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ROR γ t limits the amount of the cytokine receptor γ c through the prosurvival factor Bcl-x_L in developing thymocytes

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Abstract

The cytokine receptor subunit γ c provides critical signals for T cell survival and differentiation. We investigated the molecular mechanism that controls the cell surface abundance of γ c during T cell development in the thymus. We found that the amount of γ c was low on CD4⁺CD8⁺ double-positive (DP) thymocytes before their positive selection to become mature T cells. The transcription factor ROR γ t was abundant in immature DP thymocytes, and its loss resulted in an increase in the abundance of surface γ c, specifically on preselection DP cells. Rather than directly repressing expression of the gene encoding γ c, ROR γ t acted through the antiapoptotic protein Bcl-x_L to reduce the abundance of surface γ c, which resulted in decreased cytokine signaling and was associated with inhibition of cell metabolism and mitochondrial biogenesis. Accordingly, overexpression of Bcl-x_L in ROR γ t-deficient thymocytes restored the amount of surface γ c to that present on normal preselection DP cells. Together, these data highlight a previously unappreciated role for ROR γ t and Bcl-x_L in limiting γ c abundance at the cell surface and reveal a signaling circuit in which survival factors control cytokine signaling by limiting the abundance and surface distribution of a receptor subunit shared by several cytokines.

INTRODUCTION

T cell development in the thymus is driven by the concerted action of T cell receptor (TCR) and cytokine receptor signaling. Whereas TCR signaling is necessary to select and shape a self-major histocompatibility complex (MHC)-restricted T cell repertoire that is not self-reactive (referred to as “self-MHC-restricted”), cytokine signaling is critical for cell survival and differentiation and for the lineage commitment of thymocytes (1-4). Specifically,

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SUPPLEMENTARY MATERIALS

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cytokines of the common γ chain (γ_c) family are essential for thymopoiesis and also for the generation of various T cell subsets in the thymus, such as CD8⁺ T cells, Foxp3⁺ T regulatory cells (T_{regs}), and natural killer T (NKT) cells (4, 5). The γ_c family members are defined as cytokines that bind to receptor complexes containing the γ_c protein as a subunit, and they include interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, and IL-21 (6). In the absence of γ_c , T cell development is severely impaired, and thymic Foxp3⁺ T_{regs} and NKT cells fail to develop (6, 7). The γ_c subunit stabilizes and increases the binding of the individual cytokines to their cognate cytokine receptors and initiates downstream signaling by activating the tyrosine kinase Janus kinase 3 (8-10). Despite these critical roles, the regulatory mechanism that controls the γ_c cytokine receptor during T cell development is poorly understood.

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Thymic T cell development proceeds along a well-characterized path that can be mapped by the presence or absence of the cell surface molecules CD4 and CD8 (3). The most immature thymocytes display neither CD4 nor CD8 on their surface and are known as double-negative (DN) cells. DN cells produce an immature TCR containing the β chain without the α chain, and activation of this TCR drives their differentiation into CD4⁺CD8⁺ double-positive (DP) thymocytes, which are the first cells to have a functional $\alpha\beta$ TCR on the cell surface. In parallel to TCR signaling, IL-7 receptor (IL-7R) signaling enhances survival and promotes proliferation during the progression from DN to DP cells. Mice lacking either the IL-7 proprietary IL-7R α subunit or the shared γ_c subunit show markedly diminished thymocyte numbers, and thymocytes are arrested at the DN stage of development (11-13). DP cells with self-MHC-restricted TCR specificities are rescued from programmed cell death through a process called “positive selection,” and signaling by intrathymic γ_c cytokines in positively selected cells imposes lineage fate and induces the expression of genes encoding lineage-specific transcription factors, such as Runx3 and Foxp3 (14-17). Between the proliferative burst at the DN stage and the positive selection of DP cells, γ_c receptor signaling must be suppressed. Otherwise, the prosurvival effects of intrathymic γ_c cytokines could result in the survival of cells with inappropriate TCR specificities and, thus, impede the establishment of a functional TCR repertoire (18).

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The abundance of γ_c at the cell surface is markedly reduced on preselection DP thymocytes, and it is reinduced at the cell surface only upon positive selection (7, 16). This reduction in γ_c abundance on DP cells is specific to γ_c because other cytokine receptors, such as IL-4R α and IL-21R, are found in large amounts on DP thymocytes before positive selection (19-22). Thus, the reduction in γ_c abundance is a developmentally controlled event, but the molecular basis for such transient loss of γ_c from developing thymocytes is unclear. Here, we focused our attention on a potential role of the transcription factor ROR γ_t in controlling γ_c because ROR γ_t abundance inversely correlates with surface γ_c abundance during thymocyte development (23, 24). We found that ROR γ_t deficiency was associated with a marked increase in surface γ_c proteins on DP thymocytes. Our data suggest that ROR γ_t acted through the antiapoptotic protein Bcl-x_L, rather than through direct repression of the gene encoding γ_c , to reduce the amount of surface γ_c proteins. Consistently, overexpression of the prosurvival factor Bcl-x_L, which is a downstream effector molecule of ROR γ_t (23, 24), reduced γ_c abundance in the absence of ROR γ_t . Together, these results reveal a causal

relationship between ROR γ t and reduction in the surface abundance of γ c in developing thymocytes that is mediated by Bcl-x_L.

RESULTS

ROR γ t reduces γ c abundance on DP thymocytes before their positive selection

Compared to DN thymocytes and both CD4 single-positive (SP) and CD8 SP thymocytes, developmentally immature preselection DP cells display markedly lower amounts of surface γ c (Fig. 1A) (7, 16). The molecular basis for decreased γ c abundance on DP thymocytes, however, is unclear. Among all populations of thymocytes, the transcription factor ROR γ t is exclusively present in immature DP cells (fig. S1A) (23, 24). Thus, we hypothesized that ROR γ t might be responsible for reducing γ c abundance on DP thymocytes. To test this idea, we quantitated surface γ c on thymocytes from wild-type (WT) mice and ROR γ t-deficient (ROR γ t^{KO}) mice by flow cytometry. DP cells with low amounts of TCR β (TCR β ^{low}) are pre-positive selection thymocytes, and SP cells with high amounts of TCR β (TCR β ^{hi}) correspond to post-positive selection cells. Using the relative amount of TCR β rather than only relying on surface staining for CD4 and CD8 ensured proper segregation of pre- and post-selection thymocytes for analysis.

