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RESULTS

Unaltered pre-TCR signaling in crt$^{-/-}$ thymocytes

Early in αβ T cell development, pre-TCR expression determines the β selection of CD4$^-$8$^-$ double negative (DN) thymocytes, which is characterized by down-regulation of CD25 and transition of cells from the CD44$^-$25$^+$ (DN3) to the CD44$^-$25$^-$ (DN4) stage as well as transition of DN cells to the CD4$^+$8$^+$ double positive (DP) stage. The subset distribution of DN cells with respect to CD44 and CD25 expression revealed a slight decrease of the most immature CD44$^+$25$^-$ DN1 subset. However, DN2, DN3, and DN4 representations were unaffected by calreticulin deficiency, implying unaltered pre-TCR signaling in *crt*$^{-/-}$ chimeras (Fig. S1).

Unaltered deletion of crt$^{-/-}$ thymocytes

Because fetal liver hemopoietic progenitors from *crt*$^{+/+}$ and *crt*$^{-/-}$ embryos were transferred to a BALB/c background in RAG/γ chain double KO mice, we tested whether TCR Vβ-specific negative selection mediated by superantigens encoded by endogenous mouse mammary tumor viruses (MMTVs) was taking place with the same efficiency. In BALB/c mice, MMTV proteins are presented in association with the class II molecule I-E^d and result in the deletion of CD4$^+$ thymocytes expressing Vβ3, Vβ5, Vβ11, and Vβ12, but not Vβ8. Indeed, the analysis of TCR Vβ3 and Vβ11 expression on CD4 single positive thymocytes demonstrated complete deletion of cells bearing these MMTV-reactive Vβ regions in both *crt*$^{+/+}$ and *crt*$^{-/-}$ chimeras. Moreover, the same partial deletion of TCR Vβ5-bearing cells and relative expression of TCR Vβ8 were observed in both chimeras (Fig. S2 a). We concluded that superantigen-mediated clonal deletion was occurring with the same efficiency in *crt*$^{-/-}$ as well as *crt*$^{+/+}$ thymocytes. Further assays addressing the efficiency of thymocyte clonal deletion included: (a) CD4/CD8 down-regulation as well as annexin V staining in DP cells from single cell suspensions of thymocytes treated with CD3ε antibodies (5 μg/ml; not depicted), (b) deletion of DP cells in FTOCs treated with CD3ε antibodies (10 μg/ml; not depicted), and (c) deletion of sorted DP cells with fixed concentrations of TCR-β–specific antibodies and decreasing concentrations of CD28 antibodies (Fig. S2 b). Indeed, it was shown that CD28 costimulation can stimulate apoptosis in an anti-CD28 concentration-dependent manner (1): (d) deletion of *crt*$^{-/-}$ as well as *crt*$^{+/+}$ DO11.10tg fetal thymocytes in FTOCs in which one lobe was cultured in medium and the other lobe was in medium supplemented with 1 μM OVAp (Fig. S2 c). None of these assays revealed a significant impairment of the clonal deletion process in *crt*$^{-/-}$ thymocytes.

MATERIALS AND METHODS

Detection of antinuclear, anti–double-stranded DNA, and anti-cardiolipin antibodies.

For detection of ANA, sera dilutions (1:40) from mice (at weeks 7–28 after reconstitution) were incubated with fixed Hep-2 ANA slides (Menarini). ANA were revealed by FITC-conjugated goat anti–mouse IgG (SouthernBiotech). Sera from NZB/NZW F_1 lupus-prone (25-wk-old) and naive BALB/c mice were used as positive and negative controls, respectively. Slides were read at a magnification of 400 and scored as either homogenous, nuclear speckled, or cytoplasmic staining patterns by a reader blinded to the genotype of the mice.

