Nck adaptor proteins modulate differentiation and effector function of T cells

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ABSTRACT
Understanding the molecular mechanisms regulating T cell reactivity is required for successful reprogramming of immune responses in medical conditions, characterized by dysfunctions of the immune system. Nck proteins are cytoplasmic adaptors mediating diverse cellular functions, including TCR signaling. By enhancing TCR signal strength, Nck proteins influence thymic selection and regulate the size and sensitivity of the peripheral T cell repertoire. Here, we investigated the contribution of Nck proteins to CD4+ T cell differentiation and effector function using Nck.T−/−mice. Impaired GC formation and reduced Tfh were observed in Nck.T−/−mice after immunization with T cell-dependent antigens. Th2/Tfh-related cytokines, such as IL-4, IL-10, and IL-21, were decreased in Nck.T−/−mice as well. Moreover, an increased susceptibility to cell death of Tfh cells in Nck.T−/−mice was associated with decreased levels of Akt phosphorylation. As a result of this dysregulation in Tfh cells of Nck.T−/−mice, we found impaired production and affinity maturation of antibodies against T cell-dependent antigens. Thus, Nck proteins not only participate in thymic selection and generation of the peripheral T cell repertoire but also are involved in the differentiation and effector functions of CD4+ T cells.


Introduction
Regulation of TCR signal strength is central to T cell development and activation. Finely tuned TCR signaling has an essential role in positive and negative selection processes in the thymus and defines the lineage fate of αβ/γδ T cells [1-3]. In the periphery, besides homeostatic maintenance [4], TCR signaling controls activation [5], differentiation [6-10], and memory formation [11, 12] of T cells during immune responses. For example, the strength and temporal length of TCR signaling influence the balance between Th1 and Th2 CD4+ T cell differentiation [10, 13]. Strong TCR signaling supports the Th1 fate with robust production of IFNγ [9]. In contrast, weak TCR signaling can favor the differentiation to the Th17 phenotype [8]. The role of T cell-extrinsic factors, such as the avidity of peptide-MHC complexes, in thymic selection, as well as in vivo T cell activation and differentiation, has been clearly established [14-16]. More recently, T cell-intrinsic mechanisms, modulating TCR signal strength, have also been recognized as a major factor orienting the functional outcome of T cell stimulation [5].

Nck adaptor proteins are key players to enhance and tune TCR signaling strength [17, 18]. Nck1 and Nck2 are highly homologous, widely expressed adaptor proteins, which contain 3 SH3 domains and a single SH2 domain [19]. In many cell types, the Nck proteins link phosphotyrosine signals to actin cytoskeleton reorganization through the WASP and the PAK [19]. The involvement of Nck in TCR signaling [5] has been suggested based on its binding to CD3ε [20-22] and SLP-76 [23]. Using a conditional genetic approach, it was demonstrated that in vivo Nck deletion induced severe impairment in thymic selection of low-avidity T cells [18]. In peripheral lymphoid organs, Nck deletion resulted in profound T cell lymphopenia and hyporeactivity to TCR-mediated stimulation. Nck-deficient T cells expressing TCRs with low avidity for self-antigens were strongly reduced, whereas T cell proliferation was defective upon weak antigenic stimulation [17]. Thus, Nck adaptors reduce the threshold of TCR responsiveness in both developing and mature T cells.

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Abbreviations: 7-AAD = 7-aminoactinomycin D, DPBS = Dulbecco phosphate-buffered saline, GC = germinal center, ICOS = inducible T cell co-stimulator, KLH = keyhole limpet hemocyanin, KO = knockout, LEAF = low-endotoxin azide buffered saline, GC = germinal center, ICOS = inducible T cell co-stimulator, KLH = keyhole limpet hemocyanin, KO = knockout, LEAF = low-endotoxin azide buffered saline, pAkt = Akt phosphorylation, SH = Src homology, Tfh = T follicular helper cell, WASP = Wiskott-Aldrich syndrome protein, WT = wild-type