Here, we found that ROR γ t was necessary to reduce γ c abundance on preselection thymocytes because ROR γ t^{KO} DP cells showed a substantial increase of surface γ c at amounts similar to those observed on mature SP cells of WT mice (Fig. 1B and fig. S1B). Although ROR γ t is a transcription factor, we found that ROR γ t did not reduce γ c abundance by suppressing the expression of *Ii2rg* mRNA, which encodes γ c. Previously, we identified two different species of *Ii2rg* mRNA transcripts that were generated by alternative splicing to produce either a membrane-bound (m γ c) or a soluble form (s γ c) of γ c protein (25). Neither of these γ c-encoding transcripts was increased in the absence of ROR γ t, and the abundance of soluble *Ii2rg* mRNA was rather decreased (Fig. 1C and fig. S1C). These results suggested that ROR γ t does not suppress *Ii2rg* expression and that ROR γ t reduces γ c surface abundance through posttranscriptional mechanisms.

ROR γ t^{KO} mice are impaired in thymopoiesis because their thymocytes have a defect in cell survival and are partially blocked in thymocyte maturation (Fig. 1D, top) (23, 24). To assure that the abundance of surface γ c was assessed on developmentally comparable thymocyte populations, we first divided WT and ROR γ t^{KO} thymocytes into five distinct subsets (populations I to V) based on surface staining for CD24 and TCR β (Fig. 1D and fig. S2A). Note that populations II and III correspond to DP thymocytes (fig. S2A). We confirmed that, in the absence of ROR γ t, the amount of surface γ c was substantially increased in both DP populations (Fig. 1D, bottom). To focus our analysis exclusively on preselection DP thymocytes, we further stained WT and ROR γ t^{KO} thymocytes for CD69 and CCR7, which are surface molecules induced by TCR signaling (Fig. 1E). Consequently, cells that were negative for both CD69 and CCR7 (CD69⁻CCR7⁻; population I) correspond to preselection thymocytes that have not received TCR signals (fig. S2B) (26). Among CD69⁻CCR7⁻ cells, we gated on CD4⁺CD8⁺ DP thymocytes and examined their amount of γ c at the surface (Fig. 1E, bottom). We observed a marked increase in surface γ c abundance when ROR γ t was absent (Fig. 1E). This increase was specific to γ c because the amount of surface

IL-4R α , which is normally abundant on preselection DP thymocytes (18), was substantially reduced on ROR γ t^{KO} DP cells (Fig. 1F). This reduction in surface IL-4R α abundance was associated with markedly decreased expression of *Ii4ra* mRNA, which encodes IL-4R α (fig. S2C). Thus, ROR γ t presumably controls the amount of IL-4R α through transcriptional mechanisms. Together, these results show that the presence of ROR γ t inversely correlates with the surface abundance of γ c proteins and suggest that ROR γ t limits the amount of γ c on thymocytes through a posttranscriptional mechanism.

Enforced ROR γ t expression fails to reduce γ c abundance on mature thymocytes

To directly test whether ROR γ t could reduce γ c abundance, we generated mice expressing a murine ROR γ t complementary DNA (cDNA) under the control of the proximal *Lck* promoter (ROR γ t^{Tg}). Transgenic ROR γ t expression impaired thymocyte development so that the total number of thymocytes was reduced in ROR γ t^{Tg} animals (Fig. 2A). Furthermore, CD4 versus CD8 thymocyte differentiation was altered in ROR γ t^{Tg} mice, such that the mature CD8 SP subpopulation was disproportionately increased among post-selection thymocytes (defined as TCR β ^{hi}; Fig. 2A and fig. S3A). The amount of surface γ c, however, remained unaffected on ROR γ t^{Tg} thymocytes (Fig. 2B) so that both TCR β ^{low} DP and TCR β ^{hi} SP cells displayed the same abundance of γ c between WT and ROR γ t^{Tg} mice (Fig. 2B, bottom). Thus, overexpression of ROR γ t did not further reduce the existing amounts of γ c on thymocytes (Fig. 2B, top, and fig. S3, B and C).

In the periphery of ROR γ t^{Tg} mice, we found that ROR γ t was ectopically expressed in lymph node (LN) T cells. However, the presence of ROR γ t did not alter the amount of surface γ c proteins (Fig. 2, C and D). These results suggest that ROR γ t alone is insufficient to reduce γ c abundance in mature SP thymocytes and peripheral T cells (Fig. 2, B and D). We next confirmed that transgenic ROR γ t was functionally active. ROR γ t^{Tg} CD4⁺ T cells showed increased frequencies of IL-17 production ex vivo (Fig. 2E), and they were substantially more effective in generating T_H17 cells in vitro than were CD4⁺ T cells from WT mice (Fig. 2F) (27). Together, these results demonstrate that enforced ROR γ t expression itself did not suffice to reduce γ c abundance on mature thymocytes and T cells.

Thymocytes exhibit an intrinsic requirement for ROR γ t to control γ c abundance

To demonstrate that transgenic ROR γ t proteins can reduce γ c abundance, we introduced the ROR γ t transgene into germ-line ROR γ t^{KO} mice to generate ROR γ t^{KO/Tg} mice. In these mice, ROR γ t was only expressed in T lineage cells because the transgene is driven by the T lineage-specific proximal *Lck* promoter (28). Analysis of ROR γ t^{KO/Tg} thymocytes revealed that T cell-specific ROR γ t expression restored both thymopoiesis and T cell development in ROR γ t^{KO} mice (Fig. 3, A and B). In ROR γ t^{KO/Tg} thymocytes, the percentage of post-selection (TCR β ^{hi}) mature thymocytes (Fig. 3A) and the number of total thymocytes (Fig. 3B) were markedly increased and were almost restored to the degree observed in WT mice. Note that ROR γ t encoded by the transgene was present at similar amounts to that of endogenous ROR γ t protein in DP cells (Fig. 3C), and transgenic ROR γ t was sufficient to reduce the abundance of γ c in ROR γ t^{KO} preselection (TCR β ^{low}) DP thymocytes (Fig. 3D). Consequently, the amount of γ c on ROR γ t^{KO/Tg} DP cells was comparable to that on WT DP

thymocytes. These results indicate a cell-intrinsic role for ROR γ t in reducing the amount of surface γ c on DP cells.