For anti–double-stranded DNA, sera dilutions (1:10) were applied to fixed *Crithidia luciliae* slides (Menarini) and reactive antibodies were revealed by FITC-conjugated goat anti–mouse IgG (SouthernBiotech). The presence of anti-cardiolipin antibodies was evaluated in ELISA. Cardiolipin at 50 μg/ml in ethanol was used to coat microplate wells for 16 h at 4°C. Plates were blotted on paper and blocked with PBS 10% FCS for 1 h at 22–24°C. After one wash in PBS, sera dilutions (from 1:50 to 1:1,000) were added for 1 h at 22–24°C. Plates were then washed three times in PBS and binding of IgG specific for cardiolipin was revealed by alkaline phosphatase-conjugated goat anti–mouse IgG (SouthernBiotech). As positive controls, we used sera from MRL/lpr mice.

Flow cytometry.

For FACS analysis, mAbs conjugated with either FITC, PE, CyChrome, or APC against the following antigens were used: CD8α (CD62L; MEL-14), CD25 (PC61.5), CD4 (L3T4), CD69 (H1.2F3), CD3ε (145-2C11), CD44 (IM7), TCR-β (H57-597), γδ TCR (GL3), and CD11c (N418; all from eBioscence); and I-A^b (AF6-120.1), H-2K^b (AF6-88.5), H-2K^d (SF1-1.1), TCR Vβ3 (KJ25), TCR Vβ5.1, 5.2 (MR9-4), TCR Vβ8 (F23.1), and TCR Vβ11 (RR3-15; all from BD Biosciences). Clonotype (DO11.10)-specific KJ1-26 mAb was from Caltag. Cytokine production was analyzed by stimulating T cells with PMA and ionomycin for 4 h. Brefeldin A (Sigma-Aldrich) was added for the last 2 h of incubation. APC-labeled antibodies to IL-2, IFN-γ, and TNF-α (eBioscence) were used after cell fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences). All samples were analyzed with a FACSCalibur (Becton Dickinson). To detect apoptosis, cells were stained with annexin V (BD Biosciences). Statistical analysis was performed by using a Student's *t* test. Data are reported as mean ± SD. Values of $P < 0.05$ were considered significant.

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In vitro and in vivo induction of anergy.
Sorted $CD4^+$ effector–memory T cells were treated with 1 μM ionomycin for 16 h before activation. In CFSE dilution experiments, T cell proliferation was measured 3 d after stimulation for 72 h on plates coated with CD3ε antibodies. In [^{3}H]thymidine incorporation experiments, proliferation and IL-2 production were measured 96 h after stimulation on plates coated with CD3ε and CD28 antibodies.

For induction of oral tolerance, $crt^{+/+}$ or $crt^{-/-}$ DO11.10tg chimeras were fed with 100 mg OVA protein by intragastric administration for 3 d and with 20 mg/ml OVA in drinking water for 2 d. $CD4^+$ T cells sorted from the spleen and lymph nodes were incubated for 96 h with BALB/c dendritic cells pulsed with different concentrations of $OVA_{323–339}$ peptide. The extent of proliferation was evaluated by [^{3}H]thymidine incorporation. Culture supernatants were collected 48 h after activation and IL-2 concentration was measured by ELISA (Quantikine; R&D Systems).