The online version of this paper, found at www.jleukbio.org, includes supplemental information.
Figure 1. Loss of Nck in T cells leads to impaired GC formation and reduction of GC-associated B cells and Tfh cells. (A) In contrast to Nck.T+/+ mice (N = 10), Nck.T−/− mice (N = 9) showed impaired GC formation 9–10 d after subcutaneous immunization of 100 μg KLH/CFA. Three independent experiments were carried out, and representative GL7 stainings on frozen sections are presented; 3–4 mice were used in each experiment. Arrows outline GL7+ GC. Quantitative stereological evaluation of immunostainings revealed a significantly diminished mean GC volume fraction in Nck.T−/− mice (P = 0.0025). Mean volume fractions are depicted by box plot with confidence intervals of 0.0018–0.0048 in the Nck.T+/+ group and of
Here, we address the question of whether Nck-mediated pathways are required for in vivo CD4+ T cell differentiation and acquisition of effector function. Nck.T+/− mice with systemic deletion of Nck1 and T cell-specific deletion of Nck2 showed impaired GC [24] formation after immunization with T cell-dependent antigens. Both total and GC-associated follicular helper T cells (Thh and GC-Thh) [25−27] populations were decreased in Nck.T+/− mice in comparison to WT mice. Moreover, Nck.T+/− T cells were found to be more susceptible to cell death, which was associated with decreased levels of Akt phosphorylation. Consequently, Nck.T+/− mice showed reduced antibody production and insufficient affinity maturation. Taken together, our data show that Nck proteins have an important role in the differentiation and effector functions of CD4+ helper T cells.

MATERIALS AND METHODS

Mice
Nck.T+/− mice were bred as described before [17, 18]. In short, Nck1 single-knockout mice were crossed with Nck2fl/fl mice, in which exon1 of Nck2 was flanked by loxP sites. The offspring were further crossed with Lck-Cre deleter mice, in which the Cre recombinase was under the control of T cell-specific Lck promoter. These T cell-specific Nck knockout mice were then maintained by sibling mating. Absence of Nck genes was confirmed by PCR. Lck-Cre deleter mice were used as controls and noted as Nck.T+/+.

Immunization and sampling
For subcutaneous primary immunization, TNP30-BSA or TNP50-BSA (T-5650; Biosearch Technology, Petaluma, CA, USA) was diluted in 100 μl DPBS and mixed with 100 μl CFA. Spleen and blood were collected 10 d after primary immunization or 7 d from secondary immunization.

Cell culture and restimulation
Single cells from spleens were suspended and cultured in complete RPMI 1640 medium. For restimulation, anti-CD3 antibodies (LEAF-purified clone 145-2C11, 100314; BioLegend, San Diego, CA, USA) were diluted in 500 μl 10× PBS and coated on 24 well microplates at 37°C for 24 h. Coating solution was removed right before adding 500 μl single-cell suspensions; 48 h later, cells were harvested for further analysis. For the intracellular staining of cytokines, 4 h before the harvest, 500 μl fresh-culture medium, containing PMA and ionomycin, along with monensin (GolgiStop, 554724; BD Biosciences, Franklin Lakes, NJ, USA) was added into the culture.

Flow cytometry
To stain surface markers, harvested cells were washed twice with staining buffer (3% FBS and 0.1% sodium azide in DPBS) and stained with antibody combinations diluted in staining buffer in the dark on ice for 30 min. Stained cells were washed twice before analysis on the BD FACSCanto II flow cytometry (BD Biosciences). For intracellular staining of cytokines or transcriptional factors, surface markers were stained first. The fixation, permeabilization, and cytokine staining were performed with the buffer sets 560409 (BD Biosciences) and 88882400 (eBioscience, San Diego, CA, USA), following manufacturer’s instructions. For staining of apoptotic cells, apoptosis detection kit 559793 (BD Biosciences) was used. For staining of phosphorylated Akt, BD Phospho Perm Buffer III (558050; BD Biosciences) was used, and the manufacturer’s instructions were followed.

ELISA
For the quantification and affinity assay of serum antibodies, TNP30-BSA, TNP30-KLH (T-5650; Biosearch Technology), or TNP50-KLH (T-5660; Biosearch Technology) molecules were diluted in coating buffer (50 mM sodium carbonate, pH 9.5) and then coated on the 96 well ELISA plates at 4°C overnight. For TNP50-BSA, the coating concentration was 1 μg/ml, and for TNP50-KLH or TNP50-KLH, the coating concentration was 10 μg/ml. The coating buffer was discarded, and the wells were washed 3 times with wash buffer (1× TBS with 0.1% Tween 20). Gelatin-containing blocking buffer was used to block the wells at 4°C overnight. Samples, standards, or HRP-conjugated, secondary antibodies that recognize various antibody isotypes were diluted in wash buffer and then incubated at room temperature for 1 h. Serial dilutions were made for samples and standards. Secondary antibodies were diluted at 1:1000. Signals were developed by 100 μl OPD solution (400 μg/ml in 0.1M K2PO4, pH 6.0) containing 0.05% peroxide and then stopped by adding 50 μl 1M sulfuric acid solutions. The optical density at wavelength of 495 nm (OD 495) was measured by automatic reader (VICTOR 1420 Multilabel Counter; PerkinElmer, Waltham, MA, USA). Antibody concentrations were calculated by OD 495 values gained from TNP50-BSA binding and standard curves. Affinity units were calculated by dividing the OD 495 values gained from TNP50-KLH binding with the corresponding antibody concentrations. The reduction ratio was calculated by dividing the OD 495 values gained from TNP50-KLH binding with values from TNP50-KLH binding in the same serum dilution. For quantification of cytokine concentrations in the supernatant of in vitro restimulated splenic T cells, Ready-SET-Go! kits for mouse IL-4 (88-7044; eBioscience) or IL-10 (88-7104; eBioscience) were used. The protocols were according to the manufacturer’s instructions.

