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Histone Deacetylase 3 Is Required for T Cell Maturation

Fan-Chi Hsu,* Paul J. Belmonte,* Megan M. Constans,* Meibo W. Chen,* Douglas C. McWilliams,* Scott W. Hiebert,† and Virginia Smith Shapiro*

Recent thymic emigrants are newly generated T cells that need to undergo postthymic maturation to gain functional competency and enter the long-lived naive T cell pool. The mechanism of T cell maturation remains incompletely understood. Previously, we demonstrated that the transcriptional repressor NKAP is required for T cell maturation. Because NKAP associates with histone deacetylase 3 (HDAC3), we examined whether HDAC3 is also required for T cell maturation. Although thymic populations are similar in CD4-cre HDAC3 conditional knockout mice compared with wild-type mice, the peripheral numbers of CD4+ and CD8+ T cells are dramatically decreased. In the periphery, the majority of HDAC3-deficient naive T cells are recent thymic emigrants, indicating a block in T cell maturation. CD55 upregulation during T cell maturation is substantially decreased in HDAC3-deficient T cells. Consistent with a block in functional maturation, HDAC3-deficient peripheral T cells have a defect in TNF licensing after TCR/CD28 stimulation. CD4-cre HDAC3 conditional knockout mice do not have a defect in intrathymic migration, thymic egress, T cell survival, or homeostasis. In the periphery, similar to immature NKAP-deficient peripheral T cells, HDAC3-deficient peripheral T cells were bound by IgM and complement proteins, leading to the elimination of these cells. In addition, HDAC3-deficient T cells display decreases in the sialic acid modifications on the cell surface that recruit natural IgM to initiate the classical complement pathway. Therefore, HDAC3 is required for T cell maturation. *The Journal of Immunology, 2015, 195: 1578–1590.

T cells are critical to mounting adaptive immune responses against pathogens and Ags. The generation of useful and self-tolerant T cells is tightly controlled, and developing T cells must successfully navigate several checkpoints: β-selection, positive selection, negative selection, and maturation. T cell maturation initiates after thymocytes are positively selected, continues in the periphery, and is critical for T cells to gain functional competency and enter into the long-lived mature naive T cell (MNT) pool (reviewed in Refs. 1, 2). Several functional and phenotypic changes occur during T cell maturation. Functionally, upon Ag stimulation, immature single-positive (SP) thymocytes are susceptible to apoptosis and do not produce cytokines, whereas in response to the same antigenic signals, mature peripheral T cells activate, proliferate, and produce cytokines. Phenotypically, SP thymocytes are traditionally subdivided into “semimature” CD24hi Qa2lo and “mature” CD24loQa2hi populations. The expression of chemokine receptors, CCR9, CCR7, and CCR4, changes during SP thymocyte maturation to ensure their transit from the cortex to medulla (3–6). Thymic maturation also involves upregulation of IL-7Rα, which is required for T cell survival and homeostasis in the periphery (7). In addition, CD24hiQa2hi mature SP thymocytes upregulate the transcription factor KLF2, sphingosine-1–phosphate receptor 1 (S1P1), and CD62L and downregulate CD69 for thymic egress and entry into peripheral lymphoid organs (8, 9). The successful egress of SP thymocytes and entry into the periphery is not the final step in T cell development. Rather, these recent thymic emigrants (RTEs) continue their postthymic maturation. Maturation requires physical entry of RTEs into secondary lymphoid organs (10). Although the cells and receptors that produce signals required for RTEs to mature are not known, maturation is independent of either antigenic signals through the TCR (11) or homeostasis signals through IL-7Rα (12, 13). Recent work has demonstrated that the transcriptional regulators Zpf335, Bptf, and NKAP are required for T cell maturation (14–16). Mice with a mutation in the Zinc-finger containing protein Zfp335 have a defect in accumulation of naive T cells, which result from impaired maturation in SP thymocytes and RTEs (14). Bptf, a chromatin-remodeling factor, is required for thymocyte maturation postpositive selection (15). Lck-cre Bptf conditional knockout (cKO) mice cannot generate mature TCRhiCD24loCD69loCD5hi thymocytes and have reduced numbers of peripheral T cells.

Previously, we demonstrated that the transcriptional repressor NKAP is required for T cell maturation (16). Although there are no gross alterations in the numbers or proportions of double-negative (DN), double-positive (DP), and SP thymocyte populations, there are few naive peripheral T cells in CD4-cre NKAP cKO mice. In the absence of NKAP, the naive peripheral T cell pool is composed almost entirely of phenotypically and functionally immature RTEs. NKAP-deficient RTEs do not die by apoptosis, but rather are eliminated by complement, as demonstrated by C3 deposition on the cell surface. C4 and C1q are also bound to NKAP-deficient T cells, indicating activation of the classical arm of the complement pathway (17). Because wild-type (WT) thymocytes mature before thymic egress, they increase incorporation of sialic acids into glycoproteins and glycolipids at the cell surface. This addition of sialic acid is critical to mature lymphocyte survival in the periphery, because stripping cell-surface sialic acids...
by neuraminidase in mature peripheral lymphocytes leads to the binding of natural IgM and complement fixation. We showed that there was an increase in IgM binding, and complement protein depositions were found on NKAP-deficient T cells, largely as a consequence of impaired sialic acid incorporation, especially through α2-8 linkage. As T cells mature, they also upregulate expression of the complement inhibitor DAF/CD55 on the cell surface. DAF/CD55 upregulation was also defective in NKAP-deficient RTEs, which likely contributes to the increase in complement-mediated elimination.

