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ShcA Regulates Late Stages of T Cell Development and Peripheral CD4+ T Cell Numbers

Monica W. Buckley,1 Paul C. Trampont,1 Sanja Arandjelovic, Aaron M. Fond, Ignacio J. Juncadella, and Kodi S. Ravichandran

T cell development in the thymus is a highly regulated process that critically depends upon productive signaling via the preTCR at the β-selection stage, as well as via the TCR for selection from the CD4+CD8+ double-positive stage to the CD4 or CD8 single-positive stage. ShcA is an adapter protein expressed in thymocytes, and it is required for productive signaling through the preTCR, with impaired signaling via ShcA leading to a developmental block at the β-selection checkpoint. However, the role of ShcA in subsequent stages of T cell development has not been addressed. In this study, we generated transgenic mice (CD4-Cre/ShcFFF mice) that specifically express a phosphorylation-defective dominant-negative ShcA mutant (ShcFFF) in late T cell development. Thymocytes in CD4-Cre/ShcFFF mice progressed normally through the β-selection checkpoint, but displayed a significant reduction in the numbers of single-positive CD4+ and CD8+ thymocytes. Furthermore, CD4-Cre/ShcFFF mice, when bred with transgenic TCR mouse strains, had impaired signaling through the transgenic TCRs. Consistent with defective progression to the single-positive stage, CD4-Cre/ShcFFF mice also had significant peripheral lymphopenia. Moreover, these CD4-Cre/ShcFFF mice develop attenuated disease in CD4+ T cell–dependent experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Collectively, these data identify an important role for the adapter protein ShcA in later stages of thymic T cell development and in peripheral T cell–dependent events. The Journal of Immunology, 2015, 194: 1665–1676.
Although previous studies highlighted the requirement for ShcA in the DN to DP transition (14–17), the nearly complete block in development at the β-selection checkpoint in the Lck-Cre/ShcΔΔ transgenic line has precluded us from using this mouse line to study the role of ShcA in subsequent developmental stages. In this study, we used CD4−Cre-mediated expression of the phosphorylation-defective ShcΔΔ transgene, in which the crucial ShcA tyrosine residues within the CHI domain were mutated to phenylalanine (15). The CD4−Cre transgenic mouse line expresses the Cre recombinase from the late DN4/early DP stage, allowing normal progression at the β-selection checkpoint (21). We now find that ShcA function is important during the stage, allowing normal progression at the β-selection checkpoint.

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Materials and Methods

Mice

All mice used were on the C57BL/6J background, unless otherwise noted. C57BL/6J wild-type (WT) mice, TCR-deficient mice, Rosa26STOP-EYPFP reporter mice, and CD4−Cre−, OT−ι−, OT−II−, and H−γ−transgenic mice were purchased from The Jackson Laboratory or Taconic (6, 21–25). The inducible ShcΔΔ transgenic line was described previously (14, 15). The LckF505-transgenic mouse line and the Rag2−cγ−γ− mouse line were kindly provided by Dr. Roger Perlmutter’s laboratory (Merck Research Laboratories) and Dr. Pamela Fink’s laboratory (University of Washington), respectively, and were described previously (26–28). PCR was performed on tail DNA or sorted cell populations. Mice were bred and housed in a specific pathogen–free environment, and all animal experiments were approved by the University of Virginia (UVA) Animal Care and Use Committee.

Flow cytometry

Thymocytes, splenocytes, and lymphocytes were isolated from 4–6-wk-old mice (littermates) for analysis of T cell development. DP and DN compartments were analyzed by staining thymocytes with Abs specific for CD4, CD8, CD3, Thy1.2, CD25, CD24, CD28, CD28β, CD25, CD124, and CD69, as well as lineage markers (CD11b, CD11c, B220, Gr1, Ly6G, and Ter119), as described previously (17). Expression of the transgenic TCR in the different transgenic lines was analyzed via staining for KJ1.26, V85.1/2, T3.70, and Vα3 for the DO11.10-, OT-II−, LckΔΔ−, and OT-I−transgenic mice, respectively. Absolute numbers were determined via enumeration with the hemocytometer, followed by flow cytometry analysis or by inclusion of reference counting beads (Spherotech). Viability and apoptosis were evaluated by staining with annexin V and 7-aminoactinomycin D (7AAD) (Invitrogen), according to the manufacturer’s instructions. Splenocytes and lymphocytes were stained with Abs specific for CD3, CD4, CD8, Foxp3, CD62L, CD44, and CCR7. All Abs used were obtained from eBioscience, unless otherwise noted. FACScanto (BD Biosciences) was used for flow cytometry, and the results were analyzed by FlowJo software (TreeStar).

Migration

Migration of thymocytes was performed using the Transwell system. Briefly, thymocytes were isolated from 4–6-wk-old mice and suspended in RPMI 1640 with 0.5% BSA (Sigma-Aldrich) at 106 cells/ml. One million thymocytes (100 μl) were placed in the upper chamber of a Transwell with a 5-μm pore size (Costar). Migration medium (RPMI 1640 with 0.5% fatty-acid free BSA) or various concentrations of the indicated cytokine in 600 μl migration media was placed in the bottom well. An additional well contained 106 thymocytes (in 500 μl media) and represented the “input” for determining the fraction of migrating cells. Cells were allowed to migrate for 3 h in the 37°C incubator. After migration, the cells in the bottom chamber, as well as in the input well, were collected; the bottom chambers were rinsed with ice-cold PBS; the cells were stained with cell surface markers and analyzed via flow cytometry, with inclusion of reference counting beads (Spherotech); and the percentage migration was determined.