Antibodies
For analysis of flow cytometry, the following antibodies were used: anti-mouse CD4 Pacific Blue (clone RM4-5, 100531; BioLegend), anti-mouse CD69 FITC (clone HI2F3, 553286; BD Biosciences), anti-mouse CD152 (CTLA4) PerCP-eFluor 710 (clone 11B11, 554011; BD Biosciences), anti-human/mouse/rat CD279 PE-Cy7 (clone 603406, MAB5046; R&D Systems, Minneapolis, MN, USA), anti-human/mouse/rat CD278 Alexa Fluor 647 (clone 505014; BioLegend), anti-mouse CD279 PE-Cy7 (clone 29F.1A12, 315251; Biolegend), anti-human/mouse/rat GL7 PE (clone GL7-12, 12-5902; eBioscience), anti-mouse IL-4 Alexa Fluor 488 (clone 11B11, 504111; BioLegend), anti-mouse IL-10 Alexa Fluor 647 (clone JES5-16E3, 505014; BioLegend), anti-mouse IL-21 PE (clone m3ab21, 12-7213; eBioscience), anti-pAkt(pSer473) PE (clone 58-4873PE; PE) (clone 69941, 560378; BD Biosciences); anti-human/mouse Becl6 (clone 605406, MAB5046; R&D Systems, Minneapolis, MN, USA), anti-human/mouse GA3-5 (clone 605406, MAB5046; R&D Systems, Minneapolis, MN, USA), anti-human/mouse Ga3-5 (clone 615891; BD Biosciences), and 7-AAD (clone 11B11, 504111; BioLegend). For ELISA tests, the following antibodies were used: goat-anti-mouse IgM, IgG1, IgG2a, and IgG2b HRP-conjugated antibodies (553236; BD Biosciences), and 7-AAD (51-68981E; BD). For ELISA tests, the following antibodies were used: goat-anti-mouse IgM, IgG1, IgG2a, and IgG2b HRP-conjugated antibodies (553236; BD Biosciences).
Figure 2. Loss of Nck reduces the production of Th2/Tfh cytokines. Nck.T<sup>−/−</sup> (●, N = 6) and Nck.T<sup>+/+</sup> (○, N = 6) mice were immunized with 100 µg TNP<sub>26</sub>-BSA/IFA. Splenic T cells were isolated 10 d later and restimulated with 5 µg/ml plate-bound α-CD3 antibodies for 48 h. (A) Cytokines in supernatant were analyzed by ELISA. IL-4 and IL-10 were significantly reduced in the supernatant of T cells isolated from Nck.T<sup>−/−</sup> (both P < 0.0001). (B) For intracellular staining of cytokines, 50 ng/ml PMA, and 1 µg/ml ionomycin along with monensin were added to the culture medium 4 h before (continued on next page)
Biotech, Birmingham, AL, USA); AffiniPure goat-anti-mouse IgG + IgM (H+L) (115405-068; Jackson ImmunoResearch, West Grove, PA, USA); and purified mouse IgM (550963; BD Biosciences), IgG1 (553485; BD Biosciences), IgG2a (homemade), and IgG2b (homemade).

**Immunohistochemistry and stereology**

GL7 immunohistochemistry was carried out on frozen sections with biotin-labeled, anti-human/mouse GL-7 (clone GL-7, 19-39602; eBioscience) diluted 1:100, followed by streptavidin-alkaline phosphatase and permanent red as a color substrate. Sections were counterstained with hematoxylin. The spleens of 19 mice were evaluated (10 Nck.T−/− and 9 Nck.T+/+) by means of quantitative stereology. From each spleen, 1–2 random sections were studied. Each section profile was traced interactively on the stored, digitized micrographs. Within each section, the immunohistochromically stained cell profiles were also traced interactively. The areas of the sections and of the stained cells were then measured using standard software (Adobe Photoshop, ImageTool; Adobe Systems, San Jose, CA, USA). From these data, the area fraction of the stained cells per unit reference area was estimated for each animal as the ratio of the sum of stained areas, divided by the sum of the areas for the total sections per animal. This area fraction is an unbiased estimator of the volume fraction according to the Delesse principle of classic stereology. The mean volume fraction of the stained cells per animal was compared between the 2 groups using Student’s t test.