NKAP is a transcriptional repressor that associates with DNA by chromatin immunoprecipitation (18, 19) but lacks a DNA binding domain. NKAP is a negative regulator of the Notch pathway, but the block in T cell maturation when NKAP is absent is independent of its role as a negative regulator of the Notch pathway (16). Therefore, NKAP must associate with additional transcriptional regulators to bind to DNA and regulate gene expression. NKAP associates with histone deacetylase 3 (HDAC3) and requires HDAC3 for its ability to repress transcription (18). HDACs are divided into four families (class I: HDAC1, 2, 3, and 8; class II: HDAC4, 5, 6, 7, 9, and 10; class III: SIRT1–7; class IV: HDAC11) and are important for regulating gene expression, chromatin structure, and genomic stability. HDAC3 belongs to the class I HDAC family and has 60% amino acid identity with HDAC1 and HDAC2. HDAC3 is a component of the NCoR/SMRT repressor complex, and can also associate with class II HDACs (20). Somatic deletion of HDAC3 leads to embryonic lethality, and tissue-specific deletion of HDAC3 results in profound hypertrophy in liver and heart (21, 22). Interestingly, deletion of HDAC3 in hematopoietic compartment using a Vav-cre model revealed the role of HDAC3 in maintaining genomic integrity in hematopoietic progenitor cells (23, 24), but a role for HDAC3 in T cell development and maturation has not yet been examined.

In this study, we report that HDAC3, like its binding partner NKAP, is essential for T cell maturation. Thymic development is intact in CD4-cd HDAC3 cKO mice, although there are few peripheral T cells. The majority of HDAC3-deficient naïve T cells are RTEs, suggesting a block in postthymic maturation preventing HDAC3-deficient RTEs from entering into the long-lived naïve T cell pool. As T cells continue maturation in the periphery, they acquire the capability to produce TNF upon TCR activation, referred to as TNF licensing (25). In this article, we show that HDAC3-deficient peripheral T cells have a defect in functional maturation, because these cells cannot produce TNF upon TCR stimulation. The maturation defect in CD4-cd HDAC3 cKO mice is independent of intrathymic migration, thymic egress, or homeostasis. Substantial IgM and complement protein binding and a defect in sialic acid modification were found on the cell surface of peripheral HDAC3-deficient T cells and were also found in immature NKAP-deficient T cells. Taken together, we conclude that HDAC3 is an essential regulator of postthymic T cell maturation.

Materials and Methods

Mice

The floxed HDAC3 and floxed NKAP mice were described previously (18, 22). Rag1-1GFP knock-in mice were generated and provided by Dr. Nobuo Sakaguchi (26). C57BL/6 (B6) mice were purchased from The Jackson Laboratory. B6.SJL mice were purchased from National Cancer Institute Frederick, WT littermates included either HDAC3 floxed/cre, HDAC3 WT/cre2, or HDAC3 WT/cre mice. All of these littermate controls were indistinguishable from WT B6 mice. Littermates were used whenever possible as controls, and when not available, age-matched controls were used. All animal experiments were approved by and performed under the guidelines from the Institutional Animal Care and Use Committee at Mayo Clinic. All mice were housed in a pathogen-free environment and were analyzed between 8 and 12 wk of age.

Cell preparation and flow cytometry

Single-cell suspensions of thymocyte and splenocytes were obtained from WT B6, CD4-cd HDAC3 cKO, Rag1-1GFP WT, Rag1-1GFP CD4-cd HDAC3 cKO, Rag1-1GFP HDAC3-deficient NKAP cKO, and bone marrow chimeras (BMCs). The following Abs were purchased from BD Bioscience, Biolegend, ebioscience, R&D Systems, or Toxobio Bioscience: CD4 (RM4-5; GK1.5), CD45.1 (A20), CD69 (53-67), CD24 (M1/69), CD44 (IM7), CD55 (RIK0-3), CD262L (ME1-14), CD69 (1H9), CCR4 (2G12), CCR7 (4B12), SCFR (9B1), IgM (RMM-1), IL-7Rα (AT3R4), Bcl-2 (BCL/104), TNF-α (MP6-XT22), and Qa2 (695H1-9-9). Biotinylated Abs for complement C1q (C57RCHB), complement C4 (RMC16D2), and complement C3 (RMC11H9) were purchased from Cedarlane. Biotinylated plant lectins peanut agglutinin (PNA), Sambucus nigra bark lectin (SNBL), and Maackia amurensis lectin II (MAL II) were purchased from Vector Laboratories. S1P1 and recombiant mouse Siglec E-Fc were purchased from R&D Systems, the mouse anti-rat IgG2a PE (2a-21B2; ebioscience) and goat anti-mouse IgG2a PE (Jackson ImmunoResearch) were used as secondary Abs, respectively. For the complement experiments, freshly harvested splenocytes were incubated in GVB+ buffer (Complement Technology) for 1 h at room temperature; then cells were washed once with FACS buffer and stained with indicated Abs. For intracellular staining, cells were fixed with FACS buffer with fixable viability dye (ebioscience) before surface stain. Following the surface stain, cells were fixed and permeabilized with IC Fixation Buffer (ebioscience) and then stained with Bcl-2 (BCL/104; Biolegend). All data were doublet excluded with forward light scatter width/height and side light scatter width/height, and dead cells were excluded using fixable viability dye before analysis. Samples were acquired using an LSR II cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star).

Cell stimulation and intracellular cytokine assays

Total splenocytes were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS, 100 μM penicillin-streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies), and stimulated as follows. For TNF-α intracellular staining, 5 × 10^6 thymocytes or splenocytes were stimulated with or without plate-coated anti-CD3ε (10 μg/ml, 145-2C11; BioXCell) plus soluble anti-CD28 (1 μg/ml, 37.51; BD Bioscience) in the presence of Protein Transport Inhibitor Cocktail (ebioscience) for 17 h at 37°C in 5% CO2. After incubation, cells were harvested and washed once with 1× PBS, and stained with fixable viability dye before surface stain. After the surface stain, cells were fixed and permeabilized with IC Fixation Buffer (ebioscience) and then stained for intracellular TNF-α (MP6-XT22; ebioscience).

