Regulation of peripheral T cell activation by calreticulin

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Regulated expression of positive and negative regulatory factors controls the extent and duration of T cell adaptive immune response preserving the organism’s integrity. Calreticulin (CRT) is a major Ca2+ buffering chaperone in the lumen of the endoplasmic reticulum. Here we investigated the impact of CRT deficiency on T cell function in immunodeficient mice reconstituted with fetal liver crt−/− hemopoietic progenitors. These chimeric mice displayed severe immunopathological traits, which correlated with a lower threshold of T cell receptor (TCR) activation and exaggerated peripheral T cell response to antigen with enhanced secretion of inflammatory cytokines. In crt−/− T cells TCR stimulation induced pulsatile cytosolic elevations of Ca2+ concentration and protracted accumulation of nuclear factor of activated T cells in the nucleus as well as sustained activation of the mitogen-activated protein kinase pathways. These observations support the hypothesis that CRT-dependent shaping of Ca2+ signaling critically contributes to the modulation of the T cell adaptive immune response.

Abbreviations used: [Ca2+]i, intracellular free Ca2+; CRT, calreticulin; DKO, double knockout; DP, double positive; FLP, fetal liver hemopoietic progenitor; MAPK, mitogen-activated protein kinase; OVAp, OVA peptide; SERCA, sarco-endoplasmic reticulum calcium transport ATPase; SP, single positive; T reg, T regulatory.

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in the ER where it assists the folding of glycoproteins (16). The protein may be divided into three structural and functional domains: the NH2-terminal N-domain, which is extremely conserved and likely participates in the folding of specific substrates and, together with the extended proline-rich P-domain, has a lectin-like chaperone activity. The acidic COOH-terminal C-domain with low affinity, high avidity for Ca2+ contributes to the Ca2+ storage capacity of the ER (17). The amount of CRT-bound Ca2+ in the ER may dramatically influence the cell fate; e.g., overexpression of CRT leads to increased susceptibility to apoptotic stimuli (18), whereas CRT-deficient cells are more resistant to apoptosis (19). CRT deficiency in mice is embryonically lethal because of altered cardiac development, and the majority of crt−/− embryos die at day 12–13 of gestation (20). In embryonic crt−/− cardiomyocyte, defective Ca2+ release from the ER upon agonist stimulation results in defective calcineurin-dependent activation of myocyte enhancer factor 2C and contributes significantly to embryonic lethality (21). Indeed, transgenic expression of a constitutively active isoform of calcineurin in the heart resumed myofibrillogenesis in crt−/− embryos (22).

To study the impact of CRT deficiency on lymphocyte function we generated fetal liver chimeric mice by reconstituting RAG-2/common γ chain double knockout (DKO) mice with fetal liver hemopoietic progenitors (FLPs) from crt−/− embryos. Crt−/− fetal liver chimeras displayed immunopathological traits that correlated with exaggerated responsiveness to TCR stimulation, increased cytokine production, reduced apoptosis, and altered regulation of signaling in peripheral T cells. These observations suggest that CRT plays a crucial role in regulating the effector phase of the adaptive immune response.

RESULTS

Immunopathological phenotype of crt−/− fetal liver chimeras

RAG/γ chain DKO mice reconstituted with crt−/− FLPs display alopecia and blepharitis starting at week 7 after reconstitution (Fig. 1 a). Histological analysis of the skin revealed epidermis hyperplasia and an inflammatory infiltrate (not depicted). An inflammatory infiltrate was also evident in the peribronchial space of the lung, whereas the thymus, gut, kidney, liver, and pancreas were histologically unaltered. Antinuclear, anti–dou-

Figure 1. Phenotype of chimeric mice. (a) RAG/γ chain DKO mice reconstituted with crt+/+ and crt−/− FLPs at weeks 12 (crt+/+), 8 (crt−/−, top right), 10 (crt−/−, bottom left), and 12 (crt−/−, bottom right) after reconstitution. (b) Relative distributions of chimeric mice with respect to phenotype (left) and detection of TNF-α in the serum (>2 pg/ml). WS, wasting syndrome.
Thymocyte development in crt$^-/-$ chimeric mice