Bcl-x_L acts downstream of ROR γ t to diminish γ c abundance on DP thymocytes

To understand how ROR γ t could reduce γ c abundance, we focused on the downstream effector molecules of ROR γ t. Immature DP thymocytes are destined to undergo programmed cell death unless they are rescued by positive selection (29). Mcl-1 and Bcl-x_L are nonredundant antiapoptotic proteins that maintain DP cell survival (30, 31), and the gene encoding Bcl-x_L is a direct target of ROR γ t (24). As such, ROR γ t deficiency results in the loss of Bcl-x_L and increases apoptosis of preselection DP thymocytes (23, 24). We found that transgenic ROR γ t markedly increased the abundance of Bcl-x_L in ROR γ t^{KO/Tg} DP thymocytes (fig. S4A) and increased the proportion of TCR β ^{low} preselection DP thymocytes, consistent with their enhanced survival (fig. S4B). Thus, Bcl-x_L abundance inversely correlated with the amount of surface γ c in preselection thymocytes, suggesting a potential association of the prosurvival effect of ROR γ t with a decrease in γ c abundance. To test whether Bcl-x_L was sufficient to reduce γ c abundance in immature DP thymocytes, we introduced a Bcl-x_L transgene (Bcl-x_L^{Tg}) into ROR γ t^{KO} mice to generate ROR γ t^{KO}Bcl-x_L^{Tg} mice. We found that Bcl-x_L overexpression improved the survival of ROR γ t^{KO} thymocytes (Fig. 4A) and restored the development of preselection thymocytes (Fig. 4B), as indicated by the accumulation of CD69⁻CCR7⁻ TCR intermediate DP cells, which were reduced in ROR γ t^{KO} mice (Fig. 4B and fig. S5A).

To examine whether the increased survival of ROR γ t^{KO}Bcl-x_L^{Tg} thymocytes involved changes in other pro- or antiapoptotic proteins, we further assessed the mRNA abundance for other prosurvival or proapoptotic proteins in ROR γ t^{KO} and ROR γ t^{KO}Bcl-x_L^{Tg} thymocytes. Bcl-2 is found only at very low amounts in immature DP thymocytes (30), and its gene expression was unaffected by the absence or presence of ROR γ t or the overexpression of Bcl-x_L in TCR β ^{low} DP thymocytes (fig. S5B). We also did not find any substantial changes in proapoptotic gene expression when comparing TCR β ^{low} DP thymocytes from WT and ROR γ t^{KO} mice (fig. S5C). However, transcripts for the antiapoptotic protein Mcl-1 were statistically significantly reduced in ROR γ t^{KO} cells and then were restored to amounts similar to those in WT cells upon Bcl-x_L overexpression (fig. S5B). Thus, these results suggest that Bcl-x_L provides antiapoptotic signals both directly and indirectly by positively regulating the expression of another prosurvival gene.

Together with the observation that Bcl-x_L can control expression of other molecules, we found that reconstitution of Bcl-x_L expression reduced the amount of surface γ c on ROR γ t^{KO} DP thymocytes (Fig. 4C). This Bcl-x_L-mediated reduction in γ c abundance occurred without suppressing the expression of *Il2rg*, which encodes γ c (fig. S5D). Compared to *Il2rg* transcripts in ROR γ t^{KO} DP thymocytes, overexpression of Bcl-x_L significantly increased the expression of γ c-encoding transcripts in the absence of ROR γ t (fig. S5D). These results suggest that enforced expression of Bcl-x_L was sufficient to replace an ROR γ t requirement for reducing γ c abundance, presumably through posttranscriptional mechanisms.

Bcl-x_L is mostly known as an antiapoptotic factor. However, it also plays critical roles in other cellular processes, such as cell metabolism, autophagy, assembly of the NLRP1 inflammasome, and mitochondrial dynamics (32). To discriminate the prosurvival effect of Bcl-x_L from its other functions in controlling γ c abundance, we investigated whether enforced expression of other antiapoptotic factors or the deletion of proapoptotic molecules could reduce γ c abundance. Analysis of γ c abundance on preselection DP thymocytes from antiapoptotic Mcl-1 transgenic mice (33) or mice deficient in either of the proapoptotic proteins Bim or Noxa (34, 35) suggested that regulation of γ c abundance was independent from the prosurvival effect of Bcl-x_L: None of the TCR β ^{low} DP thymocytes from these mice exhibited an increase in γ c abundance similar to that observed in the absence of ROR γ t (Fig. 4D). Moreover, conditional deletion of *Mcl1*, the gene encoding Mcl-1, at a late stage of T cell development with a *CD2-Cre* transgene (36) did not increase the amount of surface γ c either (Fig. 4E). Using a *CD2-Cre* transgene that deletes *Mcl1* after the DN stage (*Mcl1*^{fl/fl}*CD2-Cre*) (36), we avoided complications related to the requirement for Mcl-1 in early thymopoiesis (31). Successful deletion of *Mcl1* can be monitored on a single-cell basis by assessing the presence of the hCD4 (37). We found no differences in thymocyte populations between cells from WT and *Mcl1*^{fl/fl}*CD2-Cre* mice (fig. S6A). The abundance of γ c between hCD4-positive and hCD4-negative DP thymocytes was also the same (fig. S6B), indicating that the absence of the antiapoptotic protein Mcl-1 did not affect γ c abundance. Together, these data suggest that the prosurvival function of Bcl-x_L either is not responsible for or is not the only mechanism by which Bcl-x_L reduces γ c abundance. Therefore, we next examined a role for other cellular changes that were induced by the lack of ROR γ t and were restored by the Bcl-x_L^{Tg}. Notably, we found that ROR γ t^{KO} DP thymocytes were increased in size compared to WT thymocytes and that enforced Bcl-x_L expression rescued this phenotype (Fig. 4F). An increase in cell size is an indicator of increased cell metabolism (38), and Bcl-x_L reportedly limits bioenergetic and metabolic activity (32). Thus, we hypothesized that a ROR γ t-controlled Bcl-x_L circuit could alter metabolic cues to regulate γ c abundance.

Bcl-x_L reduces metabolism and mitochondrial dynamics in immature DP thymocytes

To understand the basis of increased cell size, we used electron microscopy to image TCR β ^{low} DP thymocytes from ROR γ t^{KO} mice (Fig. 5A). Compared to WT cells, which had a relatively small cytoplasm, ROR γ t^{KO} cells had substantially increased cytoplasmic areas with abundant mitochondria (Fig. 5, A and B). We assessed the number of mitochondria in two ways. We measured the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA), which revealed a substantial increase in mitochondrial numbers in preselection DP thymocytes from ROR γ t^{KO} mice (Fig. 5C). We also quantified mitochondrial mass by MitoTracker Green staining (39), which showed an increase in mitochondrial staining in ROR γ t^{KO} cells by flow cytometry (Fig. 5D). Enforced expression of Bcl-x_L reverted these phenotypes (Fig. 5, A to D), suggesting that the lack of Bcl-x_L in ROR γ t^{KO} thymocytes is the molecular basis for increased cell size and increased mitochondrial content in these cells. Thus, these findings suggest that ROR γ t deficiency in preselection thymocytes results in increased mitochondrial biogenesis, which would be predicted to enhance mitochondrial and cellular metabolic activity.