Generation of radiation chimeras

Mixed BMCs were generated by i.v. injecting 4 × 10^6 cells from either mixes of Rag1-1GFP WT (CD45.2+/+)/B6.SJL (CD45.1-/-) or Rag1-1GFP CD4-cd HDAC3 cKO (CD45.2+/-)/B6.SJL (CD45.1-/-) at a 50:50 ratio into lethally irradiated congenic B6.SJL (CD45.1-/-) recipients. Recipient mice received 1000 rad and were rested for at least 5 h before injection. Chimeric mice received the antibiotic enrofloxacin in their drinking water for 3 wk and were analyzed after 10 wk.

FACS sorting and real-time quantitative PCR analysis of relative mRNA expression

To isolate CD4+ T cells, we harvested splenocytes from Rag1-1GFP WT and Rag1-1GFP CD4-cd HDAC3 cKO mice and negatively selected them from biotin-labeled populations (the biotin-mixture contained B20, CD11b, Ter119, Gr-1, CD19, CD11c) using magnetic bead selection (Miltenyi Biotec). WT splenocytes lacking the Rag1-1GFP reporter were used to define gates for Rag1-1GFP+ (RTE) and Rag1-1GFP- (MNT) populations. All sorting was done on a FACS Aria (BD Bioscience). mRNA was later isolated from sorted CD4+ RTEs (CD44+CD62L+Rag1-1GFP+), CD4+ MNTs (CD44+CD62L+Rag1-1GFP-), and memory CD4+ T cells (CD44+CD62L+Rag1-1GFP−), with an RNeasy Mini kit (Qiagen). cDNA was generated and amplified with the Ovation PicoSL WTA kit (NuGen). mRNA expression was analyzed using TaqMan probes (Applied Biosystems) for HDAC3, Sirt5, Slit1a, Slit2a, and 18S rRNA as an internal control. An ABI RT-PCR StepOne Plus System (Applied Biosystems) was used, and gene expression was calculated via the 2-ΔΔ cycle threshold method.

Statistical analysis

Samples were analyzed with the Student t test using GraphPad Prism software. Statistical significance was defined as a p value < 0.05. Data are shown as mean ± SEM.
HDAC3 IS REQUIRED FOR T CELL MATURATION

A

Thymus

WT

CD4-cre

HDAC3 cKO

Spleen

WT

CD4-cre

HDAC3 cKO

B

TCRβ

CD24

CD69

Percent of Max

Percent of Max

Percent of Max

Percent of Max

C

WT

CD4-cre

HDAC3 cKO

D

Thymic cellularity

Spleenic cellularity

Relative HDAC3 expression

E

CD4+

naive CD4+

naive CD4+

RTEs

F

Rag1-GFP CD4 SP

Rag1-GFP CD4-cre HDAC3 cKO CD4 SP

CD4

CD62L

CD55

CD4

CD62L

CD55

CD24

CD55

CD24

CD55

CD24

CD55
Results

CD4-cre HDAC cKO mice have a block in T cell maturation leading to a severe defect in peripheral T cell numbers

To determine the role of HDAC3 in T cells, we generated CD4-cre HDAC3 cKO mice, which delete HDAC3 at the DP stage. Previously, we demonstrated that CD4-cre HDAC3 cKO mice have a block in invariant NKT (iNKT) cell development, whereas conventional T cell development in the thymus is normal (27). The proportions and absolute numbers of DN, DP, CD4 SP, and CD8 SP thymocytes were similar between WT and CD4-cre HDAC3 cKO mice (Fig. 1A). Positive selection, examined using TCRβ and CD69, and thymic maturation, examined using TCRβ and CD24, were similar between WT and CD4-cre HDAC3 cKO mice (Fig. 1B). CD5 and TCRβ are also upregulated similarly in CD4 and CD8 SP thymocytes from CD4-cre HDAC3 cKO mice (Fig. 1B). However, the proportions and absolute numbers of both CD4 and CD8 peripheral T cells were severely decreased, ~10- and 6-fold, respectively (Fig. 1C). Examination of the peripheral CD4 T cell pool demonstrated that HDAC3 was efficiently deleted in naive splenic T cells (both RTE and MNT), but a small proportion of memory T cells expressed HDAC3, indicating that these escapees from cre-mediated deletion had likely undergone homeostatic proliferation resulting in expression of memory markers (Fig. 1D).

RTEs can be distinguished from long-lived MNTs by differential expression of CD45RB and CD55, which are upregulated concurrently with peripheral T cell maturation (17). When naive splenic T cells were examined, CD55 expression was ~10-fold lower in HDAC3-deficient T cells as compared with WT (Fig. 1E). Both WT and CD4-cre HDAC3 cKO naive T cells upregulated CD45RB, indicating that HDAC3 is required for some, but not all, events that occur during T cell maturation. Because maturation is required for newly produced RTEs to survive and enter the long-lived naive T cell pool, we examined RTEs by interbreeding CD4-cre HDAC3 cKO mice with Rag1-GFP reporter mice (26), which have GFP knocked into the Rag1 locus. Because of the stability of GFP, even though its transcription ceases at the DP stage, GFP protein is detectable by flow cytometry for 2–3 wk after newly produced T cells leave the thymus (11). Thus, the Rag1-GFP reporter marks RTEs in the periphery. In contrast with Rag1-GFP reporter mice in which the majority of peripheral naive T cells were MNTs, RTEs comprised the majority of peripheral naive T cells from Rag1-GFP CD4-cre HDAC3 cKO mice (Fig. 1E). RTEs were also examined for Qa2, a marker that increases with maturation (28). Qa2 expression was higher in HDAC3-deficient RTEs as compared with WT, again indicating that HDAC3 regulates some, but not all, events that occur during T cell maturation (Fig. 1E). Given the block in T cell maturation in the periphery, we examined whether this block initiated in the thymus. As SP thymocytes mature, they downregulate CD24 and upregulate Qa2 and CD55. Consistent with the results in the periphery, a defect in the upregulation of CD55 in HDAC3-deficient CD4 SP thymocytes was observed, although CD24 downregulation was normal and Qa2 upregulation was enhanced (Fig. 1F). Therefore, HDAC3 deficiency results in a block in T cell maturation at the RTE stage that leads to a decrease in peripheral T cell numbers, even though no defect in expression of two markers typically used to define mature T cells, Qa2 and CD24, was observed.