Because pre-TCR (24) and αβTCR (25–27) signaling induce [Ca$^{2+}$] elevations during thymocyte development, we tested whether CRT deficiency interfered with T cell differentiation in the thymus. Immature thymocyte subset representation did not reveal an altered pre-TCR signaling in αβ$^+$ thymocytes (see Supplemental Results and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20051519/DC1). Next, we checked whether CRT deficiency affected positive selection of αβTCR$^+$ CD4$^+$8$^+$ double positive (DP) cells. Cell recoveries (αβ$^+$/αβ$^-$: 45.8 ± 27.8 × 10$^3$, n = 40; αβ$^-$: 42.9 ± 26.6 × 10$^3$, n = 30) and CD4/CD8 ratios (αβ$^+$/αβ$^-$: 2.79 ± 0.65, n = 24; αβ$^-$: 2.43 ± 0.97, n = 25) of thymocytes analyzed between weeks 6 and 20 after reconstitution were not significantly different. Thymocyte subset distribution with respect to CD4 and CD8 expression revealed a modest but statistically significant reduction of the DP compartment with relative increases of both CD4 and CD8 single positive (SP) cells (Fig. S1). DP cell contraction was most severe in the atrophic thymi obtained from animals affected by the wasting syndrome (not included in the statistical analysis) in which DP cells were undetectable. We imputed DP cell reduction in αβ$^-$ thymocytes to circulating proinflammatory cytokines affecting DP cell viability. Up-regulation of αβTCR and CD69 in DP cells as well as CD69 expression in CD4$^+$ and CD8$^+$ SP cells were unaffected, suggesting that positive selection was occurring analogously in αβ$^-$ as well as αβ$^+$ thymocytes (Fig. S1). It was possible that a thymocyte expressing a given TCR responded differently to positively selecting signals in the absence of CRT. Then, H-2$^b$ αβ$^+$ T cells were crossed with H-2$^b$ mice transgenic for the I-A$^b$-restricted DO11.10 TCR specific for the OVA peptide (OVAp) 323–339 (28). The αβ$^-$/αβ$^+$DO11.10tg progeny was intercrossed and H-2$^b$αβ$^-$/αβ$^+$DO11.10tg embryos were identified. Analysis of T cell development in fetal thymus organ culture with thymi from H-2$^b$αβ$^-$/αβ$^+$DO11.10tg embryos did not reveal differences in CD69 expression in both DO11.10tg DP cells and DO11.10tg CD4$^+$ SP cells (Fig. S1), implying unaltered positive selection of DO11.10tg thymocytes in the absence of CRT. CRT deficiency decreases the sensitivity of a cell to apoptosis (19); thus, we checked whether this behavior was also common to thymocytes undergoing negative selection in the thymus. To assess thymocyte sensitivity to apoptosis we analyzed in vivo deletion by endogenous superantigens and in vitro induction of apoptosis in different assays (see Supplemental Results and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20051519/DC1). None of these experiments revealed a significant impairment of the clonal deletion process in αβ$^-$/αβ$^+$ thymocytes. We concluded that CRT deficiency was not manifestly affecting either pre-TCR– or αβTCR–driven responses in the thymocyte.

Normal functional activity of T regulatory (T reg) cells from αβ$^-$/αβ$^+$ chimeras, but reduced sensitivity of αβ$^-$/αβ$^+$ T cells to T reg cell-mediated suppression

The CD4$^+$25$^+$ T reg cell subset exerts a crucial role in controlling anti-self immune responses (29) as well as the extent of T cell effector responses, thus inhibiting possible tissue damage resulting from antimicrobial immune responses (30). The abnormal activation of T cells in the periphery of αβ$^-$/αβ$^+$ chimeras could be due to inefficient function of the T reg cell subset. CD4$^+$25$^+$ cells were purified from αβ$^-$/αβ$^+$ as well as αβ$^+$ chimeras and challenged for immunoregulatory competence in inhibition of the mitogenic response of naive T cells to CD3ε antibodies. The same efficiency in inhibiting the proliferative response of αβ$^+$ naive T cells was detected for...
were nonmitogenic for $\alpha^+\alpha^+$ cells (Fig. 4 a). To analyze the impact of CRT deficiency on the T cell response to antigen, H-2b/d $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ DO11.10tg FLPs were used to reconstitute H-2d RAG/$\gamma$ chain DKO mice. Naive CD4$^+$ DO11.10tg T cells from the lymph nodes and spleens of reconstituted mice were sorted and stimulated in vitro with I-A$^d$ DCs loaded with different concentrations of OVAp. Fig. 4 b shows that whereas DCs pulsed with 2 $\mu$M OVAp generated comparable responses in $\alpha^+\alpha^-$ versus $\alpha^+\alpha^+$ cells, pulses with 0.2 $\mu$M OVAp generated robust proliferation of $\alpha^+\alpha^-$ but not $\alpha^+\alpha^+$ T cells. To analyze the response of DO11.10tg cells in vivo, splenocytes from reconstituted mice containing 10$^6$ CD4$^+$ DO11.10tg T cells were labeled with CFSE and injected into H-2b/d mice. After 48 h mice were challenged with I-A$^d$ DCs loaded with OVAp into the footpad. Inspection of the draining lymph nodes of mice adoptively transferred with $\alpha^+\alpha^-$ cells revealed more prominent swelling than those of mice transferred with $\alpha^+\alpha^+$ cells. Moreover, $\alpha^+\alpha^-$ DO11.10tg cells underwent more robust expansion and displayed increased cytokine secretion with respect to the $\alpha^+\alpha^+$ counterpart (Fig. 4, c and d). These data demonstrate that naive T cells from $\alpha^+\alpha^-$ chimeras undergo exaggerated responses upon antigen encounter.