Experimental autoimmune encephalomyelitis

EAE immunization was performed as previously described (29). For optimal EAE induction, 10-wk-old female mice were immunized s.c. into the lower back with 100 μg myelin oligodendrocyte gp35–55 (MOG35–55) peptide (CS Bio), emulsified in an equal volume of CFA (Sigma-Aldrich) supplemented with heat-killed Mycobacterium tuberculosis (clone H37RA; Difco), for a total of 400 μg H37RA/mouse. Mice received 200 ng pertussis toxin (List Biologicals) i.p. on days 0 and 1 after immunization. For suboptimal EAE induction, mice were immunized s.c. with 75 μg MOG35–55 in CFA supplemented with H37RA but received only a single i.p. injection of 200 ng pertussis toxin on day 0. The mice were weighed and scored daily on a 5-point scale: 0, no clinical signs; 1, paralyzied tail; 2, mild hindlimb paresis; 3, severe hindlimb paresis; 4, hindlimb paralysis; and 5, quadriplegia/moribund. Brain and spinal cord leukocytes were isolated on day 28 postinjection using Percoll (GE Healthcare) gradient centrifugation. Isolated cells were identified via staining with Abs specific for CD4, CD45, CD11b, and B220, followed by flow cytometry.

Th17 and Th1 in vitro differentiation

Th17 and Th1 skewing in vitro was performed by using total lymphocytes or selecting CD4+ T cells from spleens and lymph nodes of 4–wk-old mice (Mice and Biotopes). The cells were stirred toward the Th17 inducible state on day 1 in 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28−coated plates along with 0.3 ng/ml TGF-β1 (R&D Systems), 20 ng/ml IL-6 (R&D Systems), 10 ng/ml IL-23 (eBioscience), 10 μg/ml anti-IL-6 (eBioscience), and 10 μg/ml anti-IFN-γ (eBioscience) in IMDM supplemented with 10% FBS, 50 μM 2-ME, 2 mM l-glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 10 mM HEPES. After 4 d, the cells were collected for analysis. Cells were activated by intracellular cytokine staining were stimulated with 50 ng/ml PMA and 1 μM ionomycin, along with GolgiStop (BD Pharmin- gen), for 5 h prior to staining. Intracellular staining for IL-17A (BD Pharminingen) and IFN-γ (eBioscience) was performed by fixing the cells in 4% paraformaldehyde, followed by permeabilization with 0.1% saponin. Th1 skewing was performed by culturing total lymphocytes or CD4+ cells on 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28−coated plates along with 100 U/ml IL-2 (PeproTech), 10 ng/ml IL-12 (eBioscience), and 10 μg/ml anti-IL-4 (eBioscience); analysis was performed on day 7, as described above for Th17 cells.

T cell stimulation and proliferation

For CD3/CD28 stimulation, 80,000 purified CD4+ T cells (purified using a MACS kit; Miltenyi Biotec) were stimulated with anti-CD3/anti-CD28 beads (Dynabeads; Life Technologies), according to the manufacturer’s protocol, for the indicated times. T cells were stained with 5 μM CFSE (Molecular Probes) prior to stimulation, and proliferation was assessed via CFSE dilution. Stimulations also were performed by culturing cells with 50 ng/ml PMA with 500 ng/ml ionomycin (both from Calbiochem), 5 μg/ml anti-CD3, or with 5 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (all from BD PharminGen). All stimulations were performed in 200 μl RPMI 1640 medium (supplemented with 10% FBS, 50 μM 2-ME, 2 mM l-glutamine, and 1% penicillin/streptomycin) in round-bottom 96-well plates and cultured at 5% CO2 at 37°C.

Immunohistochemistry and immunofluorescence

For immunohistochemistry, thymi were fixed by immersion in 10% neutral buffered formalin (Fisher) and embedded in paraffin blocks. For histological analysis of the spinal cord, mice were perfused with 4% paraformaldehyde in PBS, and parts of the spinal cord were fixed in 4% paraformaldehyde in PBS and embedded in paraffin blocks. Sections were processed for immunohistochemistry using standard techniques. Images were acquired on an Olympus SZX12 low-magnification microscope equipped with an Olympus DP70 digital camera. Quantification of cell number was performed using National Institutes of Health ImageJ software.

Immunoblotting and immunoprecipitation

Immunoprecipitation was performed from thymocytes or spleenocytes lysed using RIPA buffer containing protease inhibitors (Calbiochem). Lysates were incubated with anti-Flag agarose beads (Sigma-Aldrich) or anti-ShcA

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Ab (BD), followed by Protein A/G Agarose beads (Santa Cruz Biotechnology). Beads were washed and eluted by boiling in SDS sample buffer containing 2-ME and analyzed via SDS-PAGE and immunoblotting for ShcA (BD) or anti-tyrosine.

**Ex vivo survival assays**

Thymocytes isolated from DO11.10 mice were incubated with the A20 B cell line along with OVA peptide (aa 323–339). After 8 and 20 h, thymocytes were stained with CD4, CD8, annexin V, and 7AAD, according to the manufacturers’ instructions.

**Quantitative PCR**

Total RNA was extracted from thymocytes and selected CD4⁺ T cells using a QIAshredder and RNeasy kit (QIAGEN), followed by reverse transcription using the SuperScript III Kit (Invitrogen). Quantitative PCR was performed using TaqMan Gene Expression assays on a StepOnePlus system (both from Applied Biosystems). TaqMan gene expression probes were used for gene analysis of mouse IL-2 and HPRT. Each sample was performed in duplicate, target transcripts were normalized to HPRT mRNA as an internal control gene, and the relative expression of each target gene was calculated using the comparative cycling method with StepOne v2.1 software (Applied Biosystems).