HDAC3 is required for TNF licensing during maturation

As T cells mature in the periphery, they also acquire functional competency to produce cytokines upon stimulation through TCR and CD28, including TNF-α (“TNF licensing”) (25). SP thymocytes produce little TNF-α when stimulated, as compared with peripheral MNTs, with RTEs having an intermediate level of TNF-α production. Splenocytes from Rag1-GFP WT or Rag1-GFP CD4-cre HDAC3 cKO mice were either left unstimulated or stimulated overnight with TCR/CD28 and examined for TNF-α production, to determine whether there was a functional block in maturation. As a result of fixation and permeabilization before intracellular staining, GFP fluorescence was diminished as compared with unfixed and permeabilized controls. Thus, the gating for RTEs based on GFP was based on WT mice that lacked the Rag1-GFP reporter, as shown in Fig. 2. Although the majority of CD4 and CD8 RTEs and MNTs from Rag1-GFP WT mice produced high levels of TNF-α upon stimulation, significantly fewer HDAC3-deficient RTEs produced TNF-α under these conditions (Fig. 2). Therefore, there is also a functional block in T cell maturation in the absence of HDAC3.

The defect in T cell maturation in CD4-cre HDAC3 cKO mice is cell intrinsic

To determine whether the defect in T cell maturation in HDAC3-deficient T cells was cell intrinsic, we generated mixed BMCs with a combination of CD45.2+Rag1-GFP WT or CD45.2+Rag1-GFP CD4-cre HDAC3 cKO mice mixed with CD45.1+ B6.SJL total bone marrow cells. The mice were analyzed 10 wk later. As shown in Fig. 3A, the relative chimerism in the control CD45.2+Rag1-GFP

FIGURE 1. Block in T cell maturation in CD4-cre HDAC3 cKO mice. (A) Thymocytes and splenocytes were stained with CD4 and CD8 to examine T cell development and peripheral T cell numbers. Shown are representative FACS plots from a total of 12 WT and 7 CD4-cre HDAC3 cKO mice from seven independent experiments. (B, left two columns) Total thymocytes from Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice were stained with TCRβ, CD69, and CD24 to examine positive selection. Cells were pregated on the Rag1-GFP+ population to exclude recirculating mature T cells. CD69mid TCRβmid are denoted as preselected thymocytes; CD69hi TCRβhi are denoted as postselected thymocytes; CD69 med TCRβmed are denoted as mature thymocytes. Right two columns, The expression levels of CD5 and TCRβ were examined in DP, CD4 SP, and CD8 SP thymocytes from Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice. Cells were pregated on the Rag1-GFP+ population, and the thymocytes were gated by CD4 and CD8 as shown in (A), left. (C) The absolute number of DN, DP, CD4 SP, CD8 SP thymocytes, and peripheral CD4+ and CD8+ T cells in the spleen are shown. Please note that thymic cellularity of DN, DP, and SP populations is shown as a log plot. Numbers shown are the average of 12 WT and 7 CD4-cre HDAC3 cKO mice, from seven independent experiments. Error bars represent SEM, and significance was determined using an unpaired Student t test. (D) CD4+ RTEs, MNTs, and memory cells from Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice were sorted and examined for the expression level of HDAC3 mRNA by Q-PCR. Light gray bar indicates cells from Rag1-GFP WT, whereas dark gray bar indicates cells from Rag1-GFP CD4-cre HDAC3 cKO mice. Numbers shown are the average of three WT and three CD4-cre HDAC3 cKO mice from two independent experiments. (E) Naive splenic CD4+ T cells from WT and CD4-cre HDAC3 cKO mice were examined for T cell maturation using CD45RB and CD55. Percentage RTEs in the peripheral naive T cell pool was examined using Rag1-GFP reporter. The expression of Qa2 was examined in Rag1-GFP+ RTEs. Shown are representative FACS plots from a total of 11 WT and 6 CD4-cre HDAC3 cKO mice from six independent experiments. (F) T cell maturation was examined in the newly generated CD4 SP thymocytes in Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice using CD24, Qa2, and CD55. For CD55 overlays, the solid gray histogram represents semimature and the black histogram represents mature CD4 SP thymocytes. Shown are representative FACS plots from a total of five WT and four CD4-cre HDAC3 cKO mice from five independent experiments.
WT/CD45.1+B6.SJL mice was similar across all populations examined. However, in the CD45.2+Rag1-GFP CD4-cre HDAC3 cKO/CD45.1+B6.SJL mixed radiation chimeras, the relative chimerism of the Rag1-GFP CD4-cre HDAC3 cKO mice decreased with T cell maturation. The relative chimerism of HDAC3-deficient T cells in the CD4+ naive T cell pool was 10-fold lower than the relative chimerism in the granulocyte pool. Relative chimerism in the CD4-cre HDAC3 cKO mixed chimera was reduced from 78.1% in DP to 56.2% in CD4 SP thymocytes. However, we previously demonstrated that HDAC3 is required for development of iNKT cells (27). Thus, the decrease in chimerism from DP to CD4 SP in CD4-cre HDAC3 cKO mice decreased with T cell maturation. The relative chimerism of HDAC3-deficient T cells in the CD4+ naive T cell pool was 10-fold lower than the relative chimerism in the granulocyte pool. Relative chimerism in the CD4-cre HDAC3 cKO mixed chimera was reduced from 78.1% in DP to 56.2% in CD4 SP thymocytes. However, we previously demonstrated that HDAC3 is required for development of iNKT cells (27). Thus, the decrease in chimerism from DP to CD4 SP in CD4-cre HDAC3 cKO mice decreased with T cell maturation. The relative chimerism of HDAC3-deficient T cells in the CD4+ naive T cell pool was 10-fold lower than the relative chimerism in the granulocyte pool. Relative chimerism in the CD4-cre HDAC3 cKO mixed chimera was reduced from 78.1% in DP to 56.2% in CD4 SP thymocytes.

