, bottom). Upregulation of CD5 and CD69 has been linked to positive selection (26). Notably, both markers were prematurely increased on Nck-deficient DP thymocytes (Fig. 1F, top). However, no obvious phenotypic alterations were found in mature SP thymocytes from Nck.T−/− mice (Fig. 1F, bottom), which also exhibited comparable VB-chain frequency and distribution to Nck.T+/+ thymocytes (Supplemental Fig. 8). Taken together, the phenotypic association of altered TCR and CD5 expression on Nck-deficient DP thymocytes points toward a potential dysregulation in TCR signal strength with possible effects on thymic selection.

The Nck adapters are required for deletion of thymocytes expressing a “weak” TCR

To assess the impact of Nck on thymocyte selection, we used the HY-TCR transgenic model. HY-TCR T cells recognize a peptide derived from the male minor histocompatibility Ag HY in the context of H-2Dβ (18). The presence of the target Ag in the thymi of male mice results in HY-TCR thymocyte deletion. If, as in the current study, the transgenic HY–TCRα-chain and HY–TCRβ-chain are concomitantly expressed at the DN stage, negative selection occurs in the thymic cortex, prior to the DP stage (18). In
contrast, if expression of the transgenic TCRα-chain is more physiologically induced at the DP stage, HY-TCR T cells are eliminated at the single-positive or medullary stage (27). One of the key features of the HY-TCR is its relatively low affinity for the HY peptide–H2-D^b complex, which is responsible for its exquisite sensitivity to alterations of the TCR signal strength (28). The “weakness” of the HY-TCR leads to inefficient deletion and peripheral “leakage” of HY-TCR T cells, which remain anergic and express reduced levels of the CD8αβ coreceptor. Unlike non-TCR-transgenic Nck.T^-/- mice, HY-TCR RAG^-/-Nck.T^-/- males exhibited a thymic cellularity comparable with that of wild-type mice (Supplemental Fig. 9A). Their CD4/CD8 profile was characterized by a marked increase in DP thymocytes (Fig. 2A). In the periphery, Nck-deficient HY-TCR T cells were reduced (Fig. 2B) and exhibited increased surface levels of the CD8αβ coreceptor (Fig. 2C, left). Notably, the increase in CD8 expression on Nck-deficient mature T cells never exceeded the maximal levels of male wild-type T cells, remaining lower than that of HY-TCR T cells in female mice (Fig. 2C, right). Taken together, these alterations point toward a crucial role for the Nck adapters in shaping the selection and phenotypic profile of HY-TCR T cells. To better understand the role of the Nck adapters in the selection of the polyclonal repertoire, thymocytes from Nck.T^-/- and Nck.T^+^/+ mice were stimulated in vitro with graded doses of SEA or of CD3/CD28. Both treatments induce apoptosis of DP thymocytes upon TCR engagement, which, in the case of SEA, is selective for thymocytes expressing Vβ11 (29). Apoptosis of Nck-deficient Vβ11^+^ DP thymocytes was less efficient than that of Nck.T^-/+^ thymocytes at low but not high doses of SEA (Fig. 2D, bottom). No deletion was observed in the case of DP thymocytes expressing Vβ8.1/2 (Fig. 2D, top). Similarly, Nck deletion conferred increased resistance to the induction of apoptosis by low doses of CD3/CD28 but did not alter the response to high doses of CD3/CD28 (Fig. 2E), as assessed by annexin V/propidium iodide staining. Taken together, these findings indicate that in the absence of Nck, DP thymocytes lose sensitivity to weak TCR-mediated stimulation and suggest that Nck is required for thymocyte deletion by low-affinity ligands.