**Statistics**

An unpaired Student’s t test was applied throughout the studies, except for the analysis on antibody-affinity maturation, for which 2-way ANOVA was applied.

**RESULTS**

**Nck deletion results in impaired GC formation and reduction of Tfh cells**

To study the role of Nck adaptors in helper T cell function, Nck.T−/− and Nck.T+/+ mice were immunized with KLH in CFA, and GC formation was investigated in the respective spleens. The GC architecture was disrupted in Nck.T−/− mice (Fig. 1A). In all WT animals, GL7-positive volume fractions of the stained cells were found. In the KO group, 7 animals had positive-volume fractions of stained cells, whereas 2 animals had no stained cell at all. The mean volume fraction of GL7-positive cells per reference area was significantly diminished in the Nck.T−/− mice. In addition, the cellularity of GC B cells [28] with the phenotype [29, 30] (220 GL7+CD95dim) was decreased in Nck.T−/− mice in comparison with Nck.T+/+ mice (Figure 1B). Based on these initial findings, we performed a detailed analysis of the splenic Tfh cell compartment in both types of mice after immunization with the T cell-dependent antigen TNP26-BSA. Splenic CD4+CXCR5+ Tfh cells [26] as well as GC-associated Tfh cells (GC-Tfh) [25] expressing the GC marker GL7 [31, 32] were reduced in Nck.T−/− mice. Furthermore, Tfh cells, which expressed the activation marker CD69 or the costimulatory proteins ICOS and PD-1 [28, 33] were also reduced (Fig. 1D). In addition to the changes in cell populations, reduced expression levels of ICOS were found at the single-cell level (Supplemental Fig. 1). Similar alterations were observed after secondary immunization (Supplemental Fig. 2). Taken together, loss of Nck function results in impaired GC formation and in a reduction of GC-associated B and Tfh cells.

**Nck adapters are required for efficient cytokine production by CD4+ helper T cells**

To determine the capacity of CD4+ helper T cells deficient for Nck adapters to produce cytokines, we immunized Nck.T−/− and Nck.T+/+ mice with TNP26-BSA. Splenic Tfh cells were isolated 10 d later and restimulated with α-CD3 antibodies for 48 h. Protein levels of IL-4 and IL-10, both of which can be produced by Tfh cells and support germinal center formation [26, 34], were strongly reduced in the supernatant of T cells isolated from Nck.T−/− mice (Fig. 2A). When single Tfh cells were further assessed by intracellular staining, IL-10 production was significantly reduced in Tfh cells from Nck.T−/− mice, whereas IL-4 or IL-21 [26] production was slightly reduced without reaching statistical significance (Fig. 2B). However, strong reduction of all 5 cytokines was observed in PD-1+ Tfh cells (Fig. 2B). Thus, Nck adapters are required for efficient cytokine production by Tfh cells.

**Nck is not essential for the expression of Th2/Tfh transcriptional factors**

To investigate whether the observed decrease in Tfh cellularity and the reduction in cytokine production might be attributed to impaired T cell differentiation, we determined the expression of the transcription factors GATA-3 [35] and Bcl-6 [28, 26, 36]. Nck.T−/− and Nck.T+/+ mice were immunized with TNP26-BSA, and splenic Tfh cells were isolated 10 d later. Expression of both transcription factors was assessed in total Tfh and GC-Tfh populations after isolating single cells from the spleen. GATA-3 expression was decreased in the total Tfh population in Nck.T−/− compared with Nck.T+/+ mice. In contrast, neither the expression intensity (Fig. 3A-B) nor the population percentage of expressing cells (Fig. 3C and D) was found to be different in the GC-Tfh populations for both types of mice for Bcl-6 and GATA-3. In addition, restimulation of isolated splenic Tfh cells with α-CD3 did not cause a difference on the expression profiles of Bcl-6 or GATA-3 (Supplemental Fig. 3). Therefore, Nck adapters do not significantly influence expression of transcription factors promoting Th2/Tfh cell differentiation.