Thymic egress is normal in CD4-cre HDAC3 cKO mice

Mice with a defect in thymic egress, such as S1P1-deficient mice, aberrantly accumulate CD24loQa2hi mature thymocytes within the SP population (8). Because CD4-cre HDAC3 cKO mice have increased proportions of CD24loQa2hi mature SP thymocytes, genes critical for thymic egress were examined. The upregulation of CD62L and S1P1 and the downregulation of CD69 concurrent with decreasing CD24 expression were similar between WT and CD4-cre HDAC3 cKO mice (Fig. 4A). The expression of CCR4, CCR7, and CCR9, which change during thymic maturation to regulate movement from the cortex to medulla, was also similar between WT and CD4-cre HDAC3 cKO mice (Fig. 4B). Thus, expression of proteins that regulate chemotaxis and egress in SP thymocytes was not altered in the absence of HDAC3. To confirm that egress occurs with similar timing, we compared Rag1-GFP reporter expression in DP, semimature, and mature CD4 SP thymocytes. If the increased frequency of mature CD4 SP thymocytes

FIGURE 2. HDAC3 is required for functional maturation and TNF licensing. Splenocytes from Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice were either left unstimulated or stimulated overnight with TCR and CD28. The following day, naive (CD44low) CD4 and CD8 Rag1-GFP+ MNTs and Rag1-GFP+ RTEs were examined by flow cytometry for TNF-α production by intracellular staining. Top row, Gating strategy for MNTs and RTEs within the naive CD4 T cell pool. For examination of TNF-α production, the filled gray histogram represents unstimulated and the solid line represents TCR/CD28 stimulated for each population examined. The gate indicates the percent in each stimulated sample that expresses TNF-α. Data are representative of five Rag1-GFP WT and five Rag1-GFP CD4-cre HDAC3 cKO mice from five independent experiments.
was due to defective egress, then the relative expression of the Rag1-GFP reporter should have been lower in HDAC3-deficient mature CD4 SP thymocytes as compared with WT. GFP expression was similar in HDAC3-deficient and WT thymocytes, indicating that the increase in Qa2hiCD24lo mature SP thymocytes was not due to a block in egress (Fig. 4C), but may simply reflect dysregulation of the Qa2 marker rather than an effect on the thymic maturation program.

**FIGURE 3.** The defect in T cell maturation in the absence of HDAC3 is cell intrinsic. (A) BMCs were generated using either a 1:1 mixture of CD45.2+Rag1-GFP WT/CD45.1+B6.SJL or CD45.2+Rag1-GFP CD4-cre HDAC3 cKO/CD45.1+B6.SJL whole bone marrow cells and analyzed after 10 wk. The relative chimerism as compared with the granulocyte population was calculated. Shown is the average from four CD45.2+Rag1-GFP WT/CD45.1+B6.SJL chimeras and four CD45.2+Rag1-GFP CD4-cre HDAC3 cKO/CD45.1+B6.SJL chimeras from four independent FACS analyses. Error bars are SEM, and significance was determined using an unpaired Student t test. (B) RTEs were examined in splenocytes from CD45.2+Rag1-GFP WT/CD45.1+B6.SJL and CD45.2+Rag1-GFP CD4-cre HDAC3 cKO/CD45.1+B6.SJL mixed radiation chimeras. Representative FACS analysis from four mice in each group is shown. (C) Qa2 and CD55 expression were examined in MNTs and RTEs from CD45.2+Rag1-GFP WT/CD45.1+B6.SJL and CD45.2+Rag1-GFP CD4-cre HDAC3 cKO/CD45.1+B6.SJL mixed radiation chimeras, using the gating strategy shown in (B) to define RTEs and MNTs. The filled histograms denote Qa2 or CD55 expression in cells from Rag1-GFP WT mice, and the solid lines denote expression from Rag1-GFP CD4-cre HDAC3 cKO mice as shown in the figure. Representative FACS analysis from four mice in each group is shown.
FIGURE 4. Thymic egress is not altered in CD4-cre HDAC3 cKO mice. (A) The expression of CD62L, CD69, and S1P1 relative to CD24 was examined in Rag1-GFP+ CD4 SP thymocytes from Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice. Representative FACS analysis from at least four mice in each group is shown. (B) The expression of CCR4, CCR9, and CCR7 relative to CD24 was examined in Rag1-GFP+ CD4 SP thymocytes from Rag1-GFP WT and Rag1-GFP CD4-cre HDAC3 cKO mice. Representative FACS analysis from at least four mice in each group is shown. (C) The expression of a Rag1-GFP reporter in DP, semimature (CD24<sup>hi</sup>Q<sup>a<sub>2</sub>lo</sup>) CD4 SP, and mature (CD24<sup>lo</sup>Q<sup>a<sub>2</sub>hi</sup>) CD4 SP thymocytes was examined in CD4 SP thymocytes from Rag1-GFP WT (filled histograms) and Rag1-GFP CD4-cre HDAC3 cKO mice (solid line). Representative FACS analysis from at least four mice in each group is shown.
Lack of peripheral T cells is not due to a defect in IL-7–mediated homeostasis