**Increased representation in vivo and hyperresponsiveness of $\alpha^+\alpha^-$ effector–memory T cells**

Analysis of peripheral T cells for representation of effector–memory cells as determined by the CD44$^+$CD62L$^-$ phenotype revealed an increase of this subset in both CD4 and CD8 $\alpha^+\alpha^-$ T cell populations (Fig. 5 a). To test whether effector–memory T cells derived from $\alpha^+\alpha^+$ and $\alpha^+\alpha^-$ fetal liver chimeras might differ in their activation threshold and dependence on costimulation for proliferation and differentiation into cytokine-secreting cells, we isolated CD4$^+$CD44$^+$CD62L$^-$ cells from the spleen and lymph nodes. Purified cells were labeled with CFSE and stimulated for 16 h with immobilized CD3e antibodies in the absence of costimulation as suboptimal stimulus. Stimulated cells were cultured in medium alone or medium containing IL-2, IL-7, or IL-15 and followed by analysis for cell division (31). $\alpha^+\alpha^+$ cells did not proliferate in response to suboptimal CD3 triggering, whereas the same stimulus was sufficient to determine significant expansion of $\alpha^+\alpha^-$ cells (Fig. 5 b). IL-2 and IL-7 further implemented this cell proliferation, whereas IL-15 was without any effect, as expected for murine CD4$^+$ memory cells (32). Stimulation with CD3 and CD28 antibodies for 40 h promoted increased responses in $\alpha^+\alpha^-$ effector–memory T cells; nevertheless, we detected robust proliferation of both $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ effector–memory T cells that was not significantly influenced by cytokine addition (Fig. 5 b). Analysis of CFSE dilution and annexin V staining at different times after CD3e stimulation without CD28 revealed increased percentages of proliferating cells as well as reduced percentages of apoptotic cells in $\alpha^+\alpha^-$ samples when compared with $\alpha^+\alpha^+$ samples. In contrast, no differences between $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ samples were

**In vitro– and in vivo–enhanced responsiveness of $\alpha^+\alpha^-$ naive T cells**

To determine whether $\alpha^+\alpha^-$ T cells were more responsive to TCR triggering we purified CD4$^+$CD44$^+$CD25$^-$CD62L$^+$ naive T cells from chimeric mice. Both $\alpha^+\alpha^+$ and $\alpha^+\alpha^-$ naive T cells were stimulated with decreasing concentrations of immobilized CD3e antibodies. $\alpha^+\alpha^-$ naive T cells responded robustly to concentrations of CD3e antibodies that

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**Figure 4. Enhanced in vitro and in vivo responsiveness of $\alpha^+\alpha^-$ naive T cells.** (a) [3H]thymidine incorporation in purified naive CD4$^+$ T cells stimulated with decreasing doses of plate-bound CD3e mAb. (b) DCs were loaded with the indicated concentrations of OVAp and used to stimulate purified naive CD4$^+$ T cells from H-2b/d mice immunized with OVAp-loaded DCs at day 7 after immunization. This experiment was repeated three times with at least two animals/groups/time points with comparable results. (c) CD4$^+$ DO11.10tg H-2b/d T cells stimulated with decreasing doses of plate-bound CD3e mAb. (d) In vitro T cell proliferation was assayed by [3H]thymidine incorporation in purified naive CD4$^+$ T cells from chimeric mice. Both $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ naive T cell suspensions in all experiments (from 75 to 90%). T cell proliferation was determined by [3H]thymidine incorporation in purified naive CD4$^+$ T cells from chimeric mice. Both $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ naive T cell suspensions in all experiments (from 75 to 90%). T cell proliferation was determined by [3H]thymidine incorporation in purified naive CD4$^+$ T cells from chimeric mice. Both $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ naive T cell suspensions in all experiments (from 75 to 90%).

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Both $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ T reg cells, indicating that T reg cells were not intrinsically affected by CRT deficiency. However, the proliferation of $\alpha^+\alpha^-$ naive T cells was less efficiently inhibited by T reg cells purified from both $\alpha^+\alpha^-$ and $\alpha^+\alpha^+$ chimeras (Fig. 3), suggesting that $\alpha^+\alpha^-$ T cells were less susceptible to T reg cell–mediated suppression than $\alpha^+\alpha^+$ T cells.

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detected when cells were stimulated with CD3ε and CD28 antibodies (Fig. 5 c). Analysis of IL-2 secretion after suboptimal TCR stimulation revealed a prominent increase in \( \text{crt}^{-/-} \) versus \( \text{crt}^{++} \) cells (Fig. 5 d). These results indicate that effector-memory T cells from \( \text{crt}^{-/-} \) chimeras display a lower activation threshold and less dependence on costimulation to mount a productive response. Furthermore, quantification of IL-4 in the supernatant of effector-memory T cells stimulated with CD3ε and CD28 antibodies revealed a prominent increase of IL-4 production in \( \text{crt}^{-/-} \) cultures (Fig. 5 d).

**Induction of unresponsiveness in \( \text{crt}^{-/-} \) T cells**

Induction of T cell unresponsiveness or anergy is supposed to play an important role in maintaining peripheral tolerance (33). Anergy can be induced in vitro through a massive increase in cytosolic Ca\(^{2+}\) before T cell stimulation (34). Therefore, we tested whether sorted effector-memory T cells from FLP chimeras became unresponsive to TCR stimulation after pretreatment with ionomycin (Supplemental Materials and methods). Fig. 6, a and b, shows the results of two independent experiments in which either untreated or ionomycin-pretreated cells were stimulated with CD3ε antibodies. Both cell proliferation scored by CFSE dilution or \(^{3}H\) thymidine incorporation and IL-2 production in \( \text{crt}^{-/-} \) cells were significantly inhibited by ionomycin pretreatment with a relative efficiency undistinguishable from that observed with \( \text{crt}^{++} \) cells. To further substantiate this phenomenon in vivo, we fed DO11.10tg mice with OVA (100 mg/day) for 5 d to assess anergy induction, as described previously (34). Sorted CD4 cells from untreated or OVA-fed mice were stimulated in vitro with DCs pulsed with decreasing doses of OVAp. Fig. 6 c shows that both \(^{3}H\) thymidine incorporation and IL-2 production were significantly reduced in \( \text{crt}^{-/-} \) cells from OVA-fed animals, implying that unresponsiveness could be enforced in \( \text{crt}^{-/-} \) cells.