**Nck-deficient T cells are more susceptible to apoptosis**

Although Nck-deficient Tfh cells express the respective transcription factors for successful differentiation, it is possible that cell harvesting and antibody staining. IL-10-producing CD4+CXCR5+ Tfh cells were significantly decreased in Nck.T−/− mice (P = 0.0096), whereas IL-4 and IL-21 producers were decreased on average but without reaching statistical significance (P = 0.1096, P = 0.0922). PD-1+ Tfh cells, producing IL-4, IL-10, or IL-21 were all significantly reduced in Nck.T−/− mice (P = 0.0248, P = 0.0066, P = 0.0086, respectively). When specifically investigating the cytokine-producing populations in PD-1+ Tfh cells from Nck.T−/− mice, the MFIs of IL-10 and IL-21 were both significantly reduced (P = 0.0107, P = 0.0453). The MFI of IL-4 was reduced but reached no significance (P = 0.1602). When analyzing the total Tfh cells in the same way, a similar reduction was found for 3 respective cytokines, but it only reached significance in the case of IL-10 (P = 0.0084). An unpaired Student’s t test was applied.
Figure 3. Nck is not essential for the expression of Th2/Tfh transcriptional factors. Nck.T2/2 (N = 10) and Nck.T+/+ (N = 10) mice were immunized with 100 µg Thymus tumor protein (TNP)26-BSA/IFA i.p., and splenic cells were isolated 10 d later. After isolating cells from the spleen, expression of Bcl-6 and GATA-3 were assessed by flow cytometry. (A) In total Tfh cells, MFI of Bcl-6 were comparable (P = 0.0867), whereas the MFI of GATA-3 was reduced (P = 0.0257) in Nck.T2/2 mice. (B) The MFI of either Bcl-6 or GATA-3 was comparable in GC-Tfh cells (P = 0.3609 and P = 0.2536). (C) Although Bcl-6 Tfh cells showed no difference (P = 0.3597), GATA-3 Tfh cells were reduced in Nck.T2/2 mice (P = 0.0291). (D) Both Bcl-6 and GATA-3 GC-Tfh cells showed no difference (P = 0.2310 and P = 0.1626) between Nck.T+/+ and Nck.T2/2 mice. An unpaired Student’s t test was applied.
their complete differentiation or survival is impaired. Therefore, we investigated apoptotic cell death of CD4+ T cells isolated from TNP26-BSA–immunized Nck.T<sup>−/−</sup> and Nck.T+/+ mice. Cell staining with 7-AAD and fluochrome-conjugated annexin V did not show a significant difference in cell death for total CD4+ T cells isolated from both types of mice (Fig. 4A). However, 60% of Nck-deficient GL-7+ GC-T cells entered the late phase of apoptosis, whereas WT T cells mostly remained alive or entered only the early phase of apoptosis within the tested time frame (Fig. 4A). The same phenomena were identified after restimulating the isolated splenic cells with anti-CD3 antibody (Supplemental Fig. 4). Because Nck adaptors influence Akt signaling [37] and because Akt signaling takes part in anti-apoptotic pathways [38, 39], we studied pAkt in the activated Nck-deficient and Nck-sufficient CD4+ T cells. pAkt levels were significantly decreased in total CD4+ T cells and in Tfh cells from Nck.T<sup>−/−</sup> mice (Fig. 4B). Taken together, the loss of Nck leads to enhanced apoptosis of Tfh cells, which is associated with decreased antiapoptotic signaling by pAkt.

Nck.T<sup>−/−</sup> mice show reduced antibody production and insufficient affinity maturation

Finally, we extended our studies on the role of Nck adaptors in helper T cell function by measuring the production and quality of antibodies in Nck.T<sup>−/−</sup> and Nck.T+/+ mice. The basal levels of natural IgM, IgG1, IgG2a, and IgG2b were significantly decreased in the serum of Nck.T<sup>−/−</sup> mice (Fig. 5A). This phenomenon was consistent with reported T cell-dependency of natural antibody production [40]. Similarly, primary antibody responses against TNP<sub>26</sub>-BSA were also impaired in all 4 measured isotypes in Nck.T<sup>−/−</sup> mice (Fig. 5B). Moreover, when Nck.T<sup>−/−</sup> mice experienced secondary and tertiary immunization, the production of IgG2a remained significantly lower, whereas IgG1 reduced on average but without reaching significance (Fig. 5C). Thus, Nck ablation in T cells leads to a reduction in antibody production of both primary and secondary responses.