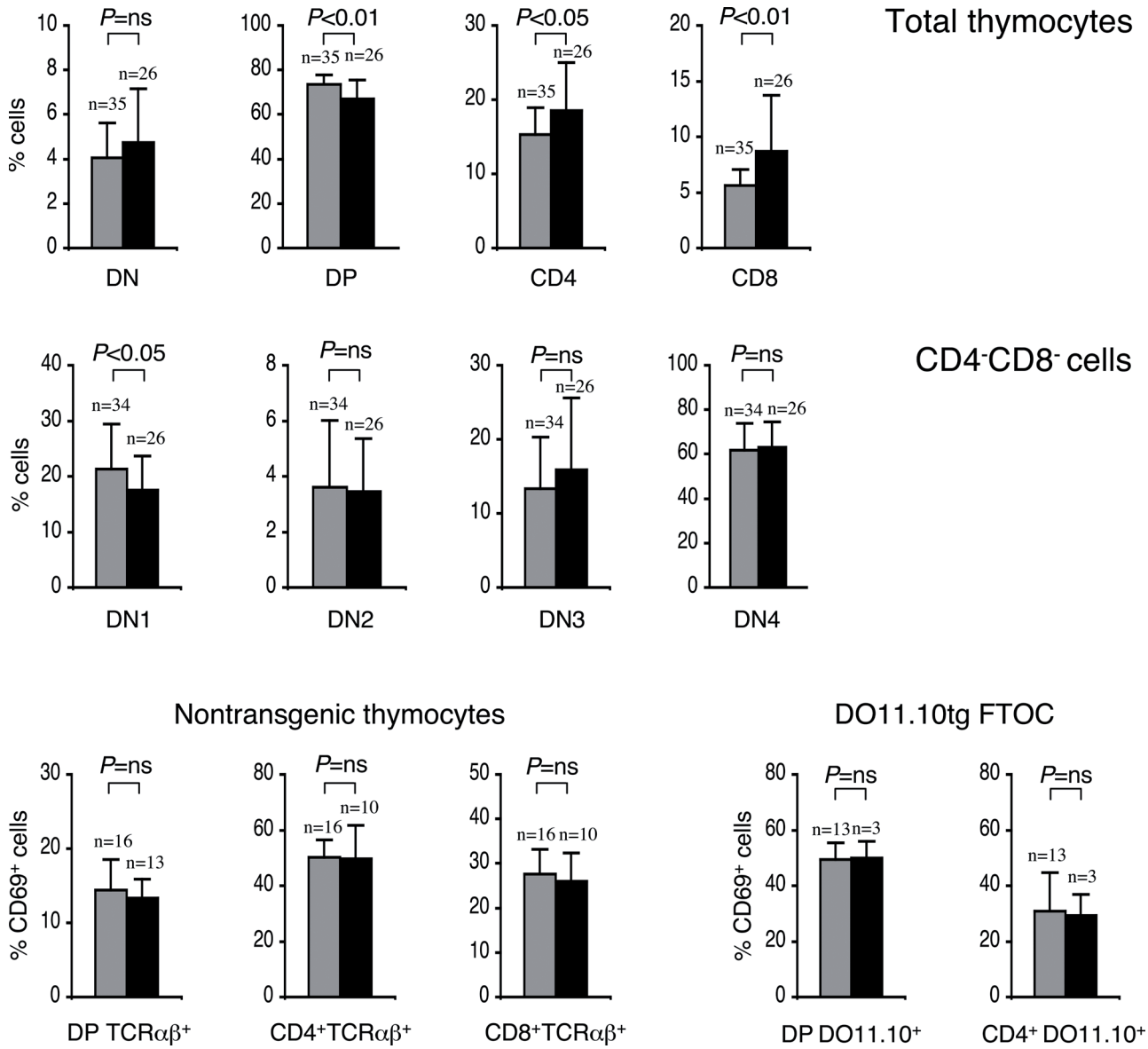

Figure S1. Thymocyte subset representations in $crt^{+/+}$ (gray bars) and $crt^{-/-}$ (black bars) mice. The two bottom right panels display percentages of $CD69^+$ cells in DP and single positive subsets expressing the DO11.10 TCR obtained from the FTOCs of $crt^{+/+}$ (gray bars) and $crt^{-/-}$ (black bars) DO11.10tg embryos.

Calcium imaging.
Cultured T cell clones or ex vivo–isolated effector–memory T cells were loaded for 30 min at room temperature with 5 μM FURA-2 pentacetoxy-methylester in RPMI with FCS, washed in the same solution, and plated on polylysin-coated coverslips in RPMI with FCS containing 2.5 μg/ml biotinylated CD3ε mAb for 15 min. Coverslips were then washed and transferred to the recording chamber of an inverted microscope (Axiovert 100; Carl Zeiss MicroImaging, Inc.) equipped with a calcium imaging unit. The experiments were performed in a static bath (155 mM NaCl, 4.5 mM KCl, 10 mM glucose, 5 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 2 mM $CaCl_2$) at 28–30°C. For the assays, a modified CAM-230 dual wavelength microfluorometer (Jasco) was used as a light source. The experiments were performed using an Axon Imaging Workbench 2.2 equipped with a PCO SuperVGA SensiCam (Axon Instruments). The ratio values in discrete areas of interest were calculated from sequences of images to obtain temporal analyses. The images were acquired at 1 340/380 ratios/s. For stimulation, surface-bound CD3ε antibodies were cross-linked by the addition of 2.5 μg/ml avidin.