**Deregulated signal transduction in \( \text{crt}^{-/-} \) T cells**

To investigate whether CRT deficiency affected Ca\(^{2+}\) signaling in T cells, sorted CD4\(^{+}\) effector-memory cells were loaded with fura-2 and analyzed by single cell calcium imaging (Supplemental Materials and methods). Stimulation with CD3ε antibodies was followed by capacitive calcium entry in both \( \text{crt}^{-/-} \) and \( \text{crt}^{++} \) cells, thus indicating that ER calcium release and Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel activation function normally in both cell types. Interestingly, increased frequencies of responding \( \text{crt}^{-/-} \) versus \( \text{crt}^{++} \) cells (48.4 ± 5%, \( n = 120 \) vs. 39.5 ± 4%, \( n = 156 \)) were observed, indicating that the threshold for activation of individual cells may be lowered by CRT deficiency. Because ex vivo cells did not allow prolonged analyses, we performed a more detailed investigation of Ca\(^{2+}\) signaling with OVAp-specific T helper clones derived from DO11.10tg chimeras. Three different clones for each group of \( \text{crt}^{-/-} \) and \( \text{crt}^{++} \) chimeras stimulated with CD3ε antibodies displayed increased frequencies...
of responding crt−/− versus crt+/+ cells as observed with ex vivo–isolated effector–memory cells (Fig. 7 a). Although the most common pattern of response observed in CD3ε-stimulated crt+/+ cells was a single [Ca2+]i spike followed by a sustained, slowly decreasing phase of [Ca2+]i, elevation, repetitive [Ca2+]i elevations leading to an oscillatory pattern were more frequently detected in crt−/− cells (Fig. 7 b). Indeed, in crt−/− clones fewer cells responding to the stimulus with only one or two [Ca2+]i spikes were detected over the time frame of analysis with respect to crt+/+ clones. Also, cells responding to the stimulus with six or more [Ca2+]i spikes were only detected in crt−/− clones (Fig. 7 c). Comparable [Ca2+]i patterns were observed when the same T cell clones were stimulated with OVA-loaded DCs (not depicted).

Because oscillatory [Ca2+]i elevations efficiently induce NFAT dephosphorylation and nuclear translocation (13), we analyzed NFAT dephosphorylation and nuclear translocation in crt−/− as well as crt+/+ T helper clones at different times after CD3ε stimulation. Of the three calcium-regulated NFAT members expressed in T cells (NFAT1, NFAT2, and NFAT4), NFAT1 is highly expressed at baseline (35) and its activation by TCR signaling is relatively short-lived. In fact, the bulk of activated NFAT1 reverts to a phosphorylated, cytoplasmic form within a few hours of stimulation (36). This effect is mediated by NFAT nuclear export kinases such as glycogensynthase kinase-3 (37), which is inhibited in CD3-stimulated human T cells by CD28 costimulation (38). 30 min after CD3 cross-linking with avidin, NFAT1 was efficiently dephosphorylated and translocated to the nucleus in both crt+/+ and crt−/− clones. At 3 h after stimulation, in crt+/+ cells NFAT1 was found in the cytosol mainly in its phosphorylated forms, as described previously (36), whereas in crt−/− cells faster migrating dephosphorylated NFAT1 was still detectable. At 16 h after stimulation, dephosphorylated NFAT1 could be detected in the cytosol only upon prolonged exposure of crt−/− blots, but a prominent band corresponding to dephosphorylated NFAT1 was readily detected in the nuclear fraction of crt−/− but not crt+/+ T cell clones (Fig. 8 a). The prolonged nuclear translocation of NFAT1 in crt−/− T helper clones may be the result of different calcium signaling between crt+/+ and crt−/− cells. Stimulation of T helper clones with DCs loaded with decreasing doses of OVA revealed in crt−/− cells a dose-dependent decrease in NFAT1 nuclear translocation at 16 h after activation, whereas the same low amount of NFAT1 was recovered from nuclear fractions of crt+/+ cells regardless of the OVA concentration used for stimulation (Fig. 8 b). These data further confirm the lower threshold of activation in crt−/− cells and suggest that CRT deficiency positively influences NFAT activation after TCR stimulation.