We next assessed phosphorylation of mTOR as an indicator of increased cellular metabolic activity (40). Compared to WT DP thymocytes, ROR γ t-deficient thymocytes contained significantly increased amounts of phosphorylated mTOR. We found that enforced Bcl-x_L expression suppressed this increase (Fig. 5E). Moreover, the surface abundance of CD71, which is a marker for T cell metabolism and activation (41), was also increased in ROR γ t^{KO} cells compared to that in WT cells but was restored to normal amounts in ROR γ t^{KO}Bcl-x_L^{Tg} cells (Fig. 5F). Together, these results reveal a role for Bcl-x_L as a suppressor of cell metabolism and mitochondrial biogenesis. Thus, we hypothesized that diminished metabolic activity was a mechanism by which Bcl-x_L reduced the cell surface abundance of γ c in these cells.

To test this hypothesis, we assessed the effect of the metabolic suppressor 2-DG on γ c abundance in mature SP thymocytes. T cell activation by antibodies against TCR and CD28 increases metabolic activity and increases γ c abundance in mature T cells (25). The addition of 2-DG during TCR/CD28 stimulation suppressed cell metabolism, as indicated by a reduction in cell size (Fig. 5G), and further impaired the increase in cell surface abundance of γ c (Fig. 5H). To exclude the possibility that 2-DG would interfere with TCR signaling, we assessed the increased abundance of the cell surface molecule CD69, a marker of T cell activation (42). T cells stimulated in the presence or absence of 2-DG showed a similar increase in CD69 abundance, indicating that 2-DG did not impair T cell activation (Fig. 5H and fig. S7A). Together, these results suggest a mechanism to increase γ c abundance that is associated with increased metabolic activity.

Bcl-x_L negatively regulates the cell surface abundance of γ c

To examine whether Bcl-x_L was sufficient to reduce γ c abundance, we assessed the relative amount of γ c on Bcl-x_L^{Tg} thymocytes (43). Intracellular staining for Bcl-x_L showed an increase in Bcl-x_L in both preselection DP and mature SP thymocytes of Bcl-x_L^{Tg} mice (Fig. 6A, top). Notably, Bcl-x_L overexpression was associated with reduced γ c abundance even in the absence of ROR γ t so that mature SP thymocytes of Bcl-x_L^{Tg} mice had less γ c at the cell surface than did WT cells (Fig. 6A, bottom, and fig. S7, B and C). Together, these results place Bcl-x_L as an upstream regulator of γ c surface abundance in T cells.

To understand how overexpression of Bcl-x_L (Fig. 6A), but not overexpression of ROR γ t (Fig. 2B), reduced γ c abundance on mature SP thymocytes, we determined the amount of Bcl-x_L proteins in ROR γ t^{KO/Tg} cells. Whereas transgenic ROR γ t induced an increase in Bcl-x_L in TCR β ^{low} DP thymocytes, it did not do so in mature TCR β ^{hi} SP thymocytes (Fig. 6B, top and right). Consistent with this lack of an effect on Bcl-x_L abundance, the abundance of surface γ c on ROR γ t^{KO/Tg} SP thymocytes was identical to that on WT cells (Fig. 6B, bottom). Thus, because ROR γ t did not induce an increase in Bcl-x_L abundance, it was unable to reduce γ c abundance in mature thymocytes. Our results thus suggest that the antiapoptotic protein Bcl-x_L is required for ROR γ t to reduce γ c abundance at the surface of developing thymocytes and that this pathway is developmentally limited to preselection DP thymocytes.

DISCUSSION

The molecular mechanisms that regulate the amount of γc proteins on T cells remain poorly understood. Here, we report an unexpected role for the transcription factor ROR γt in controlling γc abundance during T cell development. We found that ROR γt was necessary to suppress the cell surface expression of γc on immature DP thymocytes and that enforced expression of Bcl-x_L was sufficient to replace ROR γt in down-regulating the abundance of γc . Because γc signaling increases the expression of genes encoding antiapoptotic factors (6), loss of γc expression results in impaired cell survival. Thus, reducing the abundance of surface γc by ROR γt deprives preselection thymocytes of their ability to respond to prosurvival cytokine signals. Consequently, the ROR γt -induced decrease in γc abundance is a mechanism that promotes the programmed cell death of preselection thymocytes, unless they are positively selected.

The generation of an immunocompetent TCR repertoire depends on the selective survival of immature thymocytes that have self-MHC–restricted TCR specificities (2, 44). The survival of thymocytes with nonreactive TCR specificities needs to be avoided. Consequently, several mechanisms act in a redundant manner to insulate preselection thymocytes from exposure to indiscriminate prosurvival cytokine signaling. In the mouse thymus, these mechanisms include reductions in the amounts of γc and IL-7R α proteins (7, 20, 45), an increase in the abundance of suppressor of cytokine signaling 1 (46), and a paucity of IL-7 in the thymic cortex (47, 48). Whereas apoptosis and the removal of nonselected DP thymocytes are essential for thymic selection, it is also important that preselection thymocytes can survive long enough to be tested for their reactivity to self-MHC. In this regard, it is well documented that the premature death of DP thymocytes results in an altered TCR repertoire and impaired T cell development (49). Survival of immature thymocytes depends on Bcl-x_L (30), and ROR γt is necessary for Bcl-x_L production in preselection DP cells (23, 24). Thus, ROR γt is a critical factor in T cell development that bridges the survival of immature thymocytes between their entrance into the DP compartment and their exit through positive selection. We consider it not a coincidence that ROR γt , which promotes thymocyte survival, also suppresses the surface expression of γc . We propose that ROR γt reroutes the survival mechanism from a dependency on extrinsic cytokine signals to a Bcl-x_L–mediated, cell-intrinsic survival pathway that is developmentally controlled. According to this scenario, ROR γt would suppress surface γc expression to prevent undesired prosurvival cytokine signaling but, at the same time, would compensate for the loss of cytokine signaling by inducing the production of Bcl-x_L.

Although ROR γt is evidently required to induce Bcl-x_L production (23, 24), the mechanistic details of the ROR γt -mediated expression of *Bcl2l1*, which encodes Bcl-x_L, remain unclear. *Bcl2l1* transcription can be induced and controlled by multiple factors, and at least in DP thymocytes, the transcription factors c-Myb, T cell factor–1 (50), and the enzyme liver kinase B1 are required for its expression (51). Whether ROR γt directly regulates *Bcl2l1* expression by binding to an ROR γt -responsive element in the *Bcl2l1* gene or whether it acts indirectly by controlling the expression and action of other factors that induce *Bcl2l1* expression has yet to be established (52). The results from our ROR γt transgenic T cell study indicate that ROR γt alone is not sufficient to increase the abundance of Bcl-x_L in

mature T cells, although it promoted IL-17 production and the generation of T_H17 cells. Thus, in post-selection thymocytes and in mature T cells, *Bcl2l1* expression is presumably controlled by distinct mechanisms than in immature thymocytes.