**Impaired negative selection does not trigger spontaneous autoimmunity in Nck.T^-/- mice**

We next investigated whether HY-TCR T cells that had escaped negative selection in HY-TCR RAG^-/-Nck.T^-/- males induced spontaneous autoimmunity. HY-TCR RAG^-/-Nck.T^-/- males did not lose weight (Supplemental Fig. 9B) or exhibit any signs of runting syndrome. Histologically, there were no signs of immune

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**FIGURE 2.** The Nck adapters are essential for negative selection of HY-TCR T cells. A, The percentage of DP thymocytes was markedly increased in HY-TCR RAG^-/-Nck.T^-/- males (right, n = 5) compared with that of HY-TCR RAG^-/-Nck.T^+^/+ controls (left, n = 5). B, In the spleen, the percentage of HY-TCR T cells, as assessed by staining with the transgenic TCRα (T3.70) and TCRβ (Vβ8.1/2)-chains, was reduced in HY-TCR RAG^-/-Nck.T^-/- males compared with that of wild-type controls. C, In HY-TCR RAG^-/-Nck.T^-/- males, HY-TCR T cells exhibited a less profound downregulation of the CD8αβ coreceptor than that in HY-TCR RAG^-/-Nck.T^+^/+ males. The expression of CD8αβ in HY-TCR RAG^-/-Nck.T^-/- males (right) is shown for comparison. D, Upon in vitro stimulation with graded doses of SEA, thymocytes from Nck.T^-/- and Nck.T^+^/+ mice were harvested and stained with Abs specific for CD4, CD8, Vβ11 (bottom), or Vβ8.1/2 (top). SEA induces apoptosis of thymocytes expressing Vβ11 but has no effect on thymocytes expressing Vβ8.1/2. Deletion of Vβ11^+^ DP thymocytes from Nck.T^-/- mice was less efficient than deletion of those from Nck.T^+^/+ mice at low doses of SEA (0.1 μg/ml, p = 0.049; 0.01 μg/ml, p = 0.049). In contrast, no differences were found in the levels of Vβ11^+^ deletion at high SEA concentrations (10 μg/ml, p = 0.079). *p < 0.05. The levels of Vβ8.1/2^+^ DP thymocytes remained unaltered upon exposure to SEA. Cumulative data from four independent experiments are shown. E, Thymocytes from Nck.T^-/- (n = 6) and Nck.T^+^/+ (n = 7) mice were stimulated with graded doses of plate-bound CD3/CD28 and analyzed by flow cytometry after 18 h of culture. As assessed by annexin V/propidium iodide staining, the levels of specific apoptosis were reduced in DP thymocytes from Nck.T^-/- mice at low (p = 0.0066) but not high doses of CD3/CD28. *p < 0.05. Cumulative data from three independent experiments are shown.
infiltration or tissue damage in the digestive tract, lungs, kidneys, muscles, brain, or testes of HY-TCR RAG−/− Nck.T−/− males (data not shown). However, a mild perivascular infiltration in the absence of tissue damage was found in the liver of HY-TCR RAG−/− Nck.T−/− males, irrespective of their gender (Supplemental Fig. 9C, 9D). The similarity of the liver infiltrate in male and female HY-TCR RAG−/−Nck.T−/− mice and its benign and nondestructive characteristics argue against its autoimmune origin. Thus, our findings rule out the hypothesis that Nck deletion interferes with self-tolerance in HY-TCR RAG−/−Nck.T−/− males.

**Nck is required for positive selection of low-avidity HY-TCR T cells**

In HY-TCR transgenic females, the absence of HY expression allows maturation of HY-TCR T cells. In comparison with wild-type mice, HY-TCR RAG−/−Nck.T−/− females exhibited a 3- to 5-fold reduction in the percentage of mature SP8 thymocytes (Fig. 3A), associated with TCR upregulation on DP thymocytes (Fig. 3B), and a reduction in the absolute number of splenic HY-TCR T cells (Fig. 3C). Thus, Nck is required for positive selection of HY-TCR T cells.