Nuclear translocation of NFAT1 may trigger the transcription of genes involved in T cell activation as well as genes leading to tolerance and anergy (39). A requisite for the productive immune response consists in the parallel activation of mitogen–activated protein kinases (MAPKs), their nuclear translocation, and phosphorylation of specific transcription factors, which regulate together with NFAT the transcription of genes involved in T cell activation. Therefore, we investigated p38MAPK, ERK, and jun phosphorylation in T helper clones stimulated with CD3 antibodies. At early time points
after stimulation we did not detect differences in the phosphorylation intensities (not depicted). However, at 16 h after stimulation although c-jun was analogously phosphorylated in both crt+/+ and crt−/− clones, more intensely phosphorylated p38MAPK and ERK could be detected in crt−/− compared with crt+/+ T helper clones, implying a more sustained activation of these MAPK pathways (Fig. 8 c). Collectively, these results support the view that CRT critically participates in the regulation of TCR-dependent gene transcription during an adaptive immune response.

DISCUSSION

CRT is a molecular chaperone and the main Ca2+ buffering protein in the ER. In vivo as well as in vitro studies with crt−/− cells have demonstrated the crucial role of CRT in the homeostasis of intracellular Ca2+, thereby regulating signal transduction and cell susceptibility to apoptosis (18, 19). However, the role CRT might play in TCR-mediated signaling was not investigated so far. Here we showed that RAG/γ chain DKO mice reconstituted with crt−/− CD4 T cell clones responding with [Ca2+]i elevations upon CD3 stimulation although c-jun was analogously phosphorylated and this binding was shown to influence inflammatory processes (43). Recently, CRT at the cell surface was implicated in inducing the clearance of apoptotic cells (44). The role of T cells in determining the phenotype of crt−/− chimeras was supported by the absence of any sign of disease at week 20 after reconstitution in chimeras generated with CD4−/− DO11.10tg T cell clones stimulated with CD3ε mAb for the indicated times. The result is representative of three different clones per group. (c) Immunoblot of phosphorylated p38MAPK, ERK, and c-jun in crt+/+ and crt−/− T helper clones, implicating a more sustained activation of these MAPK pathways (Fig. 8 c). Collectively, these results support the view that CRT critically participates in the regulation of TCR-dependent gene transcription during an adaptive immune response.

Figure 7. Recurrent calcium elevations in crt−/− T cell clones. CD4+ DO11.10tg T cell clones from crt+/+ and crt−/− chimeras were loaded with Fura-2 and monitored for changes in [Ca2+]. (a) Percentages of crt+/+ and crt−/− T cell clones responding with [Ca2+]i elevations upon CD3 stimulation (means ± SD of at least 97 single cells). (b) Temporal analysis of [Ca2+]i elevations upon CD3 stimulation of representative crt+/+ and crt−/− clones. (c) Relative distributions of cells with respect to numbers of [Ca2+]i elevations over 1,100 s. Means ± SD of three independent experiments with two different clones per group. Black bars, crt+/+, n = 89; red bars, crt−/−, n = 93.