The specific loss of peripheral, but not thymic, T cells could also be due to a defect in homeostasis. After positive selection as thymocytes mature, IL-7Rα is upregulated, which is required for T cell survival in the periphery (7, 11). IL-7 stimulation induces and maintains expression of the antiapoptotic protein Bcl-2 (29). To determine whether a defect in homeostasis was responsible for the low numbers of T cells in the periphery, we examined the expression of IL-7Rα and Bcl-2 during T cell development and maturation. IL-7Rα and Bcl-2 were induced in CD4-cre HDAC3 cKO thymocytes at levels comparable with WT thymocytes during the transition from DP to semimature to mature SP thymocytes (Fig. 5). In WT mice, IL-7Rα levels continue to increase as T cell maturation progresses. However, in CD4-cre HDAC3 cKO mice, increase in IL-7Rα during peripheral maturation was blunted, similar to peripheral T cells that lack NKAP, which cannot undergo T cell maturation. However, although NKAP-deficient peripheral T cells had lower levels of IL-7Rα on the cell surface as compared with WT, the expression of IL-7Rα was still sufficient to maintain Bcl-2 expression at levels comparable with WT (16). Similarly, although less IL-7Rα was expressed in peripheral CD4-cre HDAC3 cKO T cells, Bcl-2 levels were also comparable with WT. Therefore, the decreased number of peripheral T cell numbers in CD4-cre HDAC3 cKO mice was not due to a block in T cell homeostasis.

**FIGURE 5.** The peripheral T cell defect in CD4-cre HDAC3 cKO mice is not due to a block in T cell homeostasis. The expression of IL-7Rα and Bcl-2 in DP, semimature (CD24hiQa2lo) CD4 SP, and mature (CD24loQa2hi) CD4 SP thymocytes (A), as well as splenic CD4 MNTs (CD62L+CD44hiRag1-GFP−) and RTEs (CD62L−CD44−Rag1-GFP+) (B), was examined in CD4 SP thymocytes from Rag1-GFP WT (filled histograms) and Rag1-GFP CD4-cre HDAC3 cKO mice (solid line). For examination of thymic development, thymocytes were first gated on expression of Rag1-GFP to exclude any recirculating mature T cells from the analysis. Representative FACS analysis from at least three mice in each group is shown.
FIGURE 6. HDAC3-deficient peripheral T cells are targeted by the classical complement pathway. (A and B) RTEs and MNTs from WT, Rag1-GFP CD4-cre HDAC3 cKO and Rag1-GFP CD4-cre NKAP cKO mice were examined for deposition of IgM, C1q, C4, and C3 as denoted in the figure. Representative FACS analysis from three WT, three Rag1-GFP CD4-cre HDAC3 cKO, and three Rag1-GFP CD4-cre NKAP cKO mice from three independent experiments are shown.
A

- DP
- CD4SP semi-mature CD24\textsuperscript{hi}
- CD4SP mature CD24\textsuperscript{lo}

B

- Rag1-GFP
- Rag1-GFP CD4-cre HDAC3 cKO

C

Relative MFI of mSiglec-E binding in WT and CD4-cre HDAC3 cKO mice

<table>
<thead>
<tr>
<th></th>
<th>DP</th>
<th>CD4 SP</th>
<th>mature CD4 SP</th>
<th>CD4 RTE</th>
<th>CD4 MNTs</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>422 ± 33</td>
<td>2097 ± 260</td>
<td>7033 ± 931</td>
<td>8956 ± 117</td>
<td>7873 ± 175</td>
</tr>
<tr>
<td>CD4-cre</td>
<td>546 ± 65</td>
<td>1142 ± 235</td>
<td>5487 ± 835</td>
<td>9038 ± 487</td>
<td>5798 ± 398</td>
</tr>
<tr>
<td>HDAC3 cKO</td>
<td>NS</td>
<td>p&lt;0.001</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

D

Relative mRNA expression

- St8Sia1
- St8Sia4
- St8Sia6

- WT
- CD4-cre HDAC3 cKO

- NS
- p<0.05
HDAC3-deficient peripheral T cells are eliminated by complement

In CD4-cre NKAP cKO mice, the naive T cell pool was composed almost entirely of RTEs that are targeted for elimination through the classical complement pathway (16, 17). Therefore, peripheral T cells from CD4-cre HDAC3 cKO mice may also be similarly targeted for destruction through the classical complement pathway. Splenocytes from RAG1-GFP and RAG1-GFP CD4-cre HDAC3 cKO mice were examined for the deposition of IgM (which strongly activates the classical complement pathway), C1q (which initiates the classical complement pathway), C4, and C3. HDAC3-deficient peripheral MNTs and RTEs displayed substantial binding of IgM and deposition of C1q, C4, and C3 on the cell surface, albeit to a lesser extent than in NKAP-deficient RTEs and MNTs (Fig. 6). Therefore, similar to CD4-cre NKAP cKO mice, immature peripheral T cells from CD4-cre HDAC3 cKO mice are targeted by the classical complement pathway for elimination.