**Statistical analysis**

Statistical comparisons were performed using the Student two-tailed *t* test or a two-way ANOVA (clinical scores for EAE) using GraphPad Prism version 4.0. Results with a *p* value < 0.05 were considered significant.

**Results**

**CD4-Cre/ShcFFF mice have defects in T cell development**

Previously, we used Lck-Cre/ShcFFF⁻ and Lck-Cre/Shcfl/fl⁻ transgenic mice to demonstrate an absolute requirement for ShcA in β-selection (14–17). To determine whether ShcA phosphorylation is required in later stages of T cell development, we crossed our dominant-negative ShcFFF-transgenic mouse line to the CD4-Cre–transgenic mouse line (21). The ShcFFF-transgenic construct allows specific expression of a phosphorylation-defective ShcFFF transgene downstream of the ubiquitous EF-1α promoter; however, to keep basal expression of the ShcFFF transgene silent and to allow Cre-mediated transgene expression, a floxed STOP cassette has been inserted between the promoter and the ShcFFF-coding sequence (14, 15). To confirm the fidelity of the CD4-Cre–transgenic mouse line, we first crossed the CD4-Cre mouse line to the Rosa26STOP-EYFP mouse and analyzed eYFP expression in the different thymic subsets. We found that eYFP is expressed in late DN4, DP, CD4 SP, and CD8 SP thymocytes but not in DN3 thymocytes and, importantly, CD4-Cre affects both the CD4 and CD8 lineages (Supplemental Fig. 1B) (30). Therefore, we expected that CD4-Cre/ShcFFF mice would allow us to bypass the requirement for ShcA phosphorylation during β-selection, yet facilitate investigation of ShcA function in late T cell development.

We first observed that CD4-Cre/ShcFFF mice have altered T cell development with a decrease in overall thymic cellularity and a striking reduction in the percentage of the CD4 SP compartment, with no change in the percentage of the CD8 SP compartment (Fig. 1A, Supplemental Fig. 1E). CD4-Cre/ShcFFF mice showed a reduction in the absolute number of DP, CD4 SP, and CD8 SP thymocytes (Fig. 1A, Table I). Although there was a slight decrease in the absolute number of DP thymocytes, the...
defect in the SP compartment was more profound than the defect in the DP compartment, suggesting that the reduction in SP thymocytes is likely not entirely due to the reduced numbers of DP thymocytes.

The development of the CD4 lineage is often more sensitive to alterations in TCR signaling because CD4-lineage commitment requires stronger and more sustained signaling than does CD8-lineage commitment (7, 8, 31–33). We noted that, in CD4-Cre/ShcFFF mice, the development of CD4 SP thymocytes was more affected than the development of CD8 SP thymocytes, leading to an alteration in the ratio of CD4 SP/CD8 SP thymocytes (Supplemental Fig. 1E). Moreover, the phenotypic maturation of CD4 SP and CD8 SP thymocytes also appeared to be impaired in CD4-Cre/ShcFFF mice, based on cell surface staining for CD24 and CD5 (Fig. 1B) (34–36). Both CD4 SP and CD8 SP thymocytes had a reduced percentage of mature CD24hi SP thymocytes and an alteration in the ratio of mature (CD24lo)/immature (CD24hi) SP thymocytes (Fig. 1B, Supplemental Fig. 1F). Additionally, expression of the maturation and adhesion molecule CD5 was decreased in the CD8 SP thymocytes (Fig. 1B). CD5 levels correlate with TCR signal strength; however, it is also possible that these CD5lo CD8 SP thymocytes represent CD8 immature SP (ISP) thymocytes, and there might be additional defects in the transition from ISP to DP thymocytes in CD4-Cre/ShcFFF–transgenic mice (Fig. 1B) (36). Collectively, the impairment in CD4 SP and CD8 SP compartments, with a more profound defect in the CD4 SP compartment, suggests that the CD4-Cre/ShcFFF mice have a defect in TCR signaling and transition from the DP to SP stages of thymocyte development.

To further understand the specific developmental stages affected in CD4-Cre/ShcFFF mice, we subdivided thymocytes into five developmental stages based on the expression of TCRβ and CD69 (31–33). Populations 1 (TCRβhiCD69hi) and 2 (TCRβloCD69lo) represent the most immature prepositive-selection DN and DP thymocytes (31). CD4-Cre/ShcFFF mice have a slight increase in the percentages of both of these subsets (data not shown). Population 3 (TCRβloCD69hi) has partially upregulated CD69 and is thought to be undergoing positive selection (37). We found a slight reduction in this population in CD4-Cre/ShcFFF mice (data not shown). Additionally, CD4-Cre/ShcFFF mice had a significant reduction in the composition of postpositive-selection thymocytes (population 4, TCRβhiCD69lo) and mature thymocytes (population 5, TCRβhiCD69lo) (Fig. 1C). Collectively, these data suggested that CD4-Cre/ShcFFF mice have deficiencies in late T cell development, likely due to altered positive selection.

Signaling through transgenic TCRs is impaired in CD4-Cre/ShcFFF mice

Developmental defects in positive selection are often better revealed in mice carrying a transgenic TCR, because the normal TCR repertoire may undergo compensatory changes that can mask defects in thymocyte selection (32, 38, 39). Therefore, we crossed CD4-Cre/ShcFFF mice to two different MHC class II–restricted TCR-transgenic lines: OT-II and DO11.10. The developmental defects due to ShcFFF expression were more pronounced in these TCR-transgenic backgrounds (Table I). CD4-Cre/ShcFFF mice expressing either the OT-II or the DO11.10 transgenic TCR had an overall reduced thymic cellularity and decreased numbers in the DP and CD4 SP thymic compartments (Table I). Additionally, staining with Abs specific for the transgenic TCRs demonstrated a specific reduction in thymocytes expressing the OT-II–transgenic TCR (Fig. 2A, 2B), with fewer CD4 SP-transgenic Vβ5.1/2α cells (Supplemental Fig. 2), al-
though we did not find a defect in the percentage of cells expressing the DO11.10-transgenic TCR.