Figure 8. Protracted NFAT activation and MAPK signaling in crt−/− T cell clones. (a) Immunoblot analysis of NFAT1 in cytosolic and nuclear fractions of crt+/+ and crt−/− DO11.10tg T cell clones stimulated with CD3ε mAb for the indicated times. The result is representative of three different clones per group. (b) Immunoblot of NFAT1 in nuclear fractions of crt+/+ and crt−/− DO11.10tg T cell clones stimulated for 16 h with DCs loaded with decreasing doses of OVA. The result is representative of three experiments with two different clones per group. (c) Immunoblot of phosphorylated p38MAPK, ERK, and c-jun in crt+/+ and crt−/− DO11.10tg T cell clones before and 16 h after crt−/− stimulation. The result is representative of four different experiments with at least two different clones.
antibodies were not detected in the sera from \( \alpha \sigma ^{-/-} \) chimeras, suggesting that an autoreactive response was not implicated in the observed immunopathology. Analysis of peripheral T cell compartments revealed increased frequencies of T cells displaying markers of activation and secreting proinflammatory cytokines as well as expansion of the CD44\(^+\)CD62L\(^-\) effector–memory subset in mice reconstituted with \( \alpha \sigma ^{-/-} \) FLPs. Chimeric mice generated with \( \alpha \sigma ^{-/-} \) DO11.10tg FLPs did not display an overt pathological phenotype as “polyclonal” mice up to 24 wk after reconstitution. Peripheral colonization of lymphopenic hosts by T cells determines homeostatic expansion of positively selected T cells and a pattern of gene expression similar to that of antigen–experienced cells (45) with acquisition of the CD44\(^+\)CD62L\(^-\) phenotype (46). Because in DO11.10tg mice a fraction of peripheral T cells does not express the clonotypic TCR but TCRs with different specificities, we compared the representation of CD44\(^+\)CD62L\(^-\) cells either expressing or not the transgenic TCR. \( \alpha \sigma ^{-/-} \) and \( \alpha \sigma ^{+/+} \) chimeras did not display significant differences in CD44\(^+\)CD62L\(^-\) expression in the clonotype–positive compartment (\( \alpha \sigma ^{+/+} = 37.2 \pm 11.4\% \), \( n = 4 \); \( \alpha \sigma ^{-/-} = 38.7 \pm 12.2\% \), \( n = 4 \)); however, a significant difference in CD44\(^+\)CD62L\(^-\) cell representation was detected in the clonotype–negative fraction (\( \alpha \sigma ^{+/+} = 39.0 \pm 6.2\% \), \( n = 4 \); \( \alpha \sigma ^{-/-} = 54.8 \pm 7.5\% \), \( n = 4 \), \( P = 0.006 \)), thereby suggesting that Crt deletion altered the T cell response to environmental antigens but not homeostatic expansion. Moreover, this indicates that immunopathology in \( \alpha \sigma ^{-/-} \) chimeras depends on hyperresponsiveness to environmental antigens of a physiological peripheral T cell pool. Indeed, antigen priming of DO11.10tg \( \alpha \sigma ^{-/-} \) cells determined exaggerated swelling of the draining lymph node as well as more robust cell proliferation and cytokine secretion than observed with the \( \alpha \sigma ^{+/+} \) counterpart. These results were consistent with the reduced apoptosis and increased IL-2 production by suboptimally stimulated \( \alpha \sigma ^{-/-} \) T cells, suggesting that the threshold for TCR activation was lowered and persistence of positive signals was enforced in peripheral T cells by Crt deficiency. Calcium signaling intervenes at multiple stages during T cell development in the thymus; e.g., thymocyte \( \beta \) selection by the pre-TCR (24) and positive as well as negative selection by the \( \alpha \beta \) TCR (25–27). Moreover, the sustained [Ca\(^{2+}\)] elevation after TCR triggering of peripheral T cells is crucial for the development of the immune response (11). Crt deficiency affected T cell function manifestly in the periphery while thymocyte development appeared unaltered. In spite of analogous signal transduction pathways used by both immature and mature T cells, the magnitude of the [Ca\(^{2+}\)] increase after TCR stimulation is larger in mature T cells (references 24 and 25, and unpublished data). We hypothesize that Crt deficiency selectively affects Ca\(^{2+}\) signaling when sustained [Ca\(^{2+}\)] elevations are critical in the regulation of the T cell response, likely in the peripheral T cell upon antigen encounter. After TCR stimulation, we observed repetitive [Ca\(^{2+}\)] peaks leading to an oscillatory pattern in \( \alpha \sigma ^{-/-} \) cells, whereas sustained Ca\(^{2+}\) influx predominated in \( \alpha \sigma ^{+/+} \) T cells. Similar observations were made in Xenopus oocytes, where Crt was proposed to regulate the sarco/endoplasmic reticulum calcium transport ATPase (SERCA), which pumps Ca\(^{2+}\) from the cytosol into the ER lumen, thereby influencing the degree of ER Ca\(^{2+}\) filling (47). Indeed, Crt association with SERCA reduced pump activity through the recruitment of the ER oxidoreductase ERP57 and oxidation of critical ER facing thiol groups of SERCA (47, 48). An increased activity of the SERCA pump might contribute to the oscillatory [Ca\(^{2+}\)] pattern observed in \( \alpha \sigma ^{-/-} \) T cells. Magnitude, kinetics, and subcellular location of Ca\(^{2+}\) signal can all be deciphered by the cell and translated into distinct responses, thereby conferring specificity to an otherwise pleiotropic signal (49). Indeed, [Ca\(^{2+}\)], oscillation frequency was shown to control the activation of distinct sets of transcription factors and the expression of different genes (12, 13). [Ca\(^{2+}\)] spikes elicited by CD3\(\varepsilon\) antibodies were positively correlated with NFAT-regulated gene expression (50). Recently, oscillatory changes in [Ca\(^{2+}\)], were shown to be more efficient than a continuous increase in translocating NFAT (13). CD3\(\varepsilon\) stimulation of T cell clones derived from DO11.10tg \( \alpha \sigma ^{-/-} \) chimeric mice resulted in the reduced detection of phosphorylated NFAT1 forms in the cytosol and the prominent accumulation of dephosphorylated forms in the nucleus, suggesting that the strength of TCR signaling was enhanced in \( \alpha \sigma ^{-/-} \) cells and nuclear export kinase activity was inhibited. Similar results were obtained in the same clones stimulated with the cognate antigen presented by DCs, thus suggesting that enhanced IL-2 secretion in \( \alpha \sigma ^{-/-} \) cells could depend on aberrant NFAT regulation. The analysis of the T cell response after sustained [Ca\(^{2+}\)], elevation by ionomycin treatment revealed the Ca\(^{2+}\)–dependent up-regulation of genes involved in the targeted proteolysis of signaling proteins. This transcriptional program is controlled by calcineurin/NFAT activation and induces T cell unresponsiveness to TCR stimulation (51). Deletion of these genes in mice results in immunopathology, suggesting the physiological relevance of such a transcriptional program in T cell homeostasis. Treatment of \( \alpha \sigma ^{-/-} \) effector–memory T cells with ionomycin promoted unresponsiveness to subsequent TCR stimulation and OVA feeding of DO11.10tg chimeras promoted ex vivo T cell unresponsiveness of sorted \( \alpha \sigma ^{-/-} \) CD44\(^+\) cells, suggesting that Crt deficiency was not altering the functional efficiency of the inhibitory machinery implicated in inducing T cell unresponsiveness but maybe interferes with its up-regulation after TCR stimulation. Crt could ensure the appropriate supply of Ca\(^{2+}\) for the physiological calcium response and regulated T cell activation. Interestingly, it was shown that calcium restriction in kidney epithelial cells was associated with increased B-Raf protein levels and derepressed B-Raf/ERK pathways leading to phenotypic remodeling and conversion to cell proliferation of otherwise growth–inhibited cells (52). The more oscillatory calcium response observed in \( \alpha \sigma ^{-/-} \) cells could result in a different transcriptional program in which activation components would dominate. Positive
regulators of TCR-induced activation include p38MAPK, ERK, and jun (53). These proteins were shown to influence the NFAT transactivation potential, further contributing to the T cell activation genetic program controlled by NFAT (39, 54, 55). Analysis of p38MAPK, ERK, and jun phosphorylation after TCR stimulation revealed significantly protracted activation of p38MAPK and ERK in αT−/− clones, implying a defective modulation of these T cell activation pathways in the absence of CRT. Accordingly, [Ca2+] oscillations were shown to reduce the threshold for Ras activation potentiating ERK activation induced by suboptimal agonist concentration (56).