We then asked whether Nck expression in T cells also had an effect on the quality of the produced antibodies as measured by affinity maturation. As an assay for affinity maturation, we first determined the hapten binding per mass unit of antibodies in the serum to the 2 lower hapten conjugates on 1 carrier, TNP<sub>5</sub>-KLH and TNP<sub>16</sub>-KLH, after secondary immunization with TNP26-BSA. We then calculated the ratio of the obtained signal strength of TNP5-binding to TNP16-binding [41]. All isotypes, except for IgG1, showed a significantly lower strength ratio in the sera from Nck.T<sup>−/−</sup> mice compared with the sera from Nck.T+/+ mice (Fig. 5D). This indicated that Nck.T<sup>−/−</sup> antibody populations contained less high-affinity antibodies than did the controls because they could not bind as strongly as the control antibodies when the number of haptens on the carrier was decreased. Based on this analysis, we propose that affinity maturation is impaired in Nck.T<sup>−/−</sup> mice.
Figure 5. Nck.T\(^{−/−}\) mice show reduced antibody production and insufficient affinity maturation. (A) Basal immunoglobulin levels in the serum of 6- to 8-wk-old Nck.T\(^{−/−}\) (●, \(N = 12\)) and Nck.T\(^{+/+}\) (○, \(N = 11\)) mice were assessed by ELISA. The serum levels of IgM (\(P < 0.0001\)), IgG1 (\(P < 0.0001\)), and IgG2a (\(P = 0.001\)) were significantly reduced in Nck.T\(^{−/−}\) mice, when compared with the Nck.T\(^{+/+}\) mice. By contrast, the levels of IgG2b were comparable in both types of mice (\(P = 0.0696\)). (B) Nck.T\(^{−/−}\) (●, \(N = 18\)) and Nck.T\(^{+/+}\) (○, \(N = 13\)) mice were immunized with 200 μg TNP\(_{26}\)-BSA/CFA s.c. in 4 different sites of the belly. Antigen-specific antibodies were analyzed in the serum on days 7, 14, and 21. Significantly reduced levels of IgM (\(P = 0.0163\)), IgG1 (\(P = 0.0007\)), IgG2a (\(P = 0.0002\)), and IgG2b (\(P = 0.0008\)) were observed on day 14. (C) Nck.T\(^{−/−}\) (●, \(N = 15\)) and Nck.T\(^{+/+}\) (○, \(N = 13\)) (continued on next page)
DISCUSSION

Here, we provide evidence that Nck proteins are essential for survival and effector functions of Tfh cells. Loss of Nck proteins leads to impaired GC formation along with reduced cellularity of splenic Tfh cells and GC-Tfh cells. Production of key Th2 and Tfh cytokines, such as IL-4, IL-10, and IL-21 [26, 42], was also diminished. These signs of dysfunction were associated with decreased levels of pAkt and intensified cell death of the Tfh cells. Consequently, T cell-dependent antibody responses were reduced in quantity as well as quality by affinity maturation.

In our Nck.T⁻/⁻ model Nck1 was deleted among all somatic cells, including B cells. We recently studied antibody responses in mice with a deletion of Nck1 in all somatic cells (Nck1-KO) or of both Nck homologs specifically in B cells (Nck.B⁻/⁻) and similar phenotypes were found in both types of mice [37]. By contrast, titers of all these isotypes were reduced in the Nck.T⁻/⁻ mice compared with the control animals. Furthermore, Nck1-KO as well as Nck.B⁻/⁻ mice showed no difference from WT control mice in T cell-dependent antibody production and affinity maturation [37]. In contrast, we observed an impairment of both T cell-dependent antibody production and affinity maturation in the Nck.T⁻/⁻ mice studied, with Nck2 deleted in T cells in addition to complete Nck1 deficiency. Therefore, we assume that the deletion of both Nck1 and Nck2 in Nck.T⁻/⁻ mice may contribute to the observed, impaired humoral responses, rather than the Nck1-deficiency in B cells. These observations support the view of an important and specific role for Nck proteins in T helper cell function.

In previous studies, Nck.T⁻/⁻ mice showed impaired thymic selection and peripheral survival on T cells expressing low-avidity TCR [17, 18]. Therefore, reduced cellularity and developmental defects, rather than the direct involvement of Nck proteins, might be responsible for the impaired effector function and T cell-dependent humoral responses. However, the reduced cellularity was found to be restricted to T cells expressing low-avidity TCR. T cells expressing TCR with optimal avidity showed normal thymic selection and survival in the periphery in Nck.T⁻/⁻ mice. Because we used BSA or KLH, conjugated with many immunogenic TNP haptens [43] as T cell-dependent antigens, we assume that most of the responding T cells may not have been those T cells expressing low-avidity TCR. Hence, reduced cellularity may be less likely to account for the observed changes in T cell effector function. Indeed, we did not observe significant differences in cellularity or in the quality of the splenic CD4⁺ T cells between naive WT and Nck.T⁻/⁻ mice (Supplemental Fig. 5). Therefore, we suggest that Nck proteins may be directly involved in the differentiation or effector function of Tfh cells.