Previously, we demonstrated that the block in early T cell development in mice expressing the ShcFFF transgene is essentially complete, such that any DP, SP, or peripheral T cells observed in Lck-Cre/ShcFFF mice arise as the result of incomplete Cre-mediated recombination at the \( \text{loxP} \) sites and, in turn, fail to express the ShcFFF transgene (i.e., essentially WT cells) (14–16). To determine whether peripheral T cells in DO11.10/CD4\(^{-}\)-Cre/ShcFFF mice were similar escapees without Cre-mediated deletion of the STOP cassette, we assessed the expression of the transgene-encoded, FLAG-tagged ShcFFF protein by immunoblotting. We were able to detect expression of FLAG-ShcFFF in the lysates from the thymus but not in the lysates of peripheral CD4\(^{+}\) T cells (Supplemental Fig. 2D). This suggested that, in DO11.10/CD4\(^{-}\)-Cre/ShcFFF mice, ShcA phosphorylation is required for the development of CD4\(^{+}\) T cells and that the peripheral T cells detected in these animals either do not express the ShcFFF transgene or express low levels of the protein that are undetectable via immunoblotting.

DO11.10 mice have an MHC class II–restricted TCR that recognizes the OVA peptide, and it was shown that in vivo i.p. injection of OVA peptide or ex vivo treatment of thymocytes with the OVA peptide leads to apoptosis of DP thymocytes (23, 40). To test a potential defect in signaling through the transgenic TCR in DO11.10/CD4\(^{-}\)-Cre/ShcFFF mice, we assessed peptide-induced apoptosis of DP thymocytes from these mice. In an ex vivo assay, thymocytes from DO11.10/CD4\(^{-}\)-Cre/ShcFFF mice had reduced apoptosis of DP thymocytes after 8 h and, in turn, increased recovery of DP thymocytes after 20 h (Fig. 2E), suggestive of reduced signaling via the transgenic TCR in these thymocytes. Collectively, these data suggest a defect in signaling through MHC class II–restricted TCRs in CD4\(^{-}\)-Cre/ShcFFF mice. These transgenic-TCR studies further implied a crucial role for ShcA phosphorylation during the DP to SP stage of development, because even the enhanced signaling through the transgenic TCR failed to rescue the developmental defects in CD4\(^{-}\)-Cre/ShcFFF mice.

Next, we investigated whether ShcA phosphorylation is required for CD8-lineage commitment and positive selection. In CD4\(^{-}\)-Cre/ShcFFF mice, we found a less profound defect in the development of CD8 SP thymocytes compared with CD4 SP thymocytes, with no apparent change in the percentage and a reduction in the absolute numbers of CD8 SP thymocytes (Table I, Supplemental Fig. 1E). To further address this in the context of transgenic TCRs, we crossed our CD4\(^{-}\)-Cre/ShcFFF mice to two different MHC class I–restricted mouse lines: OT-I– and H-Y–transgenic TCR lines. We found a defect in CD8 SP T cell development due to impaired ShcA-mediated signaling, with a slight decrease in overall thymic cellularity in CD4\(^{-}\)-Cre/ShcFFF mice expressing either the H-Y– or OT-I–transgenic TCR and a decrease in DP and CD8 SP thymocytes (Table I), with fewer transgenic-TCR\(^{+}\) CD8 SP thymocytes (Fig. 2C, 2D, Supplemental Fig. 2A).

To assess negative selection in thymocytes expressing the ShcFFF transgene, we analyzed thymocyte development in male H-Y/CD4\(^{-}\)/ShcFFF mice. The H-Y–transgenic mouse line encodes an MHC class I–restricted transgenic TCR that recognizes a male Ag (24). Although positive selection leads to the generation of CD8 SP thymocytes in female H-Y–transgenic mice, negative selection in male H-Y–transgenic mice leads to a de-
crease in overall thymic cellularity and very few DP and SP thymocytes (24). Negative selection appeared normal in H-Y/CD4-Cre/ShcFFF mice, with greatly reduced thymic cellularity due to negative selection and a decrease in the expression of CD4 and CD8 comparable to control male H-Y mice without ShcFFF (Supplemental Fig. 2C). Collectively, these data suggest that there is also a defect in the CD8 T cell lineage, although it is less profound than the one observed in the CD4 lineage, in the context of endogenous and transgenic TCRRs.

**CD4-Cre/ShcFFF mice do not display obvious defects within the DP thymocyte subset**

Defects in TCR signaling and positive selection often lead to a relative accumulation and increased percentage of DP thymocytes. However, we found that there was a reduction in the absolute number of DP thymocytes in CD4-Cre/ShcFFF mice (Table I). Therefore, we next assessed whether there was an additional defect within the DP compartment. First, we crossed the transgenic CD4-Cre/ShcFFF mouse line to the TCRα-deficient mouse line. Previous studies showed that thymocytes from TCRα-deficient mice can undergo normal DN → DP transition, but they are blocked at the DP stage by their inability to generate the αβTCR and undergo positive selection (6). We found only a slight difference in the total cellularity between TCRα-deficient CD4-Cre/ShcFFF mice and control TCRα-deficient mice (Fig. 2F). This suggested that the reduced thymic cellularity in CD4-Cre/ShcFFF mice is not likely to be reflective of a major defect in DP thymocytes and is mainly due to defects in the DP to SP transition. Furthermore, no defects in the survival of DP thymocytes isolated from CD4-Cre/ShcFFF-transgenic mice were revealed by annexin V and 7AAD staining of freshly isolated thymocytes or thymocytes undergoing spontaneous and anti-CD3–mediated apoptosis under ex vivo conditions (Supplemental Fig. 2E). Based on these data, we conclude that the primary defect in CD4-Cre/ShcFFF mice is at the stage of DP to SP transition, although a modest reduction in the number of DP thymocytes is observed.

