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Cutting Edge: HDAC3 Protects Double-Positive Thymocytes from P2X7 Receptor–Induced Cell Death

Rachael L. Philips, Shaylene A. McCue, Matthew J. Rajcula, and Virginia S. Shapiro

Intricate life-versus-death decisions are programmed during T cell development, and the regulatory mechanisms that coordinate their activation and repression are still under investigation. In this study, HDAC3-deficient double-positive (DP) thymocytes exhibit a severe decrease in numbers. The thymic cortex is rich in ATP, which is released by macrophages that clear apoptotic DP thymocytes that fail to undergo positive selection. We demonstrate that HDAC3 is required to repress expression of the purinergic receptor P2X7 to prevent DP cell death. HDAC3-deficient DP thymocytes upregulate the P2X7 receptor, increasing sensitivity to ATP-induced cell death. P2rx7/-HDAC3 double knockout mice show a partial rescue in DP cell number. HDAC3 directly binds to the P2rx7 enhancer, which is hyperacetylated in the absence of HDAC3. In addition, ROR\(\text{g}\)t binds to the P2rx7 enhancer and promotes P2X7 receptor expression in the absence of HDAC3. Therefore, HDAC3 is a critical regulator of DP thymocyte survival and is required to suppress P2X7 receptor expression. The Journal of Immunology, 2019, 202: 1033–1038.
resulting from HDAC3 deficiency (ROryt-KO Bcl-xl transgenic [Tg] HDAC3-cKO, hereafter called “RB3”). In addition, DP cellularity was restored in RB3 mice, although the mechanism was not known. The focus of this paper is the mechanism by which HDAC3 regulates DP thymocyte survival.

In this study, we find one cause for the survival defect in DP thymocytes from HDAC3-cKO mice. HDAC3-deficient DP thymocytes exhibit increased expression of the purinergic receptor P2X7 (encoded by the P2rx7 gene). Cells that express P2X7 receptor are more sensitive to high concentrations of extracellular ATP, which results in large pore formation and loss of membrane integrity (reviewed in Ref. 11). The regulation of P2rx7 expression is coordinated by HDAC3 and ROryt at the P2rx7 enhancer. HDAC3 deletion leads to an increase in histone acetylation at the P2rx7 gene locus and deletion of ROryt normalizes P2X7 receptor expression in HDAC3-deficient DP thymocytes. Therefore, HDAC3 is required to suppress P2X7 receptor expression in DP thymocytes and promote DP survival.

Materials and Methods

Mice

HDAC3 b/b mice were provided by S. Hiebert (Vanderbilt (12)). Human Bcl-2 Tg mice were generated by S. Korsmeyer (13) and provided by A. Singer (National Institutes of Health). Bcl-xl Tg mice (14), ROR γt-KO mice (15), HDAC3-cKO mice because mice that lack TCR-β signaling molecules (e.g., Zap70) do not have compromised thymocyte development. HDAC3-cKO mice had ~80% fewer DP thymocytes as compared with WT mice (7), Fig. 1A). The reduction was unlikely to be due to the positive selection block in HDAC3-cKO mice because mice that lack TCR-α, HMC, or key TCR signaling molecules (e.g., Zap70) do not have compromised DP thymocyte numbers (22–24). To determine whether the DP reduction was cell intrinsic, 50:50 mixed bone marrow chimeric mice were generated. Chimerism frequency was slightly reduced (7), Fig. 1B) demonstrating that the effect is cell intrinsic. Although HDAC3 protein deletion starts at DN3 in HDAC3-cKO mice, there was not a decrease in CD4 or CD8 T cells. The deficit in DP cell number is due to a defect prior to the DP stage.

FACS analysis was performed on an Attune NXT Flow Cytometer (Thermo Fisher Scientific) and analyzed with FlowJo (Tree Star). Experiments were acquired live or fixed (BD Cytofix/Cytoperm Fixation and Permeabilization Kit; BD Biosciences). Bcl-xl staining used the Foxp3/Transcription Factor Staining Buffer Kit (Tonbo Biosciences). All analyses included size exclusion (forward scatter [FSC] area) and dead cell exclusion (Annexin V binding; Apoptosis Detection experiments using A438079 (Abcam), cells were pretreated with 10 or 100 μM M2

Statistical analysis

Two-tailed unpaired Student t test (GraphPad Prism) was used to compare groups. Box plots encompass the 25th to 75th percentile, and whiskers extend to the minimum and maximum values.