During humoral immune responses GCs are formed in secondary lymphoid organs and provide a microenvironment for differentiation and activation of B cells [24]. GCs were normally generated in mice with Nck-deficient B cells [37]. In contrast, we found impaired GC formation in Nck.T⁻/⁻ mice. Tfh cells are essential for GC formation, affinity maturation, and the development of high-affinity antibodies and memory B cells [24, 27]. The function of GC-Tfh has been reported to be regulated by the strength of the TCR binding to peptide-MHC complexes [7]. Our findings show that, in addition, T cell-intrinsic modulation of the TCR signal strength by Nck proteins can regulate the function of GC-Tfh and thereby the formation of GC. Tfh cells provide help to GC B cells by cell surface receptor interactions and by secretion of cytokines [27]. CD4óż, PD-1, and ICOS expressed on Tfh cells transmit help by binding to their respective ligands on GC B cells. For example, very recently, it has been shown that costimulation provided by ICOS led to up-regulation of CD40L by B cells and thereby to higher ICOSL surface expression. The consequently more efficient interactions between Tfh cells and B cells resulted in positive selection of higher-affinity plasma cells [33]. We found reduced numbers of GC-Tfh cells expressing ICOS and decreased ICOS expression per cell in Nck.T⁻/⁻ mice. This observation could be explained by the reported impairment of ERK phosphorylation and Ca²⁺ flux in Nck.T⁻/⁻ T cells [17] as ERK signal is essential for ICOS expression [44]. Furthermore, Tfh cells from Nck.T⁻/⁻ mice produced lesser amounts of the relevant cytokines IL-10, IL-4, and IL-21 per cell than the respective cells from WT mice. Akt activity is reported to support the production of different cytokines by T cells, including IL-4, IL-10, and IL-21 [45–47]. However, although ERK signaling promotes IL-10 production [48], it also negatively regulates the production of IL-4 or IL-21 in certain situations [49–51]. Therefore, the reduction of IL-10 after the loss of Nck may be explained by synergistic effects of decreased Akt and ERK signaling. The observed changes of IL-4 and IL-21 in Nck.T⁻/⁻ T cells may be the result of opposite activities by decreased Akt activities and decreased ERK signaling. This may explain why the reduction of IL-4 and IL-21 is not universally significant among all Tfh but is specifically pronounced in PD-1⁺ Tfh cells. The detailed balance between decreased Akt and ERK signaling after the loss of Nck proteins requires further investigation.

mice were immunized with 100 mg TNP2₇-BSA/CFA s.c. on day 0 and boosted twice with 100 mg TNP2₇-BSA/IFA s.c. at 42 and 84 d after the primary stimulation. Serum was collected on day 7 after the secondary boosting. The levels of TNP-specific IgG1 and IgG2a were assessed by ELISA. The levels of IgG1 were comparable between Nck.T⁻/⁻ and Nck.T⁻/⁺ mice (P = 0.0708), whereas the levels of IgG2a were significantly decreased (P = 0.0015) in the absence of Nck. (D) Nck.T⁻/⁻ (N = 8) and Nck.T⁻/⁺ (N = 10) mice were immunized with 100 µg TNP2₇-BSA/CFA s.c. in the tail base and boosted with TNP2₇-BSA/IFA i.p. 10 d later. Sera were collected on day 7 after the secondary immunization. The levels of antigen-specific IgM, IgG1, IgG2a, and IgG2b were assessed by ELISA; 10 µg of TNP2₋KLH or TNP16-KLH was coated on ELISA plates. Sera were 4-fold diluted from 1/100 serially. Binding signal (OD values) of every serum dilution was acquired by ELISA. The affinity index was calculated by dividing the OD values with the corresponding serum concentrations of the antibody isotypes. Affinity indices from TNP2₋KLH detection were then divided by corresponding affinity indices from TNP16-KLH detection. All isotypes showed a significantly stronger decrease in the ratio of affinity indices (P < 0.0001, P = 0.0104, P < 0.0001, respectively), except for IgG1 (P = 0.2525). An unpaired Student’s t test was applied for analyzing (A) and (B); 2-way ANOVA was carried out for analyzing (C) and (D).
Together, our findings and previously published data point to an important role for Nck proteins in fine-tuning TCR signaling strength during all T cell stages, including development, maintenance, activation, and effector function. Nck adaptor proteins contribute to shaping the preimmune T cell repertoire during thymic selection, contribute to the size and sensitivity of the peripheral T cell repertoire, and, as shown here, regulate helper T cell functions during T cell-dependent antibody responses in GCs.