**ShcA is downstream of p56Lck in DP thymocytes**

The protein tyrosine kinase p56Lck (Lck) is an important signaling molecule in T cells and signals downstream of both the preTCR and the TCR (16). Lck activity is required for preTCR and TCR signaling, because the constitutively active LckF505 transgene can rescue thymic development defects resulting from the absence of either the preTCR or TCR (41–44). Previous studies showed that, during TCR stimulation, Lck phosphorylates ShcA on the same three crucial tyrosine residues that are mutated in the ShcFFF transgene (16). To determine whether ShcA acts downstream of Lck and whether a constitutively active version of Lck (LckF505) can bypass the need for ShcA-mediated signaling, we crossed CD4-Cre/ShcFFF mice to the LckF505-transgenic mouse line. Despite the known augmented signaling in the LckF505 context (42–44), LckF505/CD4-Cre/ShcFFF mice also displayed significantly impaired T cell development compared with control mice expressing LckF505 alone, suggesting that LckF505 transgene is unable to fully rescue T cell development in the context of impaired ShcA signaling (Fig. 2G, Table I). These data suggest that ShcA acts downstream of Lck in DP thymocytes and that ShcA phosphorylation is necessary for Lck-mediated downstream signaling.

**CD4-Cre/ShcFFF mice display defects in thymic organization and cell trafficking**

Next, we performed H&E staining on thymic sections and found that the thymic architecture was considerably altered in CD4-Cre/ShcFFF mice, with a reduction in the area representing both the cortex and the medulla (Fig. 3A, 3B). Furthermore, the medulla was fragmented and disorganized, as is often seen in mice with defects in positive selection (31, 45), and the cortex/medulla area ratio was higher in CD4-Cre/ShcFFF mice (7.9) compared with control mice (3.6) (Fig. 3A).

Previous studies showed that ShcA acts downstream of CXCR4 and is required for CXCR4-mediated migration in T cells and thymocytes (17, 46). After positive selection, thymocytes upreg-
ulate the CCR7 receptor and migrate from the cortex toward the medulla via a gradient of CCL19 and CCL21 expressed by the medullary epithelial cells (47, 48). First, we found that ShcA was phosphorylated in thymocytes after stimulation with a CCR7 ligand (Supplemental Fig. 3A). We then assessed the migration of DP thymocytes from CD4-Cre/ShcFFF mice. Interestingly, the thymocytes from CD4-Cre/ShcFFF mice had reduced migration toward the CCR7 ligands CCL19 and CCL21 (Fig. 3D). Notably, this was not due to altered CCR7 expression on these thymocytes, because the postpositively selected TCR hi DP thymocytes from CD4-Cre/ShcFFF mice had expression levels of CCR7 that were comparable to those in control mice (Fig. 3C). These findings suggest that the thymic architecture is likely altered as a result of both defects in positive selection and the subsequent migration of DP thymocytes. It is noteworthy that the defect in CCR7 migration is likely underestimated in the above assay as many of the postpositively selected DP thymocytes may represent ‘escapees’ that still retain the STOP cassette and do not express the ShcFFF transgene.

**CD4-Cre/ShcFFF mice have a reduced peripheral T cell compartment**

Next, we investigated the peripheral T cell compartment in CD4-Cre/ShcFFF mice. They had a significant lymphopenia and a reduction in both the number and percentage of CD4+ and CD8+ T cells in the lymph nodes and spleen (Fig. 4A, 4B, Table I). Additionally, although the CD4/CD8 ratio was 2:1 in WT mice, it was ∼1:1 in CD4-Cre/ShcFFF mice (Fig. 4B). Lymphopenia did not appear to be due to improper accumulation of T cells in nonlymphoid peripheral tissues, as we also saw reduced numbers of T cells in other tissues, such as the lung and blood (data not shown). As seen previously in lymphopenic conditions, we also found a slight increase in the percentage of ‘memory-like’ CD62L hi CD44 hi CD4 T cells, which is often the result of homeostatic proliferation of T cells (Supplemental Fig. 3B). We next investigated whether the peripheral T cells in CD4-Cre/ShcFFF mice were Cre “escapees” by assessing the deletion of the STOP sequence. Although this PCR-based approach was nonquantitative, we were able to detect some excision of the STOP cassette in peripheral T cells from CD4-Cre/ShcFFF mice (Supplemental Fig. 1G). Thus, although some peripheral T cells have undergone Cre-mediated excision of the STOP cassette, many of them likely do not express the ShcFFF transgene at protein levels detectable via immunoblotting (Supplemental Fig. 1H). Additionally, we did not observe a proliferative defect in these