Although ROR γ t overexpression did not induce *Bcl2l1* expression or suppress surface γ c expression in mature thymocytes, enforced expression of *Bcl2l1* was sufficient for quantitative reduction of γ c abundance in the same cells. These results suggest that it is the increase in the abundance of Bcl-x_L downstream of ROR γ t that suppresses γ c expression. Moreover, we found that Bcl-x_L interfered with γ c expression through an unexpected mechanism that involved suppression of metabolic activity and mitochondrial biogenesis. Immature DN thymocytes that undergo β selection receive both activating TCR signals and pro-metabolic Notch signals that drive their differentiation into DP cells (53). Consequently, β -selected thymocytes vigorously proliferate and show highly active metabolism. We found that ROR γ t expression in DP cells was required to constrain such high metabolic activity and mitochondrial dynamics because ROR γ t-deficient DP cells displayed features consistent with persistent T cell activation. Therefore, ROR γ t suppresses metabolism and induces T cell quiescence on freshly selected and proliferating immature thymocytes that enter the DP cell pool. Furthermore, we found that such a role of ROR γ t could be replaced by Bcl-x_L, and our data revealed an underappreciated role of Bcl-x_L in suppressing cell bioenergetics and metabolism. Conventionally, Bcl-x_L is known as an antiapoptotic protein, which promotes cell survival by protecting the integrity of the mitochondria (54). Consistent with this notion, Bcl-x_L is predominantly found in the mitochondrial membrane (55), but it is also present in the endoplasmic reticulum and within the cytosol (56). Outside of the mitochondria, Bcl-x_L exerts functions distinct to its role in survival, such as controlling Ca²⁺ transport, metabolite consumption, and bioenergetics (57). Here, we demonstrated a previously uncharacterized function of Bcl-x_L in inhibiting cell metabolism and mitochondrial biogenesis, which suppressed mTOR activation and led to a reduction in cell surface abundance of γ c. Thus, these data suggest that increased metabolic activity is the posttranscriptional mechanism that enhances γ c surface expression in ROR γ t-deficient DP thymocytes and that it is metabolic suppression by Bcl-x_L that suppresses the increase in γ c expression in normal preselection thymocytes.

Although we identified a posttranscriptional mechanism of γ c regulation, little is known about the regulatory mechanism of *Il2rg* mRNA expression. Mapping the *Il2rg* promoter region suggested a role for GA-binding proteins and the transcription factor Elf-1 in the tissue-specific expression and transcription of γ c (58). However, few studies about *Il2rg* mRNA expression during T cell differentiation have been reported. We previously found a posttranscriptional mechanism that suppresses *Il2rg* expression upon T cell activation and during T cell development in the thymus (20, 25). We found that *Il2rg* pre-mRNA transcripts can be alternatively spliced into a new splice isoform that lacks the transmembrane domain and results in the production of s γ c at the expense of membrane γ c receptors. Thus, alternative splicing into s γ c-encoding transcripts is an effective means to reduce the amount of cell surface γ c protein. s γ c-encoding transcripts are highly enriched in DP thymocytes (20, 25) so that preferential splicing into s γ c could potentially serve as a mechanism that suppresses cell surface γ c expression on DP cells. On the basis of our observations, we considered a scenario in which ROR γ t would increase s γ c expression and reduce the cell

surface abundance of γc ; however, we found that lack of ROR γt did not affect the abundance of γc -encoding transcripts in DP thymocytes. Therefore, these results are inconsistent with a role for ROR γt in controlling alternative splicing of *Il2rg* transcripts. Together, our data suggest that the transcription factor ROR γt controls the abundance of γc subunits without affecting its gene expression but through regulating the prosurvival factor Bcl-x_L, and thus, we document a regulatory circuitry of ROR γt , Bcl-x_L, and γc expression during T cell development in the thymus.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories. ROR γt ^{KO} and Bcl-x_L^{Tg} mice were previously described (43, 59) and were obtained from the Jackson Laboratory. Bcl-2^{Tg} (60), Mcl-1^{Tg} (33), Bim^{KO} (34), and NOXA^{KO} (35) mice were provided by A. Singer [National Cancer Institute (NCI), NIH]. Mcl-1 floxed (*Mcl1*^{fl/fl}) mice (37) and *CD2*-Cre transgenic mice (36) were gifts from D. Gray (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and P. Love (National Institute of Child Health and Human Development, NIH), respectively. The ROR γt ^{Tg} mouse was produced by placing a mouse ROR γt -encoding cDNA under the control of the proximal *Lck* enhancer and promoter and injecting the construct into fertilized B6 oocytes. All animal experiments were approved by the Animal Care and Use Committee of the NCI. Mice were cared for in accordance with NIH guidelines.

Quantitative real-time polymerase chain reaction

TCR β ^{low} DP thymocytes were sorted from thymi of WT, ROR γt ^{KO}, and ROR γt ^{KO}Bcl-x_L^{Tg} mice. Total RNA was isolated with a NucleoSpin kit (Clontech) and an RNeasy kit (Qiagen). RNA was reverse-transcribed into cDNA by oligo(dT) priming with the QuantiTect Reverse Transcription kit (Qiagen). qRT-PCR was performed with a QuantStudio 6 Real-Time PCR machine (Applied Biosystems), ABI PRISM 7900HT (Life Technologies), and QuantiTect SYBR Green PCR kits (Qiagen). Primer sequences are as follows: *Bcl2*, 5'-GGATAACGGAGGCTGGGATGCCT-3' [forward (F)] and 5'-GGGAAGGCCAGGATTCGA-3' [reverse (R)]; *Bak*, 5'-CCGTCCCCTTCTGAACAGC-3' (F) and 5'-TGTGTCGTAGCGCCGGT-3'; *Bax*, 5'-AGGGTTTCATCCAGGATCGA-3' (F) and 5'-CCACCCGGAAGAAGACCTC-3' (R); *Actb*, 5'-GAGAGGGAAATCGTGCGTGA-3' (F) and 5'-ACATCTGCTGGAAGGTGG-3' (R); *Il4ra*, 5'-AAGGAACCCAGGCTGAGCTTC CC-3' (F) and 5'-AATGATGATGGCCACCAA GGGACT-3 (R); *Mcl1*, 5'-AGACGGCCTTCCAGGGC-3 (F) and 5'-CCAGTCCCGTTTCGTCCTT-3 (R); membrane *Il2rg*, 5'-CATGAACCTAGATTCTCCCTGCC-3 (F) and 5'-CCAACCAACAGTACACAAAGATCAG-3' (R); soluble *Il2rg*, 5'-CATGAACCTAGATTCTCCCTGCC-3' (F) and 5'-TGATGGGGGAATTGGAGIIICCTCTAC A-3' (R).