αT−/− chimeras were characterized histologically by normal thymus, gut, kidney, liver, and pancreas. Inflammatory infiltrates were evident in the skin and in the peribronchial space of the lung. Furthermore, IgG, and IgE levels were significantly increased. These features of αT−/− chimeras are reminiscent of NFAT1/4 DKO mice described by Ranger et al. (41), in which the unbalanced activation of NFAT2 induced up-regulation of Th2-dependent cytokine responses. Another study demonstrated that NFAT1 and NFAT2 were necessary for TCR-mediated T cell effector functions, suggesting that the functions of NFAT1 and NFAT2 may be largely redundant in the regulation of most effector cytokines (57) and indicating that NFAT1 might participate with NFAT2 in the generation of T cell effector functions. The phenotype of αT−/− chimeras could derive from a dominance of Th2 differentiation in vivo resulting from hyperresponsiveness to TCR stimulation (58). Indeed, considerable amounts of IL-4 were detected in the supernatants of αT−/− but not αT+/+ primary T cells stimulated with CD3 and CD28 antibodies. In conclusion, our study suggests that CRT modulates T cell activation by regulating calcium signaling. CRT deficiency results in a lower stimulation threshold, enhanced TCR signaling through MAPK pathways, and protracted NFAT activation in peripheral T cells, thereby endowing the adaptive T cell response with harmful potential for the organism.

MATERIALS AND METHODS

Mice. αT−/− (H-2b) mice (20), BALB/c Rag2−/− (H-2b) mice provided by M. Ita (Central Institute for Experimental Animals, Maymune, Kawasaki, Japan), B6D2F1 (BALB/c × C57BL/6 J, H2b) mice from Charles River Germany, and transgenic DO11.10 (H-2b) mice from The Jackson Laboratory were bred and treated in accordance with the Swiss Federal Veterinary Office guidelines. Experiments were approved by “Dipartimento della Santità e della Società.” To generate fetal liver chimeras, timed pregnant αT−/− female mice were killed at day 13.5 and the embryos were explanted under sterile conditions (genotype frequencies were as follows: αT−/−, 16%; αT+/−, 31%; αT+/+, 53%; n = 553). The following two sets of primers were used to screen the progeny: 5′-CTCCAGGTCCCGTAA- AATTTGCC-3′ and 5′-AGTCTAAACGCTCAAAAGGACC-3′ for the detection of the αT WT gene and 5′-TGGTGTTCATTACGATGCC- CGCTTCCGATT-3′ and 5′-CAGGAGTAGCCACCTCCATAGCACCC- ATTATG-3′ for identification of the αT mutant gene. Rag2−/− chimeras were generated at 6 wk of age as recipients for lymphoid reconstitution. Mice were irradiated with 4 Gy from a 137Cs source (Biobeam 8000; STS GmbH) at 6 wk of age were used as recipients for lymphoid reconstitution. Mice were crossed with DO11.10tg mice and the progeny were screened for αT mutation as described above and for the TCR transgene with the following primers: 5′-CAGAGGGGATCCAGTGGCCACG-3′ and 5′-TGGGCTCT- ACAGGGGTTGT-3′. H-2b αT−/− and αT+/+ DO11.10tg embryos from F1 intercrosses were identified for αT and TCR transgenes in the PCRs described above and for H-2 haplotype by generating DCs from fetal livers in the presence of recombinant granulocyte-macrophage colony-stimulating factor (R&D Systems), and by flow cytometry of the in vitro–generated DCs with CD11c and H-2–specific antibodies.