**FIGURE 4.** Phenotype of peripheral T cells in CD4-Cre/ShcFFF mice. (A) CD4 and CD8 cell surface staining of cells isolated from the lymph node or spleen of CD4-Cre/ShcFFF or control mice (left panel). Total cellularity and absolute numbers of CD4 and CD8 T cells from the spleen (right panel). n > 12 mice/genotype. (B) Percentage of CD4 and CD8 T cells in the spleen from control or CD4-Cre/ShcFFF mice (n > 18 mice/genotype). (C) Cell surface staining of CD4 and CD8 on cells isolated from the spleen of CD4-Cre/ShcFFF mice or control mice expressing the OT-I-transgenic TCR (left panels). Cell surface staining of CD4 and CD8 after gating on Vα3 hi splenocytes (middle panels). Staining of splenocytes with Vα3 to assess expression of the OT-I-transgenic TCR (right panels). n > 2 mice/genotype. (D) Generation of Rag 2p-eGFP CD4-Cre/ShcFFF mice and equation used to calculate the emigration ratio. (E) Quantification of the emigration ratio (GFP+ CD4 or CD8 splenocytes/GFP+ CD4 or CD8 SP thymocytes) from control or CD4-Cre/ShcFFF mice (n > 15 mice/genotype). *p < 0.05, **p < 0.01, ***p < 0.001.
peripheral T cells from CD4-Cre/ShcFFF mice after optimal anti-CD3/anti-CD28 stimulation ex vivo (Supplemental Fig. 3C). Furthermore, we did not observe any defects in IL-2 production after ex vivo stimulation of CD4\(^+\) T cells with anti-CD3, anti-CD3/anti-CD28, or PMA/ionomycin for 6 h (Supplemental Fig. 3D) or in a time-course of anti-CD3/anti-CD28 stimulation (Supplemental Fig. 3E).

A similar impairment was seen in peripheral T cells in CD4-Cre/ShcFFF mice on the background of MHC class I- and MHC class-II restricted transgenic TCRs, with reduced splenic cellularity and a reduction in CD8\(^+\) or CD4\(^+\) peripheral T cells (Table I). We found a substantial reduction in the numbers of CD8\(^+\) T cells in OT-I/CD4-Cre/ShcFFF mice, as well as a reduction in the percentage of cells staining for the OT-I-transgenic TCR (Fig. 4B).

Because CD4-Cre/ShcFFF mice have an overall reduction in thymic cellularity, with a further reduction in CD4 SP thymocytes, we initially thought it not too surprising that CD4-Cre/ShcFFF mice have a defect in the peripheral T cell compartment. Many genetic mouse models with disruption in genes required for positive selection, such as Themis- and Tespa-deficient mice, also have defects in the peripheral T cell compartment (31–33, 45). However, we found that the defect in peripheral T cell compartment appeared to be disproportionate and could not be due exclusively to the reduced thymic cellularity (Table I). We hypothesized that CD4-Cre/ShcFFF mice have an additional defect in the emigration of CD4 and CD8 SP thymocytes from the thymus into the periphery. Previously, it was shown that Rag2-eGFP-transgenic mice (which express enhanced GFP under the expression of the Rag2 promoter) can be used to identify recent thymic emigrants (RTEs) in the periphery (26, 49, 50). Furthermore, thymocytes from Rag2-eGFP mice express GFP in the late DN and DP stages of development, but GFP expression persists briefly in peripheral T cells and, therefore, GFP\(^+\) T cells in the periphery represent RTEs (Supplemental Fig. 3F) (26, 49, 50). To identify RTEs in the context of impaired ShcA signaling, we crossed CD4-Cre/ShcFFF mice to Rag2-eGFP–transgenic mice (Fig. 4D). We calculated the emigration ratio for Rag2-eGFP CD4-Cre/ShcFFF mice and Rag2-eGFP control mice by dividing the absolute number of GFP\(^+\) CD4\(^+\) or GFP\(^+\) CD8\(^+\) splenocytes by the number of GFP\(^+\) CD4 SP or GFP\(^+\) CD8 SP thymocytes (Fig. 4C). The emigration ratio for both CD4\(^+\) and CD8\(^+\) T cells was reduced in CD4-Cre/ShcFFF mice; therefore, CD4-Cre/ShcFFF mice appeared to have an additional defect in the emigration of mature SP thymocytes into the periphery (Fig. 4D, 4E). However, we did not observe an accumulation of these SP subsets in the thymus, which is often found in mice with defects in thymic emigration (51), most likely as a consequence of the concurrent defects in positive selection and a reduction in the numbers of CD4 SP and CD8 SP thymocytes.

**Lymphopenia persists in adult CD4-Cre/ShcFFF mice, and these mice display attenuated disease in the EAE model**

T cell developmental defects are often more striking in younger animals, and certain phenotypes in ShcA mutant mice (in the context of brain) are only present in young mice (52). Therefore, we asked whether the peripheral lymphopenia is transient or persists in adult CD4-Cre/ShcFFF mice. When we analyzed 10–12-wk-old CD4-Cre/ShcFFF mice, the reduced numbers of CD4\(^+\) and CD8\(^+\) T cells in the spleen and lymph nodes remained (Fig. 5A). Next, we immunized CD4-Cre/ShcFFF mice s.c. with MOG\(_{35–55}\), and collected the draining lymph nodes on day 8 after immunization. We found a reduced number and percentage of CD4\(^+\) and CD8\(^+\) T cells in CD4-Cre/ShcFFF mice after immunization with MOG\(_{35–55}\) (Fig. 5B). Therefore, the peripheral lymphopenia in these mice persists with age, as well as after immunization.