Defective sialylation in CD4-cre HDAC3 cKO mice

One potential cause for complement targeting of immature peripheral T cells is defective sialylation during T cell maturation. Peripheral T cells stripped of cell-surface sialic acids by neuraminidase bind natural IgM and are targeted by complement (30, 31). As thymocyte development progresses, there is increased incorporation of sialic acid into cell-surface glycoproteins and glycolipids, which is detected by PNA that recognizes core-1-O-glycans that lack terminal sialic acid (32). As T cells develop from DP to semimature SP to mature SP, PNA binding decreased concomitantly with increasing sialylation (Fig. 7A). Similar decreases in PNA binding were observed in RAG1-GFP WT and RAG1-GFP CD4-cre HDAC3 cKO mice. PNA does not distinguish between different sialic acid linkages, which can occur through α2,3, α2,6, or α2,8 linkages. However, other lectins are highly specific for α2,6-linked sialic acids (SNBL) or α2,3-linked sialic acids (MAL II). Although no lectin is specific for α2,8-linked sialic acids, recombinant Siglec-E, which preferentially binds to α2,8-linked sialic acids, can be used (17, 33). Previously, we demonstrated that the ligands for Siglec-E are upregulated concomitantly with T cell maturation, and that ligands for Siglec-E are not produced to the same extent in NKAP-deficient T cells as compared with WT (17). Increases in all three types of sialylation occurred during T cell development in WT mice from DP to semimature SP to mature SP (Fig. 7). In CD4-cre HDAC3 cKO mice, there were similar increases in α2,6-linked sialic acids (SNBL binding) and α2,3-linked sialic acids (MAL II binding). However, the induction of mSiglec-E binding during T cell maturation was significantly decreased in CD4-cre HDAC3 cKO seminature and mature CD4 SP thymocytes (Fig. 7B, 7C). In the periphery, mSiglec-E binding to HDAC3-deficient MNTs was also significantly decreased. Previously, we demonstrated by quantitative PCR (Q-PCR) that mRNA expression of three α2,8 sialic acid transferases, ST8Sia1, ST8Sia4, and ST8Sia6, was decreased in NKAP-deficient RTE (17). When expression of these genes was examined in HDAC3-deficient RTEs (Fig. 7D), expression of ST8Sia6 was significantly decreased as compared with WT. Although decreases were observed in ST8Sia1 and ST8Sia4 expression as well, they were not statistically significant. Therefore, similar to NKAP-deficient peripheral T cells, there is less α2,8 sialylation in HDAC3-deficient T cells.

Discussion

In this article, we demonstrate a unique role for HDAC3 in the regulation of T cell maturation. Deletion of HDAC3 at the DP stage using CD4-cre did not alter the development or selection of conventional SP thymocytes (although iNKT cell development is blocked) (27), chemokine receptor expression for intrathymic migration, thymic egress or entry into peripheral lymphoid organs. Instead, a defect in peripheral T cell maturation was observed. HDAC3-deficient RTEs fail to mature and enter the long-lived naive T cell pool, and thus the majority of the few naive T cells present in the periphery are RAG1-GFP^+ RTEs. HDAC3-deficient RTEs and peripheral naive T cells also fail to gain functional competency as demonstrated by an inability to produce TNF-α after TCR/CD28 stimulation. The block in T cell maturation is cell intrinsic, as shown using mixed BMCs.

Although T cells express many HDAC family members, the requirement for HDAC3 in peripheral T cell maturation demonstrates that it has unique functions that cannot be performed by any other HDAC family member. Similar to NKAP-deficient peripheral T cells, we found that HDAC3-deficient peripheral T cells are targeted for elimination by the classical arm of the complement pathway, and also have decreased incorporation of α2,8-linked sialic acids in cell-surface glycans. HDAC3-deficient RTEs and MNTs have increased deposition of IgM, C1q, C3, and C4 as compared with WT peripheral T cells. However, we consistently observe less complement deposition on HDAC3-deficient RTEs and MNTs than on NKAP-deficient RTEs and MNTs. Decreased complement deposition may explain why HDAC3-deficient T cells persist longer and why there is a greater frequency of MNTs within the diminished peripheral T cell pool in CD4-cre HDAC3 cKO mice as compared with CD4-cre NKAP cKO mice, where the naive peripheral pool is composed almost entirely of RAG1-GFP^+ RTEs. Nevertheless, the similarities in the block in peripheral maturation and phenocopy between CD4-cre HDAC3 cKO mice and CD4-cre NKAP cKO mice indicates that NKAP and HDAC3 work together to regulate postthymic T cell maturation. However, CD4-cre NKAP cKO and CD4-cre HDAC3 cKO mice do not have identical phenotypes. In the absence of NKAP, Qa2 and CD45RB are not upregulated in SP thymocytes and RTEs, whereas Qa2 and CD45RB are expressed at higher levels in CD4-cre HDAC3 cKO mice as compared with WT. This implies

FIGURE 7. Defective sialylation in CD4-cre HDAC3 cKO mice. (A) Sialylation using PNA, SNBL, MAL II, and recombinant mSiglec-E was examined in DP (red histogram), seminature (CD24^hiQa2^hi) CD4 SP thymocytes (blue histogram), and mature (CD24^loQa2^hi) CD4 SP thymocytes (green histogram) from RAG1-GFP WT (upper panel) and RAG1-GFP CD4-cre HDAC3 cKO mice (lower panel). For examination of thymic development, thymocytes were first gated on expression of RAG1-GFP to exclude any recirculating mature T cells from the analysis. Representative FACs analysis from at least three mice in each group from three independent experiments is shown. (B) Sialylation using PNA, SNBL, MAL II, and recombinant mSiglec-E was examined in splenic CD4^+ RTEs (upper panels) and MNTs (lower panels) from RAG1-GFP WT (orange histogram) and RAG1-GFP CD4-cre HDAC3 cKO mice (purple histogram). Representative FACs analysis from at least three mice from each group from three independent experiments is shown. (C) The relative mean fluorescence intensities of recombinant mSiglec-E binding in DP, seminature (CD24^hiQa2^hi) CD4 SP, mature (CD24^loQa2^hi) CD4 SP, CD4 RTE (RAG1-GFP^+CD62L^-), and CD4 MNT (RAG1-GFP^-CD62L^-) from RAG1-GFP WT and RAG1-GFP CD4-cre HDAC3 cKO mice are shown. Data shown are averages from six RAG1-GFP WT and six RAG1-GFP CD4-cre HDAC3 cKO mice from five independent experiments. Error bars shown are SEM, and significance was determined using an unpaired Student t test. (D) CD4^+ RTEs from RAG1-GFP WT and RAG1-GFP CD4-cre HDAC3 cKO mice were sorted and examined for the mRNA expression levels of ST8Sia1, ST8Sia4, and ST8Sia6 by Q-PCR. Data shown are averages from five RAG1-GFP WT and five RAG1-GFP CD4-cre HDAC3 cKO mice from three independent experiments. Error bars are SEM, and significance was determined using an unpaired Student t test.
that neither the loss of Qa2 nor CD45RB expression contributes to the block in T cell maturation in the absence of NKAP, because NKAP-deficient and HDAC3-deficient peripheral T cells have the same block in functional maturation and are targeted similarly for elimination by complement.