Flow cytometry

Single-cell suspensions were prepared from the thymus and LNs of mice and then stained with antibodies of the following specificities: CD4 (GK1.5), CD8 α (53-6.7), CD62L (MEL-14), CD44 (IM7), CD25 (PC61.5), CD71 (C2), hCD4 (OKT4), CD132 (4G3, TUGm2), CCR7 (4B12), CD69 (H1.2F3), TCR β (H57-597), ROR γ t (AFKJS-9), IFN- γ (XMG1.2), and IL-17 (eBio17B7), all from eBioscience; CD24 (M1/69), IL-4R α (mIL-4R-M1), and p-mTOR (O21-404), all from BD Biosciences; and Bcl-x_L (54H6) from Cell Signaling Technology. Cells were analyzed on LSR II, LSRFortessa, or FACSCalibur flow cytometers (BD Biosciences). Dead cells were excluded by forward-light scatter-gating and PI staining. For intracellular cytokine staining, LN T cells were stimulated for 3 hours with phorbol 12-myristate 13-acetate (25 ng/ml) and ionomycin (1 μ M) in the presence of brefeldin A (eBioscience). Cells were surface-stained followed by fixation and permeabilization with an IC fixation buffer (eBioscience). ROR γ t expression was detected using the FoxP3 intracellular staining buffer set according to the manufacturer's instruction (eBioscience). Intracellular Bcl-2 and Bcl-x_L expression was assessed by fixation and permeabilization with the IC Fixation Buffer Kit (eBioscience). For intracellular phosphoprotein staining, cells were fixed using the Foxp3 Transcription Factor Buffer Set (eBioscience) and permeabilized using True-Phos Perm Buffer (BioLegend) according to the manufacturer's instruction.

mtDNA content analysis

Total DNA from TCR β ^{low} DP thymocytes was extracted using QIAamp DNA Blood Mini kits (Qiagen). The relative expression of the cytochrome *c* oxidase subunit I (*Cox1*) gene from the mitochondrial genome and the *Ndufv1* gene from the nuclear genome was quantified by qPCR with a QuantiTect SYBR Green PCR kit (Qiagen). The following primers were used for analysis: *Cox1*, 5'-TGCTAGCCGCAGGCATTAC-3' (F) and 5'-GGGTGCCCAAAGAATCAGAAC-3' (R); *Ndufv1*, 5'-CTTCCCCACTGGCCTCAAG-3' (F) and 5'-CCAAAACCCAGTGATCCAGC-3' (R).

Mitochondrial mass analysis

Thymocytes were incubated at a concentration of 2.5×10^6 cells/ml with 1 μ M MitoTracker Green dye (Invitrogen) for 20 min at 37°C in serum-free medium. Cells were then washed, stained for additional surface markers, and analyzed by flow cytometry.

Electron microscopy

Transmission electron microscopy analysis was performed by the Electron Microscopy Laboratory of the Frederick National Laboratory for Cancer Research. Briefly, electronically sorted TCR β ^{low} DP thymocytes were fixed with 2% glutaraldehyde and 0.1 M sodium cacodylate. Cell pellets were embedded, sectioned, and carbonized before undergoing imaging on a Hitachi H-7000 Transmission Electron Microscope. Image analysis and quantification were performed with ImageJ software (NIH).

In vitro CD4⁺ T helper differentiation

Naïve CD4⁺ T cells were electronically sorted by gating on CD62L⁺CD44^{lo}CD25⁻ cells. Sorted cells were stimulated with plate-bound antibodies against CD3 and CD28 (each at 1 µg/ml) for 5 days, as previously described (61). Cells were cultured for 5 days under non-skewing T_H0 conditions (medium alone) or were differentiated into T_H17 cells with human TGFβ1 (5 ng/ml; PeproTech), mouse IL-6 (30 ng/ml; BD Biosciences), antibody against mouse IL-4 (10 µg/ml; BD Biosciences), and antibody against mouse IFN-γ (10 µg/ml; BD Biosciences).

Statistical analysis

Data are shown as means ± SEM. Two-tailed Student's *t* tests were used to calculate *P* values for experiments. *P* values of less than 0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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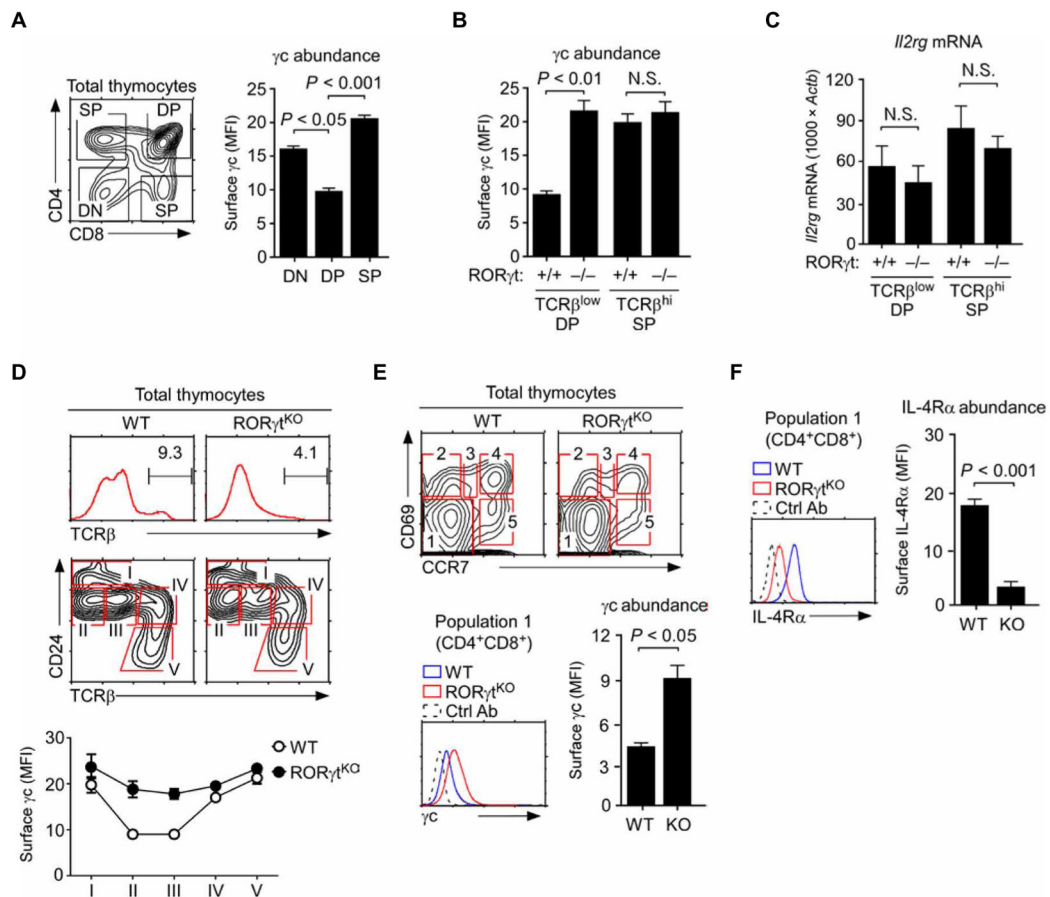