We reasoned that if the HY-TCR had become “unfit” for positive selection in the absence of Nck, rearrangement of endogenous TCRα-chains may produce thymocytes with higher TCR avidity for endogenous ligands and better chances for positive selection. To test this hypothesis, we used RAG-sufficient HY-TCR Nck.T−/− and HY-TCR Nck.T+/+ females and compared the expression of the transgenic TCRα (TCRαT) and TCRβ (TCRβ2) -chains on mature SP8 thymocytes. Because the TCRα-chain and TCRβ-chain form a heterodimer, selective downregulation of TCRαT without significant alterations of TCRβ2 expression indicates TCRαT pairing with an endogenously rearranged TCRα-chain (TCRαE). Our findings indicate a marked reduction in mature HY-TCR SP8 cells in the absence of Nck (Fig. 3D and Supplemental Fig. 10A, 10B) and a relative increase in the percentage of SP8 thymocytes expressing endogenously rearranged TCRα-chains (Fig. 3E and Supplemental Fig. 10A). Taken together, our data are compatible with the hypothesis that Nck deletion results in a reduction in the intrinsic “fitness” of the HY-TCR for positive selection, partially compensated by receptor editing.

**The thymic defects of HY-TCR Nck.T−/− mice are intrinsic to hematopoietic cells**

We next sought to define the cellular origin of the selection defects observed in Nck.T−/− mice. Because Nck1 is systemically deleted and potential alterations of thyman epithelial cells may alter thymic selection, we generated BM chimeras in sex-matched RAG2−/− recipients. The phenotypic analysis of the chimeras revealed that their phenotype faithfully reproduced the defects of negative (Fig. 4A) and positive (Fig. 4B) selection observed in the HY-TCR RAG−/−Nck.T−/− donors. Thus, the thymic selection defects observed in HY-TCR Nck.T−/− mice are due to Nck-dependent alterations intrinsic to hematopoietic cells.

**Nck is dispensable for the selection of high-avidity T cells**

Several lines of evidence suggest that the T cells have different sensitivity to mutations of the TCR signaling apparatus (28), as a function of their avidity for the selecting of self-pMHC complexes. Thus, we investigated the impact of Nck deletion on the ontogeny of high-avidity P14-TCR T cells (19). The thymic cellularity and the CD4/CD8 profile of P14-TCR Nck.T−/− mice remained unaffected by in vivo Nck deletion (Fig. 5A). Notably, the early phases of T cell development were impaired in P14-TCR Nck.T−/− females (Fig. 5D) but were comparable with those of wild-type mice in the SP8 fraction (data not shown). Within the SP8 mature thymocyte fraction, the percentage of cells expressing endogenously rearranged TCRα-chains was not increased in the absence of Nck (Fig. 5E). Taken together, these findings indicate that Nck is not required for positive selection of high-avidity T cells.

**Reduced ERK phosphorylation in Nck-deficient thymocytes**

The CD3 ε.PRS is reportedly accessible to Nck upon a conformational change induced by TCR engagement (10). The APA1/1 mAb specifically recognizes the CD3ε.PRS and has been used to detect the CD3ε conformer accessible to Nck binding (30). Freshly isolated thymocytes revealed a low but significant level of APA1/1

![FIGURE 3](http://www.jimmunol.org/) Requirement of Nck for positive selection of low-avidity HY-TCR T cells. A. The percentage of SP8 cells was strongly reduced in HY-TCR RAG−/− Nck.T+/+ females (right, n = 3) compared with that of HY-TCR RAG−/− Nck.T−/− (left, n = 2), indicating impaired positive selection. B. Expression of the transgenic TCRα (T3.70) and TCRβ (Vβ8.1/2)-chains was increased on Nck.T−/− DP thymocytes (dotted line) compared with that of Nck.T+/+ DP cells (solid line). C. The number of splenic CD8+ HY-TCR+ T cells was markedly reduced in HY-TCR RAG−/− Nck.T−/− females (n = 7) compared with that of wild-type controls (n = 6; p = 0.013). D. The percentage of SP8 thymocytes was lower in HY-TCR RAG−/−Nck.T−/− females (n = 5) compared with that of HY-TCR RAG−/−Nck.T+/+ females (n = 5). E. Association of the transgenic TCRβ-chain with endogenously rearranged TCRα-chains was associated with downregulation of the transgenic TCRα (T3.70)-chain. HY-TCR RAG−/−Nck.T−/− females were characterized by an increased percentage of SP8 cells expressing low levels of the transgenic TCRα-chain. Percentages and absolute numbers of SP8, SP8 TCRαβ−, and TCRαβ−β− are shown in Supplemental Fig. 10.
Discussion

The current study provides genetic evidence for a crucial role of the Nck adapters in T cell ontogeny using the combination of a conditional genetic system with two TCR transgenic models. Nck deletion resulted in a series of phenotypic and functional alterations including 1) a relative increase in DN3 thymocytes; 2) TCR overexpression on DP thymocytes; and 3) impairment of positive and negative selection by ligands of low antigenic potency.