To test whether the lymphopenia of CD4-Cre/ShcFFF mice leads to attenuated disease in the context of autoimmunity, we chose to use the CD4\(^+\) T cell–driven EAE model (53), which recapitulates many of the clinical, pathological, and immunological aspects of the human disease MS. In MS and EAE, infiltration of autoreactive T cells into the CNS leads to inflammation, demyelination and progressive disability (53, 54). Furthermore, 8 d after immunization with the MOG\(_{35–55}\) peptide, CD4-Cre/ShcFFF mice exhibit reduced numbers of CD4\(^+\) and CD8\(^+\) T cells in the draining lymph nodes (Fig. 5B). We immunized CD4-Cre/ShcFFF and control mice with MOG\(_{35–55}\) to induce EAE and monitored disease severity and weight loss for 28 d. We found that CD4-Cre/ShcFFF mice developed strikingly attenuated disease, with a significant reduction in disease severity and weight loss, as well as improved survival (Fig. 6A) (in three independent experiments with a combined total of n = 16 mice/genotype, p < 0.0001 for clinical scores, two-way ANOVA). Additionally, CD4-Cre/ShcFFF mice had a lower incidence of disease, with 25% never exhibiting any signs of disease (defined as score ≥ 1), as well as a lower overall maximum score and disease index (area under the curve) (Fig. 6A, Table II). Of mice that did exhibit signs of disease, the day of onset was significantly delayed from an average of 10.1 d for control mice to an average of 14.1 d for CD4-Cre/ShcFFF mice (Table II). We also found that CD4-Cre/ShcFFF–transgenic mice developed attenuated disease in a model of suboptimal EAE induction (Supplemental Fig. 4D).

At the chronic stage of disease (day 28), we assessed the composition of the immune infiltrate in the CNS by analyzing the cells isolated from the brains and spinal cords of CD4-Cre/ShcFFF and control mice. CD4-Cre/ShcFFF mice had overall fewer mononuclear cells isolated from the brain and spinal cords (Supplemental Fig. 4B). There was a decrease in the percentage of CD4\(^+\) and B220\(^+\) lymphocytes in CD4-Cre/ShcFFF mice compared with control mice (Fig. 6B, 6C). Overall, CD4-Cre/ShcFFF mice had a trend of reduced total numbers of CD4\(^+\) T cells, B cells, macrophages, and microglia in the brain and spinal cord at the chronic stage of the disease (Supplemental Fig. 4B). Histological analysis by H&E staining and immunohistochemistry confirmed that CD4-Cre/ShcFFF mice had fewer loci of immune infiltration and fewer CD3\(^+\) T cells in the spinal cords (Fig. 6E).

Previous studies showed that CD4\(^+\) T cells and, in particular, Th17 cells, play a crucial role in the initiation and development of MS and EAE. The peripheral lymphopenia in CD4-Cre/ShcFFF–transgenic mice clearly contributed to attenuated disease in the EAE model. We next investigated whether the peripheral T cells from CD4-Cre/ShcFFF–transgenic mice had a defect in ex vivo Th17 and Th1 skewing. CD4\(^+\) T cells isolated from CD4-Cre/ShcFFF–transgenic mice had an overall defect in expansion when cultured under Th17- and Th1-skewing conditions (Fig. 6E, Supplemental Fig. 4C). We found fewer absolute numbers of Th17 and Th1 cells after skewing CD4\(^+\) T cells isolated from CD4-Cre/ShcFFF–transgenic mice compared with those from lintermiate controls (Fig. 6E). Although there was an overall defect in their expansion, we found that peripheral CD4\(^+\) T cells from CD4-Cre/ShcFFF transgenic mice cells that differentiated into the Th17 and Th1 lineage had no defects in the production of IL-17 or IFN-\(\gamma\) (Fig. 6E). We next investigated whether the CD4\(^+\) T cells skewed under Th17- or Th1-skewing conditions were Cre “escapes” by assessing deletion of the STOP sequence. We detected excision of the STOP cassette, which suggests that at least some of these Th1 and Th17 cells expressed the ShcFFF transgene (data not shown). Collectively, these data demonstrated that lymphopenia and the
impaired expansion of Th17 and Th1 cells in CD4-Cre/ShcFFF mice led to a net functional deficit in CD4+ T cells that manifested as attenuated disease in the EAE model.

Discussion
In this study, we demonstrated a requirement for ShcA function in late T cell development, beyond its previously reported requirement in preTCR signaling and progression through the β-selection checkpoint (14, 15). To bypass the requirement for ShcA in early T cell development, we generated CD4-Cre/ShcFFF–transgenic mice, which express the ShcFFF transgene from the DN4/DP stage of development (21). We found that CD4-Cre/ShcFFF–transgenic mice show two notable defects in late T cell development: a defect in the absolute number and percentage of CD4 SP thymocytes and a persistent peripheral lymphopenia.

Productive signaling through the TCR in DP thymocytes is required for positive selection and the development of mature SP thymocytes. Additionally, sustained signaling through the TCR is essential for the development of CD4 SP thymocytes, and the CD4 SP compartment is more sensitive to defects in TCR signaling than is the CD8 SP compartment (1). Through several approaches, we found that impaired ShcA-mediated signaling affects the development of mature CD4 SP thymocytes. We also found that the impairment in positive selection and development of CD4 SP thymocytes due to impaired ShcA signaling is more profound than the development of CD8 SP thymocytes, although the CD8 SP compartment is also impaired. To overcome the issue that developmental defects in positive selection can be masked by compensatory changes in the TCR repertoire, we also analyzed CD4-Cre/ShcFFF mice in the context of transgenic TCR expression and found similar defects during positive selection. Furthermore, antigenic-peptide–induced signaling via the transgenic TCR in DP thymocytes also was decreased in DO11.10/CD4-Cre/ShcFFF–transgenic mice. Lastly, although the main defects in thymic development in CD4-Cre/ShcFFF–transgenic mice were found in the SP compartment, we also found a slight reduction in the number of DP thymocytes, which may be due to impairment in the transition from the ISP to the DP stage of thymocyte development. Collectively, these data demonstrated that ShcA phosphorylation is required for optimal signaling through the TCR in DP thymocytes for their progression into CD4 and CD8 SP T cells. In addition to the TCR and preTCR, many chemokine and cytokine...
receptors are critical for normal thymocyte development and survival. Previously, we demonstrated that ShcA is required for optimal signaling through CXCR4 during the DN stage of development (17), and in this study we found decreased CCR7-mediated chemotaxis in the DP compartment. Therefore, it is possible that ShcA-mediated signaling through other chemokine and cytokine receptors also contributes to the thymic development defects found in CD4-Cre/ShcFFF-transgenic mice.