T cell proliferation and proliferation assays. T cells were isolated from peripheral lymph nodes and spleens by negative selection with immunomagnetic beads followed by cells sorting. Cells were incubated with biotin-conjugated anti-CD19, anti-Ter119, anti-pan-NK, and anti-CD11b mAbs (eBioscience). T lymphocytes were enriched by removing biotin-labeled cells bound to streptavidin-conjugated magnetic beads. CD4− naive (CD4+CD44+CD25−CD62L−) and effector–memory (CD4+CD44+CD25−CD62L+) T cell subsets were sorted with a FACS Aria (Becton Dickinson). The cells were labeled with CFSE (Invitrogen) and stimulated with 10 μg/ml of plate-bound anti-CD3e mAb with or without 5 μg/ml of coimmobilized anti-CD28 mAb (eBioscience). At different times, we transferred effector–memory cells to uncoated wells to terminate TCR stimulation and continued culture for 96 h in medium alone or in the presence of 90 U/ml IL-2, 25 ng/ml IL-7, or 25 ng/ml IL-15, as described previously (31). FACs acquisitions were standardized by fixed numbers of calibration beads (BD Biosciences). IL-2 was measured in supernatants with an ELISA kit according to the manufacturer’s instructions (Quantikine; R&D Systems). CD4− naive T cell proliferation was scored on sorted cells that were cultured in anti-CD3e–coated flat-bottom microplates at 3 × 104 cells/well for 96 h. [3H]Thymidine (2 μCi/well) was added and the plates were harvested after 12 h of incubation. Bone marrow–derived DCs were generated from femurs of mice in the presence of recombinant granulocyte–macrophage colony-stimulating factor (R&D Systems). Sorted CD4+ naive T cells from DO11.10tg mice were cultured at 10 DO11.10tg cells to 1 DC ratio with DCs pulsed with various doses of OVA232-239 peptide. For adoptive transfer of TCR transgenic cells, spleen and lymph node cells from RAG2−/− chain DKO chimeric mice reconstituted with either αT−/− or αT+/+ DO11.10tg H-2b wild-type progenitors were labeled with CFSE. Cell suspensions containing 104 TCR transgenic cells were i.v. injected into B6D2F1 mice. Adoptively transferred mice were immunized s.c. into the footpad 24 or 48 h later with 2 × 105 DCs pulsed with 2 μM OVA232-239 peptide at 37°C for 1 h. Pooled lymph node cells were analyzed at various times after immunization for cell number and cytokine secretion by DO11.10tg cells.

T reg cell inhibition assay. CD4+ T reg cells (CD4+CD25+) and CD4− naive T cells (CD4+CD25−CD62L+) were isolated from peripheral lymph nodes and spleens by negative selection with immunomagnetic beads followed by cell sorting as described above. Naive CD4− T cell proliferation was scored on sorted cells in 96-well round-bottom plates at 50,000 cells/well with serial dilutions of T reg cells (from 1:1 to 1:64) in the presence of 5,000 DCs/well and with 0.2 μg/ml anti-CD3e mAb. After 48 h of incubation [3H]Thymidine (2 μCi/well) was added and the plates were harvested after 12 h of incubation.

Biochemical procedures. CD4+ DO11.10tg T cell clones were generated from the spleens of αT−/− and αT+/+ DO11.10tg chimeras by stimulation with OVA2+/-loaded DCs. They were restimulated with OVA2+/-loaded DCs every 14 d. For CD3 stimulation, clones at days 10–13 from restimulation were cocultured in 96-well round-bottom plates at 50,000 cells/well with recombinant granulocyte–macrophage colony-stimulating factor (R&D Systems). Sorted CD4+ naive T cells from DO11.10tg mice were cultured at 10 DO11.10tg cells to 1 DC ratio with DCs pulsed with various doses of OVA232-239 peptide. T cell proliferation and cytokine secretion by DO11.10tg cells was scored on sorted cells in 96-well round-bottom plates at 50,000 cells/well with serial dilutions of T reg cells (from 1:1 to 1:64) in the presence of 5,000 DCs/well and with 0.2 μg/ml anti-CD3e mAb. After 48 h of incubation [3H]Thymidine (2 μCi/well) was added and the plates were harvested after 12 h of incubation.
antibodies were provided by N.R. Race (NCI-Frederick Cancer Research and Development Center, Frederick, MD), phospho-p38 MAPK (3D7) and phospho-ERK (197G2) rabbit mAbs were from Cell Signaling Technology, phospho-c–jun (KM-1) mouse mAb was from Santa Cruz Biotechnology, Inc., and β actin mouse mAb (A-5411) was from Sigma-Aldrich.

Online supplemental material. Supplemental Results and Figs. S1 and S2 show unaltered pre-TCR signaling as well as unaltered positive selection and deletion of crt−/− thymocytes. Supplemental Materials and methods describe the detection of autoantibodies, mAbs used in flow cytometry, induction of anergy, and calcium imaging. Supplemental Results, Materials and methods, and Fig. S1 are available at http://www.jem.org/cgi/content/full/jem.20051519/DC1.

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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−/− thymocytes.

Unaltered deletion of crt−/− thymocytes
Because fetal liver hemopoietic progenitors from α/β and α−/β− 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-Eβ 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 α/β+ and α−/β− 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 α−/β− as well as α/β+ 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 cosimulation can stimulate apoptosis in an anti-CD28 concentration–dependent manner (1); (d) deletion of α−/β− as well as α/β+ 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 OVAα (Fig. S2 c). None of these assays revealed a significant impairment of the clonal deletion process in α−/β− 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 F1, 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: CD9a (CD62L; MEL-14), CD25 (PC61.5), CD4 (L3T4), CD69 (H1.2F3), CD3ε (145-2C11), CD44 (IM7), TCR-β (H57-597), γ6 TCR (GL3), and CD11c (N418; all from eBioscience); and I-Aβ (AF6-120.1), H-2Kb (AF6-88.5), H-2Kd (SF1-1.1), TCR Vβ3 (K25), 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 FACS Calibur (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](image-url)  
**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 polylysine-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 MgCl₂, 2 mM CaCl₂) 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