In both TCR transgenic and non-transgenic models analyzed in this study, the percentage of DN3 thymocytes was increased in the absence of Nck, suggesting the involvement of these adapters in TCRβ-selection. Further analysis revealed a relative decline in the CD27high DN3 fraction and a drastic reduction in ISP thymocytes, which point toward a role for Nck in TCRβ selection. Additional alterations induced by Nck in the early phases of T cell development may not be apparent in our conditional genetic model because Nck is not required for CD3ε.PRS “exposure.” The earliest step after TCR engagement is the activation of Src protein tyrosine kinases (Lck and Fyn), leading to phosphorylation of the CD3 ITAMs. Recruitment of ZAP-70 follows, leading to a cascade of events, including phosphorylation of the LAT adapter (31). Nck deletion was associated with an apparent increase in CD3ε phosphorylation under resting and stimulating conditions (Supplemental Fig. 12), which did not result in any alterations in the levels and kinetics of LAT phosphorylation (Fig. 6A and Supplemental Fig. 13A). Upon TCR ligation, binding of diacylglycerol to the Ras activators RasGRP (31) leads to activation of the Ras/MAPK pathway, which is essential for thymic selection (2). Activated thymocytes from Nck.T−/− mice exhibited lower levels of ERK1/2 phosphorylation compared with those of Nck.T+/+ controls, as assessed by immunoblotting with phospho-specific Abs recognizing the active forms of ERK1 and ERK2 (Fig. 6B and Supplemental Fig. 13B, 13C). Intracellular staining also revealed a reduction in the levels of ERK1/2 phosphorylation, which was particularly pronounced in mature SP8 cells (Fig. 6C). Taken together, our data suggest the involvement of the Nck adapters at multiple levels of the TCR signaling cascade and indicate their essential role in ERK1/2 activation.

FIGURE 4. Hematopoietic origin of the thymic defects of HY-TCR Nck.T−/− mice. BM chimeras were generated by injection of BM from HY-TCR RAG−/− (HYRAG) Nck.T−/− or Nck.T−/− mice into irradiated (4.5 Gy) sex-matched RAG−/− (HYRAG) Nck.T+/+ or Nck.T−/− mice. BM chimeras were generated by injection of BM from HY-TCR RAG−/− (HYRAG) Nck.T−/− or Nck.T−/− mice into irradiated (4.5 Gy) sex-matched RAG−/− (HYRAG) Nck.T+/+ or Nck.T−/− mice. A, In male BM chimeras, the expression of CD4/CD8−-chain was comparable in the presence/absence of Nck. Data are representative of at least three independent experiments. B, Female BM chimeras exhibited a reduction in the percentage of SP8, similar to their BM donors (Nck.T−/−, n = 13; top; Nck.T−/−, n = 10, bottom).
Altered signaling in Nck-deficient thymocytes. A, LAT phosphorylation was assessed by Western blot analysis of lysates from resting and CD3ε/CD8α-stimulated thymocytes from Nck.T+/+ and Nck.T−/− mice. Nck-deficient thymocytes did not exhibit any alterations in the levels and kinetics of LAT phosphorylation (see also Supplemental Fig. 1A). B, ERK phosphorylation was decreased upon TCR engagement in the absence of Nck as assessed by immunoblotting on thymic lysates from Nck.T+/+ and Nck.T−/− mice. The relative signal intensity is shown in Supplemental Fig. 1B and 1C. C, The levels of ERK1/2 phosphorylation were investigated on DP (top) and SP8 (bottom) thymocytes from Nck.T+/+ and Nck.T−/− mice under resting (left) and stimulating (right) conditions by intracellular staining and flow cytometric analysis. All data are representative of two to three independent experiments.