Fig. 1. RORγt deficiency increases the abundance of surface γc on preselection thymocytes. (A) Surface γc abundance and mean fluorescence intensity (MFI) were assessed in CD4, CD8 DN, DP, and TCRβ^{hi} mature CD4 and CD8 SP cells by flow cytometry. Bar graphs show means ± SEM of three independent experiments with a total of six mice. (B) Surface γc abundance (MFI) was determined on TCRβ^{low} preselection DP cells and TCRβ^{hi} mature SP cells from WT and RORγt^{KO} mice. Data are means ± SEM of eight independent experiments. (C) Sorted TCRβ^{low} DP and TCRβ^{hi} SP cells from WT and RORγt^{KO} thymocytes were assessed for *Il2rg* mRNA abundance. We performed quantitative real-time polymerase chain reaction (qRT-PCR) analysis with primers specific for the mRNA encoding γc (25), and signals were normalized to that corresponding to *Actb*. Results show means ± SEM of nine independent experiments. (D) Analysis of the change in surface γc abundance during WT and RORγt^{KO} thymocyte development, as defined by staining for CD24 and TCRβ, followed by flow cytometry. Top and middle: Representative histograms and contour plots. Bottom: Data are means ± SEM of four independent experiments. (E) Surface γc abundance on preselection DP thymocytes as defined by staining for CD69 and CCR7. Top: Representative contour plots. Bottom left: Representative histogram showing γc staining in population 1 cells from the indicated mice. Data are representative of three independent experiments. Bottom right: Data are means ± SEM of three experiments showing the MFI of γc staining on CD4⁺CD8⁺ DP cells among population 1 (CD69⁻CCR7⁻) cells. Ab, antibody. (F) Flow cytometric analysis of IL-4Rα abundance on

the surface of population 1 (CD69⁻CCR7⁻) cells from the indicated mice. Left: Representative histograms. Right: Data are means \pm SEM of two experiments showing the MFI of IL-4R α staining on DP cells among population 1 cells. N.S., not significant; KO, knockout.

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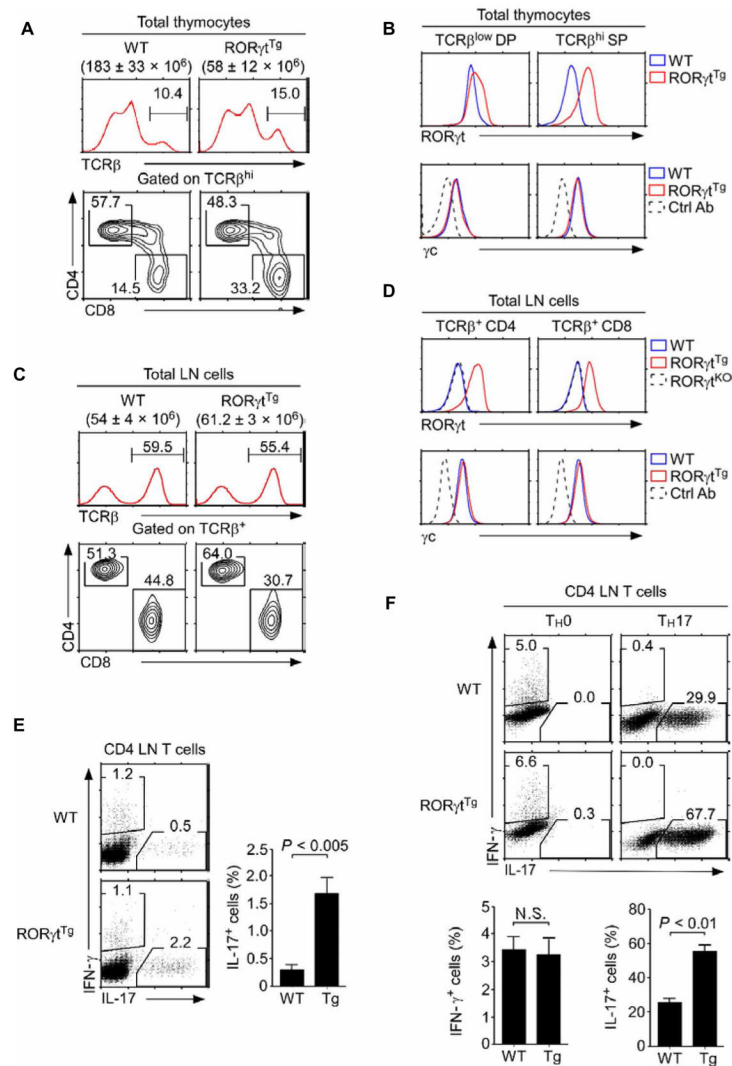


Fig. 2. RORγt overexpression does not reduce γc abundance.