REFERENCE

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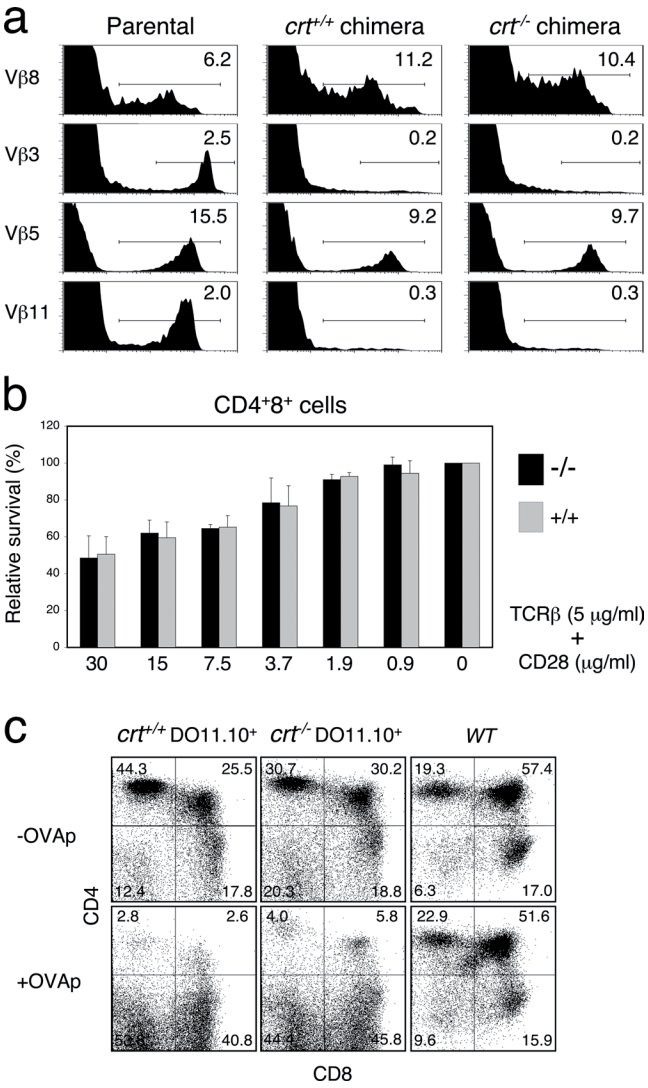

Figure S2. (a) Staining of ex vivo thymocytes from *crt*$^{+/+}$ C57BL/6 mice (Parental) and *crt*$^{+/+}$ and *crt*$^{-/-}$ chimeric BALB/c RAG/γ chain double KO mice with CD4, CD8, and the indicated TCR Vβ–specific antibodies. Histograms show TCR Vβ staining of electronically gated CD4$^+$ cells. Each histogram is representative of at least four independent mice. (b) Relative survival of sorted CD4+8+ DP cells from *crt*$^{+/+}$ and *crt*$^{-/-}$ chimeric mice incubated for 16 h in microplate wells coated with TCR-β (H57-597) mAb at 5 μg/ml and decreasing concentrations of CD28 mAb. Viable cells were scored by forward versus side scatter, propidium iodide exclusion, and absence of annexin V staining in standardized FACS acquisitions. The displayed values are mean ± SD of three independent experiments. (c) CD4/CD8 staining of cells recovered from the FTOCs of the indicated genotype that were either untreated or treated with OVAp (1 μM). The experiment displayed is representative of at least four independent FTOCs.