Traditionally, maturation has been examined using Qa2 and CD24 expression in the thymus (semimature SP thymocytes are CD24abQa2hi and mature SP thymocytes are CD24hiQa2hi) and by Qa2 expression in the periphery. However, neither Qa2 nor CD24 are required for T cell maturation, because mice deficient in either molecule have normal numbers of peripheral T cells (34, 35). We demonstrated that conditional deletion of HDAC3 at the DP stage blocks to a lead to a block in T cell maturation, resulting in a great reduction in the number of peripheral T cells, the majority of which are RTEs. However, CD24 is normally downregulated at the SP stage in the thymus, and Qa2 expression is increased in SP thymocytes and in RTEs in CD4-c4e HDAC3 cKO mice. Thus, although there is a "mature" CD24loQa2hi SP population in CD4-c4e HDAC3 cKO mice, the expression of other markers associated with T cell maturation, such as CD55 and sialylated ligands for Siglec-E, are defective in HDAC3-deficient T cells. HDAC3-deficient RTEs and MNTs are functionally immature, as demonstrated by the inability to produce TNF-α after CD3/CD28 stimulation, and are targeted for elimination by the classical complement pathway. Most importantly, there was a severe defect in peripheral T cell numbers, and RTEs comprise the majority of naive T cells in CD4-c4e HDAC3 cKO mice. Thus, the block in T cell maturation in CD4-c4e HDAC3 cKO mice would not have been uncovered if only CD24 and Qa2 were used to measure maturation. CD24 and Qa2 are therefore insufficient to determine whether a specific loss of postpositive selection peripheral T cells is due to a block in maturation. Rather, many proteins change after positive selection in either SP thymocytes or RTEs, including CD45RB, CD55, IL-7Rα, Bcl-2, CCR7, CCR9, CCR4, CD62L, CD69, and S1P1, which all need to be examined before concluding that a peripheral T cell block may be caused by defective maturation, selection, migration, egress, or homeostasis. In this study, the use of CD55 as an alternative marker to differentiate MNTs from RTEs indicated that there was a maturation defect in CD4-c4e HDAC3 cKO mice, which was confirmed using the Rag1-GFP reporter to determine that the majority of naive T cells in CD4-c4e HDAC3 cKO mice were RTEs.

Changes of chemokine receptors have been observed after thymocytes pass positive selection. Normally, SP thymocytes downregulate CCR4/CCR9 and upregulate CCR7 for cortex-to-medulla migration, and mature SP thymocytes induce S1PR1 expression for thymic egress. We found that T cell maturation was independent of migration or egress because SP thymocytes from CD4-c4e HDAC1 cKO mice regulate expression of CCR4, CCR7, CCR9, and S1P1 as expected (Fig. 4). Conversely, conventional CD4 SP thymocytes from CCR7/CCR4 double-knockout mice do not migrate into the medulla and display normal maturation, although the development of naturally occurring regulatory T cells and iNKT cells is severely curtailed (5). Mice deficient in S1PR1 or treated with FTY720 show an accumulation of CD24abQa2hi mature SP thymocytes (8). However, although there is an increased proportion of HDAC3-deficient CD24abQa2hi CD4 SP thymocytes, these neither have a defect in S1P1 expression nor accumulate intrathymically as shown by Rag1-GFP mean fluorescence intensity. Although IL-7 signaling is critical for T cell homeostasis, it is dispensable for postthymic T cell maturation (13). In addition, administration of anti-IL-7 and anti-IL-7Rα blocking Abs for 6 d did not alter T cell maturation because mice that received blocking Abs expressed comparable levels of CD24, Qa2, and CD45RB as untreated mice (12). Taken together, these findings provide evidence that T cell maturation is a unique developmental event independent of migration, egress, and homeostasis.

The transcriptome study by the Immunological Genome Project provides precise data for T cell development and differentiation (36). Surprisingly, only a few genes were significantly upregulated between mature SP thymocytes and peripheral naive T cells. One of these genes is CD55. We previously demonstrated that the expression of CD55 is induced during T cell maturation, and NKAP-deficient T cells have a defect in upregulation of CD55. Similarly, HDAC3-deficient T cells express lower levels of CD55 (17). Although CD55 deficiency alone does not lead to peripheral T cell lymphopenia (37), our results suggest that lack of CD55 expression accelerates the elimination of immature RTEs by complement attack. Whether NKAP and HDAC3 directly regulate CD55 expression or whether CD55 expression is lower due to the block in T cell maturation regulated by NKAP and HDAC3 is not known. Importantly, CD55 expression can be used as a novel marker for postthymic maturation (17).

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Disclosures
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References