FIGURE 6. CD4-Cre/ShcFFF mice are mostly resistant to EAE. (A) Mean clinical scores, survival, weight loss, and disease index of CD4-Cre/ShcFFF and control mice immunized for EAE. Compiled data are from three experiments with a total of n = 16 mice/genotype. (B) Cell surface staining of CD45, CD4, CD11b, and B220 on cells isolated from the spinal cord and brain of CD4-Cre/ShcFFF or control mice on day 28 after MOG immunization (n = 3 mice/genotype). (C) Percentage of CD4+ and B220+ cells within the CD45+ cells isolated from the spinal cord and brain of CD4-Cre/ShcFFF or control mice on day 28 after MOG immunization (n = 3 mice/genotype). (D) Immunohistochemistry for CD3 and H&E staining on spinal cord sections from CD4-Cre/ShcFFF or control mice on day 28 after immunization with EAE (left panels). Quantification of CD3+ T cells from spinal cord sections (n = 6 mice/genotype) (right panel). Original magnification ×4 and ×20 for top and bottom panels, respectively. (E) Normalized fold expansion of CD4 T cells under Th17- or Th1-skewing conditions from CD4-Cre/ShcFFF or control mice (left panels). Representative IL-17 and IFN-γ intracellular staining after Th17 or Th1 skewing (right panels). n = 5 mice/genotype. *p < 0.05, **p < 0.01, ***p < 0.001.

CD4-Cre/ShcFFF mice also had impairment in the peripheral T cell compartment, with reduced numbers and an alteration in the ratio of CD4+/CD8+ T cells. Peripheral lymphopenia can be explained, in part, by the defects in the thymic CD4 SP and CD8 SP compartments. However, we found that the defect in the peripheral compartment is disproportionate to the thymic defect. Our data suggest that the peripheral compartment in CD4-Cre/ShcFFF mice also may be partially altered as a result of reduced emigration of thymocytes into the periphery. Despite extensive investigation, we were unable to find a definitive explanation for the disproportionate peripheral defect; there was no significant defect in ex vivo sphingosine 1-phosphate-mediated migration of CD4+ CD62Lhi SP thymocytes (data not shown), and we did not observe an apparent survival or proliferative defect in peripheral T cells. However, we found that T cells isolated from CD4-Cre/ShcFFF mice had impaired expansion under Th17- and Th1-skewing conditions.

Table II. Parameters of disease in CD4-Cre/ShcFFF mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence (%)</th>
<th>Day of Onset (mean ± SD)</th>
<th>Maximum Clinical Score (mean ± SD)</th>
<th>Disease Index (mean ± SD)</th>
<th>Mortality (%)</th>
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<tr>
<td>Control</td>
<td>100 (16/16)</td>
<td>10.1 ± 3.0</td>
<td>4.5 ± 0.45</td>
<td>68.1 ± 19.8</td>
<td>37.5 (6/16)</td>
</tr>
<tr>
<td>CD4-Cre/ShcFFF</td>
<td>75 (12/16)</td>
<td>14.1 ± 5.4</td>
<td>2.4 ± 1.6</td>
<td>20.5 ± 20.6</td>
<td>6.3 (1/16)</td>
</tr>
</tbody>
</table>
conditions, suggesting that cytokine signaling required for the lineage-specific differentiation of CD4 T cells also may be dependent, at least in part, on ShcA phosphorylation. Importantly, peripheral lymphopenia and impaired expansion in CD4-Cre/ShcFFF mice result in functional impairment, because CD4-Cre/ShcFFF mice develop attenuated disease in the CD4 T cell–driven EAE mouse model. We cannot definitively discern whether the majority of peripheral T cells are Cre “escapes” or whether the peripheral CD4-Cre/ShcFFF T cells express very low levels of the transgene (detectable by the excision of the STOP cassette but not by immunoblot of the transgene-encoded protein). Because CD4 expression was reported in some cells of the CNS (55), we cannot exclude whether ShcFFF transgene expression in other cell types may have also contributed to the attenuated disease in the EAE model. However, the persistent peripheral lymphopenia and impaired expansion of T cells under Th17- and Th1-skewing conditions led us to conclude that ShcA phosphorylation directly contributes to peripheral T cell function and to the attenuated disease in the EAE model in CD4-Cre/ShcFFF transgenic mice.

Development of βα T cells critically depends on productive signaling through the preTCR in DN thymocytes and the TCR in DP thymocytes, as well as other chemokine and cytokine receptors. Disruption of the components of the preTCR and TCR or of downstream signaling molecules leads to impairment in T cell development and results in the block of developmental progression. Together with our previous studies on the essential role of the adapter protein ShcA in signaling via the preTCR, the current studies on the role of ShcA in signaling via αβTCR suggest that it critically contributes to the progression through β-selection and positive-selection checkpoints.

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Disclosures
The authors have no financial conflicts of interest.

References