Results and Discussion

Decreased viability of HDAC3-deficient DP thymocytes is cell intrinsic and not rescued by Bcl-xl or Bcl-2 transgenes

HDAC3-cKO mice had ~80% fewer DP thymocytes as compared with WT mice (7), Fig. 1A). The reduction was unlikely to be due to the positive selection block in HDAC3-cKO mice because mice that lack TCR-α, HMC, or key TCR signaling molecules (e.g., Zap70) do not have compromised DP thymocyte numbers (22–24). To determine whether the DP reduction was cell intrinsic, 50:50 mixed bone marrow chimeric mice were generated. Chimerism frequency was measured at the DN-to-DP stages, with splenic CD11b+ cells used as a control. Chimerism frequency was slightly reduced in HDAC3-deficient DN4 and immature CD8 single-positive thymocytes, whereas HDAC3-deficient DP thymocytes exhibited a large reduction in chimerism (Fig. 1B), demonstrating that the effect is cell intrinsic. Although HDAC3 protein deletion starts at DN3 in HDAC3-cKO mice, there was not a deficiency in DN cellularity, β selection, and proliferation in DN3 and DN4 thymocytes (7). Therefore, it is unlikely that the deficit in DP cell number is due to a defect prior to the DP stage.

Bcl-xl is required for DP cell survival (25); however, Bcl-xl protein expression in DP thymocytes was similar between WT and HDAC3-cKO mice (Fig. 1C). To determine whether overexpression of the Bcl-2 family anti-apoptotic protein Bcl-xl or Bcl-2 could rescue DP cell number from HDAC3-cKO mice, Bcl-xl and Bcl-2 transgenes were introduced. However, no...
increase in DP cell number from Bcl-xl Tg/HDAC3-cKO mice or Bcl-2 Tg/HDAC3-cKO mice was observed as compared with HDAC3-cKO mice (Fig. 1D). Thus, the DP survival defect in HDAC3-cKO mice cannot be compensated by overexpression of Bcl-xl or Bcl-2 (7).

**HDAC3-deficient DP thymocytes are susceptible to P2X7 receptor–induced cell death**

The purinergic receptor P2X7 induces thymocyte cell death upon stimulation with high doses of ATP (26). The thymic cortex is believed to be an ATP-rich environment, as resident macrophages release ATP as a result of phagocytosing DP thymocytes undergoing cell death (27). WT DP thymocytes express low levels of P2X7 receptor compared with DN and SP thymocytes and are thus relatively insensitive to extracellular ATP-induced cell death (Fig. 2A, Supplemental Fig. 1). However, HDAC3-deficient DP thymocytes significantly upregulated P2X7 receptor as compared with WT DP thymocytes (Fig. 2A) and were more sensitive to P2X7 receptor–induced cell death from an ex vivo culture with the P2X7 receptor ligand ATP or the P2X7 receptor agonist BzATP (Fig. 2B). Preincubation of HDAC3-deficient thymocytes with the P2X7 receptor–specific antagonist A438079 abrogated the increase in Annexin V binding caused by ATP treatment (Fig. 2C), demonstrating that the ATP-induced cell death was not due to stimulation of other purinergic receptors coexpressed by HDAC3-deficient DP thymocytes. HDAC3-deficient DP thymocytes required a higher dose of A438079 to abrogate BzATP-induced Annexin V staining (Fig. 2C), again suggesting that HDAC3-deficient DP thymocytes are more sensitive to P2X7 receptor ligands. Strong stimulation of P2X7 receptor can also induce cell membrane pore-mediated cell death (28). YO-PRO-1 is a large (~600 Da) nucleic acid stain that labels cells with compromised plasma membranes and is therefore a surrogate marker for pore formation (29). After 1 h stimulation of thymocytes with ATP or BzATP, HDAC3-deficient DP thymocytes exhibited an increase in YO-PRO-1 staining compared with unstimulated, etoposide stimulation, or WT controls (Fig. 2D, Supplemental Fig. 2), demonstrating that P2X7 receptor–induced cell death in HDAC3-cKO mice occurs via pore formation.

To understand the contribution of the P2X7 receptor to reduced DP cell survival in HDAC3-cKO mice, P2rx7/HDAC3–double knockout (DKO) mice were generated. Loss of the P2X7 receptor protected HDAC3-deficient DP thymocytes from ATP- and BzATP-induced cell death (Fig. 2E). Similarly, knocking out P2rx7 abrogated the increase in YO-PRO-1 staining in response to either ATP or BzATP in P2rx7/HDAC3-DKO mice compared with HDAC3-cKO mice (Fig. 2F), demonstrating that pore formation is specifically induced by the P2X7 receptor. Examination of DP cell number from P2rx7/HDAC3-DKO mice revealed a 2-fold increase in cell number compared with HDAC3-cKO mice (Fig. 2G); however, the number of DP thymocytes from P2rx7/HDAC3-DKO mice was still below WT mice (Fig. 2G). This indicates that there must be other causes of DP cell death in addition to increased expression of P2X7.