Increased Tfh cell numbers in peripheral blood are observed in some patients with Sjögren's syndrome [52, 53], juvenile dermatomyositis [34], and systemic lupus erythematosus [52]. Each of these diseases is associated with extensive autoantibody production [55]. Furthermore, it has been suggested that Tfh cells might support the formation of ectopic follicles in autoimmune diseases, thereby possibly serving as nucleation points for other cells with pathogenic potential in the particular autoimmune disease [56]. Based on our studies, Nck proteins in Tfh cells may be a novel target to attenuate antibody production [55]. Furthermore, it has been suggested that Tfh cells might contribute to the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. J. Immunol. 159, 5956–5963.

AUTHORSHIP

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The authors declare no competing financial interests.

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Nck adaptor proteins modulate differentiation and effector function of T cells

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**Supplemental Figure 1. Altered ICOS expression intensity in ICOS+ Tfh cells.** Mice were immunized by 100µg TNP_{26}-BSA with IFA for 10 days. After sacrificing the mice and taking out the splenocytes, the expression intensity of ICOS and PD-1 was analyzed by flow cytometry. Tfh cells which were positive for ICOS expression showed lower ICOS intensity in Nck.T^{-/-} mice (p=0.0035). The PD-1 intensity was similar between PD-1+ cells in Nck.T^{-/+} and Nck.T^{-/-} mice (p=0.073). Unpaired t-test was used for analysis.

**Supplemental Figure 2. Altered compositions of splenic Tfh cells in Nck.T^{-/-} mice during secondary immune responses.** Nck.T^{-/-} (●, N=8) and Nck.T^{-/+} (○, N=10) mice were immunized with 100µg TNP_{26}-BSA/CFA s.c. and then boosted with TNP_{26}-BSA/IFA 10 days later. Splenic cells were analyzed on day 7 after the secondary immunization. (A) The percentage of CD4^{+}CXCR5^{+} follicular helper T (Tfh) cells did not change significantly between Nck.T^{-/-} and Nck.T^{-/+} mice (p=0.3288). (B) However, the GL-7^{+} (GC-Tfh), PD-1^{+}, or ICOS^{+}PD-1^{+} Tfh cells were reduced significantly in Nck.T^{-/-} mice (p=0.0437, p=0.0083, p<0.0001), while the ICOS^{+} Tfh remained comparable (p=0.4366). Unpaired t test was used for the analysis.

**Supplemental Figure 3. Re-stimulation with α-CD3 antibody does not alter the expressional profiles of Bcl-6 and GATA-3 in splenic Nck.T^{-/-} T cells.** (A) Nck.T^{-/-} (●, N=6) and Nck.T^{-/+} (○, N=6) mice were immunized with 100µg TNP_{26}-BSA/IFA i.p. for 10 days. Splenic cells were isolated and re-stimulated with 5µg/ml plate-bound α-CD3 antibody. Neither Bcl-6 nor GATA-3 -positive populations were significantly affected among CD4^{+}CXCR5^{+} Tfh cells (p=0.8113, p=0.4717) or (B) GL7^{+} Tfh (GC-Tfh) cells (p=0.1891, p=0.6304). Neither were the expression intensities changed in Tfh or GC-Tfh cells (p=0.3432, p=0.3411, p=0.4970, p=0.7529). Unpaired t test was applied.
**Supplemental Figure 4.** Nck deficient T cells are more susceptible to cell death after re-stimulation. Ten days after immunization of 100μg TNP26-BSA/IFA i.p. splenic cells were isolated from Nck.T⁻/⁻ (●, N=6) or Nck.T⁺/+ (○, N=6) mice and re-stimulated with 5μg/ml plate-bound α-CD3 antibody. Re-stimulated cells were stained with 7-AAD and PE-conjugated Annexin V. Among GL-7⁺CD4⁺ T cells, cells in late stage of apoptosis or necrotic cell death increased significantly in Nck.T⁻/⁻ mice (p=0.0001) while cells in early apoptosis were less (p=0.0015). Unpaired t-test was applied.

**Supplemental Figure 5.** Cellularity and quality of naive CD4⁺ T cells are not changed in the spleen of unchallenged Nck.T⁻/⁻ mice. Splenic cells from naive Nck.T⁻/⁻ (●, N=5) or Nck.T⁺/+ (○, N=5) mice were analyzed by flow cytometry. No difference between Nck.T⁻/⁻ and Nck.T⁺/+ mice was observed in splenic CD4⁺ T cells (p=0.4386). CD62L-positive naive CD4⁺ T cells (p=0.9748) and CD4⁺ T cells expressing ICOS (p=0.6260) or PD-1 (p=0.5200) were not changed in Nck.T⁻/⁻ mice. Unpaired t-test was applied for the analysis.