Indeed, one of the key observations of our study is that the Nck adapters are selectively required for positive selection of low-avidity HY-TCR T cells but are dispensable for the selection of high-avidity P14-TCR T cells. Such finding parallels the observation that loss of Nck function selectively interferes with negative selection of low-avidity HY-TCR T cells and specifically impairs thymocyte deletion by weak, but not strong, TCR-mediated stimuli. These data point toward a crucial role of Nck in fine-tuning TCR sensitivity, enhancing thymocyte responses to weak TCR-mediated stimuli. Such effect is reminiscent of the activity of miR181a (39, 40), a powerful modulator of T cell sensitivity; however, a link between the two factors remains to be established. In this scenario, the reduction of DP thymocytes may simply result from death by neglect of DPs expressing low-avidity TCRs deprived of survival and differentiation signals in the absence of Nck. Because TCR rearrangement is likely to produce a large fraction of TCRs with suboptimal avidity for self-pMHC ligands, one may predict that minute changes in TCR signal strength may deeply affect survival of DP thymocytes and have a major impact on thymic cellularity. The increased expression of the CD8αβ coreceptor on HY-TCR T cell from transgenic males may be linked to its ability to enhance TCR signal strength (41), thereby optimizing maintenance of HY-TCR in the periphery. Although in vivo tolerance induction is associated with CD8αβ downmodulation in HY-TCR transgenic males, we found no evidence of autoimmunity in HY-TCR Nck.T−/− transgenic males, and HY-TCR T cells remained anergic in vitro (data not shown). This effect may be explained by a “resetting” of the threshold of TCR responsiveness in the absence of Nck. Alternatively, the reactivity of HY-TCR T cells in male transgenic mice may fall into a state of hyporesponsiveness, similar to that described in the polyclonal repertoire (15).

The selective impairment of low-avidity TCR selection is reminiscent of the phenotype of CD3ε,ITAM (43), TCRζ,ITAMs (28, 44). To this end, it is interesting that CD3ε phosphorylation is increased in Nck-deficient thymocytes under both resting and stimulating conditions. As the total CD3ε cell pool was not affected by Nck deletion, such effect is unlikely to be induced by CD3ε overexpression on Nck-deficient DP thymocytes. Notably, it has been hypothesized that the Nck-binding CD3ε,PR SpxD1 domain (11, 45) may work as a molecular switch, binding to SH3 domain-containing proteins, such as Nck, under resting conditions and to SH2 domain-containing proteins upon activation-induced phosphorylation (46). In this scenario, loss of Nck function may further perturb the phosphorylation state of CD3ε, thereby altering the threshold of TCR responsiveness. The altered ERK activation observed in the absence of Nck is likely to play a crucial role in the phenotype of Nck.T−/− mice, given the importance of ERK phosphorylation in positive and negative selection (2, 25). As a reduction in TCR-mediated ERK phosphorylation is a hallmark of clonal anergy (47), the strong reduction of ERK phosphorylation in SP thymocytes and peripheral T cells from Nck.T−/− mice (27) raises the question of the
potential link between Nck-deficient T cell hyporesponsiveness and anergy. Notably, a reduction in ERK phosphorylation was not observed in CD3e.PRS knock-in mice (13) but was present in WASP–/– (32) and Vav1−/− (33) mice, suggesting that such defect may result from altered WASP activation and formation of the Vav–SLP-76–Nck complex.

Taken together, our data suggest that the Nck adapters orchestrate a mechanism that enhances TCR signal potency in developing T cells and is decisive for the fate of thymocytes at the threshold of positive and negative selection. Because fine-tuning of TCR sensitivity to endogenous ligands (48) shapes the diversity, responsiveness, and relative size of the T cell clones composing the preimmune repertoire, the impact of the Nck adapters in T cell responses to incoming pathogens and autoimmune reactions warrants further investigation.

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Disclosures

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