**The P2rx7 gene locus is suppressed by HDAC3 in DP thymocytes**

Examination of P2X7 receptor expression during T cell development revealed that P2rx7 is specifically downregulated at DP stage compared with DN and CD4SP thymocytes (Supplemental Fig. 1). Because deletion of HDAC3 in DP thymocytes leads to P2X7 receptor upregulation (Fig. 2A), publicly available genome sequencing datasets were used to examine the chromatin state of the P2rx7 gene locus in WT thymocytes. Cldn8a and Hoxc4 gene loci were used as controls for highly expressed genes and repressed genes in thymocytes, respectively. Compared with Cldn8a, the P2rx7 locus showed a low signal for RNA polymerase II, H3K27ac, and tri-methylated histone H3 lysine-4 (Fig. 3A), indicating that the P2rx7 locus does not show chromatin marks of active gene expression in WT thymocytes. This is consistent with low P2X7 receptor expression in WT thymocytes (Fig. 2A). Interestingly, the repressive mark trimethylated histone H3 lysine-27 was not enriched at the P2rx7 locus (Fig. 3A), suggesting that P2rx7 is not actively repressed by a polycomb repressive complex–regulated mechanism. In addition, H3K4me1 ChIP-seq and CapSTARR-seq were used to identify enhancers in WT thymocytes and examine their activity (30, 31), respectively. Within intron 2 of P2rx7, an enhancer was revealed by enrichment of H3K4me1 (Fig. 3A), which is consistent with previous reports (32). The combination of H3K27ac and H3K4me1 marks identifies active enhancers (30); however, the P2rx7 enhancer lacked H3K27ac (Fig. 3A), indicating that the P2rx7 enhancer is not active in WT thymocytes. To validate P2rx7 enhancer activity in thymocytes, we used publicly available CapSTARR-seq (31). Consistent with the
absence of H3K27ac at this enhancer, the CapSTARR-seq signal was also absent at the P2rx7 enhancer (Fig. 3A), confirming that the enhancer is inactive in WT DP thymocytes. Therefore, the P2rx7 gene locus is suppressed in WT thymocytes.

HDAC3 regulates gene expression upon recruitment to gene promoters or enhancers. To determine whether HDAC3 binds to either of these regions of the P2rx7 gene, HDAC3 qChIP was performed on DP thymocytes from WT mice, with HDAC3-cKO mice used as a negative control for HDAC3 binding. HDAC3 qChIP revealed that HDAC3 bound to the P2rx7 enhancer but not the P2rx7 promoter (Fig. 3B), indicating that HDAC3 directly regulates P2rx7 expression. Because HDAC3 deacetylates histones, H3K27Ac was analyzed at the P2rx7 enhancer by qChIP in WT and HDAC3-deficient DP thymocytes. Whereas DP thymocytes from WT mice exhibited low levels of acetylation at the P2rx7 enhancer (similar to Fig. 3A), deletion of HDAC3 increased acetylation at the P2rx7 enhancer in DP thymocytes from HDAC3-cKO mice (Fig. 3C), demonstrating HDAC3 directly regulates histone acetylation at the P2rx7 gene locus.

RORγt promotes P2X7 receptor expression in HDAC3-deficient DP thymocytes

HDAC3 is required to repress RO Rγt during positive selection (7), as RO Rγt is normally downregulated at this stage, and constitutive expression of RO Rγt leads to a similar block in positive selection in RB3 mice as well as DP cellularity (7), suggesting that RO Rγt may regulate P2X7 receptor expression. A previous study identified retinoic acid response elements in the P2rx7 intronic enhancer and RARα binding to this enhancer in CD4+ T cells (32). RO Rγt belongs to the ROR family of transcription factors, which are involved in the development of thymocytes and peripheral T cells. The overexpression of RO Rγt has been associated with the development of T cell lymphomas in mice (33). Therefore, it is possible that HDAC3 regulates P2X7 receptor expression by repressing RO Rγt expression during positive selection. To test this hypothesis, we performed qPCR analysis on DP thymocytes from WT, HDAC3-cKO, P2rx7-KO, and P2rx7/HDAC3-DKO mice. The results showed that deletion of HDAC3 increased the expression of RO Rγt in DP thymocytes (Fig. 3D), supporting the idea that HDAC3 represses RO Rγt expression during positive selection.

FIGURE 3. The P2rx7 gene locus is repressed by HDAC3 in DP thymocytes. (A) ChIP-seq and CapSTARR-seq snapshots at P2rx7, Cdb8a, and Hox4. Yellow boxes identify previously characterized promoter (P) and enhancer (E) regions (32). (B) HDAC3 qChIP at the P2rx7 promoter and enhancer in DP thymocytes from WT and HDAC3-cKO mice. Plots show mean ± SEM of three to four mice per group from three independent experiments. (C) H3K27ac qChIP at the P2rx7 enhancer in DP thymocytes from WT and HDAC3-cKO mice. Plots show mean ± SEM of three mice per group from three independent experiments.
To determine whether RORγt regulates P2rx7 expression, P2X7 receptor expression in RB3 and HDAC3-deficient DP thymocytes was examined. In these experiments, RB3 and HDAC3-cKO mice contained the OT-II transgene. OT-II HDAC3-cKO DP thymocytes exhibited an increased in the frequency of P2X7 receptor-positive cells compared with OT-II thymocytes (Fig. 4C), consistent with results in HDAC3-cKO and WT DP thymocytes (Fig. 2A). Loss of RORγt expression in OT-II RB3 mice restored P2X7 receptor expression to levels comparable to WT mice (Fig. 4C). Interestingly, mice with heterozygous RORγt deficiency (OT-II RORγt-het HDAC3-cKO mice) showed an intermediate frequency of P2X7-positive cells, demonstrating that the frequency of P2X7-positive cells is exquisitely sensitive to RORγt expression (Fig. 4C). Thus, RORγt promotes P2rx7 expression in HDAC3-deficient DP thymocytes.

In summary, we have identified a novel role for HDAC3 in DP thymocytes. We demonstrate that HDAC3 is required to repress expression of the purinergic receptor P2X7 to prevent DP cell death. HDAC3-deficient DP thymocytes upregulate the P2X7 receptor, increasing sensitivity to ATP-induced cell death. P2rx7/HDAC3-DKO mice show a partial restoration in DP cell number, with twice as many DP thymocytes as HDAC3-cKO mice. Mechanistically, HDAC3 directly binds to the P2rx7 enhancer, which is hyperacetylated in the absence of HDAC3. In addition, RORγt binds to the P2rx7 enhancer and promotes P2X7 receptor expression in HDAC3-deficient DP thymocytes (model in Supplemental Fig. 3). Therefore, HDAC3 is a critical regulator of DP thymocyte survival and is required to suppress P2rx7 expression.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. P2X7 receptor expression during T cell development. (A) Expression profile of P2rx7 in thymic developmental stages (DN1-SP) and splenic naive CD4 and CD8 T cells. RNA-seq data was acquired from the Immunological Genome Consortium via the RNA-seq Gene Skyline (immgen.org). (B) P2X7 receptor protein expression in DN (CD4^+CD8^-), ISP (CD4^+CD8^+TCRβ^-), DP (CD4^+CD8^-), CD4SP (CD4^+CD8^-TCRβ^-), and CD8SP (CD4^-CD8^+TCRβ^-). FACS plot depict representative P2X7 receptor expression for each thymic stage and its corresponding gMFI. The plot below depicts mean ± SEM of gMFI of 4 mice from 3 independent experiments.
Supplementary Figure 2. Etoposide treatment of WT and HDAC3-deficient thymocytes. Thymocytes from WT and HDAC3-cKO mice were treated with or without 2.5ug/mL of etoposide for 1 hour or 18.5 hours. Plots show mean ± SEM of the frequency of YO-PRO-1+ DP thymocytes from 3-4 mice per group. Plots were gated from FVD' (to remove necrotic cells) DP thymocytes. Etoposide treatment does not increase YO-PRO-1 staining after one hour, however after 18.5 hours etoposide treatment leads to a similar frequency of YO-PRO-1+ DP thymocytes between WT and HDAC3-cKO mice.
Supplementary Figure 3. Model. In WT DP thymocytes, HDAC3 associates with the \( P2rx7 \) enhancer to repress its expression and reduce DP thymocyte sensitivity to extracellular ATP. However, when HDAC3 is absent, the \( P2rx7 \) gene locus is hyperacetylated, ROR\( \gamma \)t promotes the expression of \( P2rx7 \), and HDAC3-deficient DP thymocytes show increased cell death via ATP. Hence, HDAC3 may function to repress ROR\( \gamma \)t transcriptional activity at the \( P2rx7 \) enhancer to repress \( P2rx7 \) expression in DP thymocytes.