(A) Thymocyte development in RORγt^{Tg} mice. Total thymocyte numbers were determined, and CD4, CD8 profiles of TCRβ^{hi} cells were assessed for WT and RORγt^{Tg} mice by flow cytometry. Cell numbers are means ± SEM, whereas histograms and contour plots are representative of five independent experiments. (B) Intracellular RORγt abundance and surface γc abundance were assessed on TCRβ^{low} preselection DP and TCRβ^{hi} mature SP thymocytes of WT and RORγt^{Tg} mice by flow cytometry. Histograms are representative of five independent experiments. (C) Total lymph node (LN) cell numbers and CD4, CD8 profiles of TCRβ⁺ LN cells were determined for WT and RORγt^{Tg} mice. Cell numbers are means ± SEM, whereas histograms and contour plots are representative of five independent experiments. (D) Intracellular RORγt and surface γc abundance were assessed on WT, RORγt^{Tg}, and RORγt^{KO} LN T cells by flow cytometry. Histograms are representative of five independent experiments. (E) Left: Ex vivo interferon-γ (IFN-γ) and IL-17 production in WT and RORγt^{Tg} CD4 LN T cells. Right: Data are means ± SEM of four independent experiments with a total of four mice for each genotype. (F) In vitro differentiation of naive WT and RORγt^{Tg} CD4⁺ T cells into T helper 17 (T_H17) cells. Sorted naive CD4⁺ T cells

from the indicated mice were cultured for 5 days under T_H17-skewing conditions. IFN- γ and IL-17 production were assessed by intracellular staining. Top: Dot plots are representative of two independent experiments. Bottom: Data are means \pm SEM of two independent experiments with a total of four mice for each genotype.

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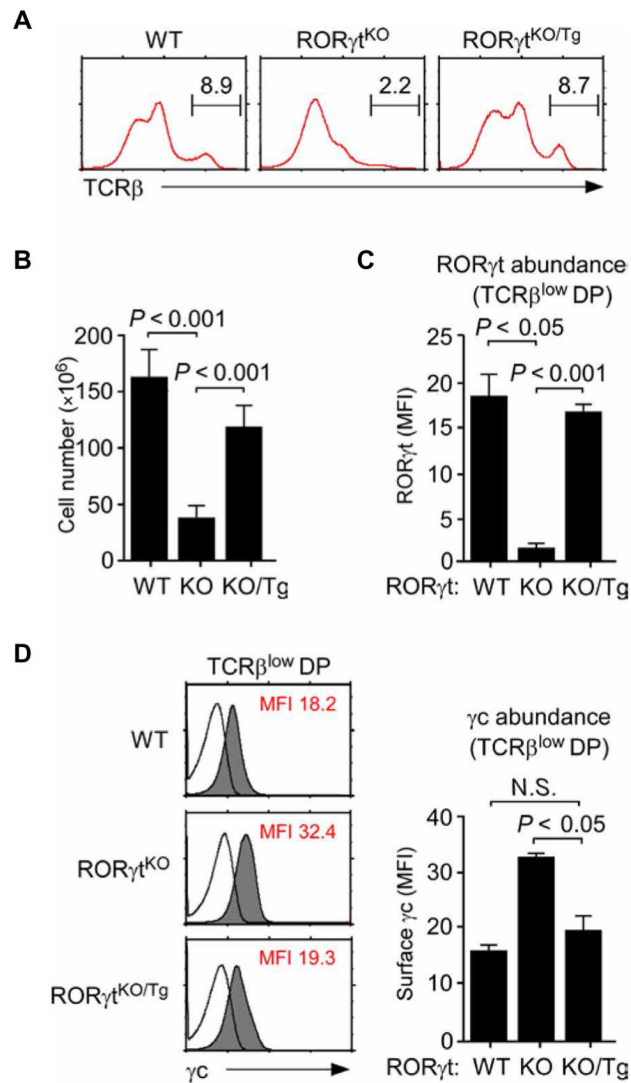


Fig. 3. Transgenic ROR γ t restores T cell development in ROR γ t^{KO} mice.

(A and B) Thymocyte development in ROR γ t^{KO/Tg} mice. Surface TCR β abundance (A) and total numbers of thymocytes (B) were determined for WT, ROR γ t^{KO}, and ROR γ t^{KO/Tg} mice. Data in (A) are representative of three experiments. Data in (B) are means \pm SEM of three independent experiments. (C) Quantitation of ROR γ t protein abundance in TCR β ^{low} preselection DP thymocytes from WT, ROR γ t^{KO}, and ROR γ t^{KO/Tg} mice. Data are means \pm SEM of four independent experiments. (D) Surface γ c abundance on TCR β ^{low} DP cells of WT, ROR γ t^{KO}, and ROR γ t^{KO/Tg} mice was assessed by flow cytometry. Left: Histograms are representative of four independent experiments. Right: Data are means \pm SEM of four independent experiments with a total of five mice for each genotype.

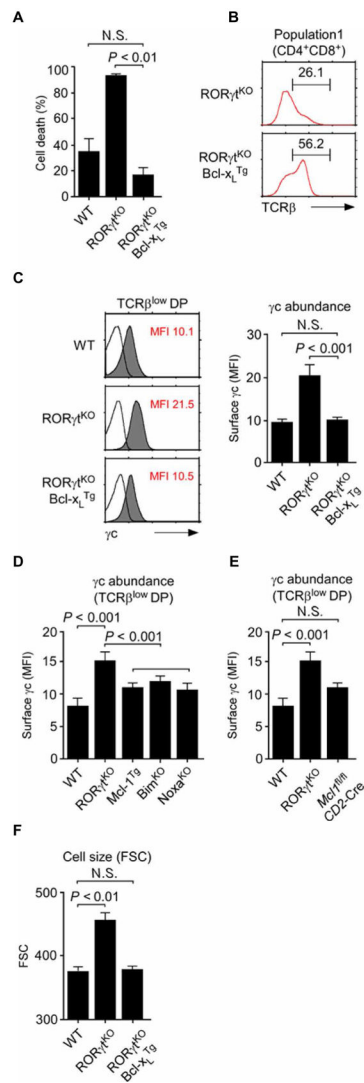


Fig. 4. Transgenic Bcl-x_L reduces γ c abundance in ROR γ t^{KO} mice.

(A) Thymocytes of the indicated mice were incubated overnight at 37°C in cell culture medium. Cell viability was determined the next day by assessing propidium iodide (PI) exclusion by flow cytometry. Data are means \pm SEM of five independent experiments with a total of five mice for each genotype. (B) TCR β abundance on CD69⁺CCR7⁻ (population I) DP thymocytes of ROR γ t^{KO} and ROR γ t^{KO}Bcl-x_L^{Tg} mice was assessed by flow cytometry. Histograms are representative of three independent experiments. (C) Surface γ c abundance on TCR β ^{low} DP cells of WT, ROR γ t^{KO}, and ROR γ t^{KO}Bcl-x_L^{Tg} mice was assessed by flow cytometry. Left: Histograms are representative of five independent experiments. Right: Data are means \pm SEM of five independent experiments with a total of eight mice for each genotype. (D) Surface γ c abundance on TCR β ^{low} DP thymocytes of the indicated mice was assessed by flow cytometry. Data are means \pm SEM of three independent experiments with five WT, four ROR γ t^{KO}, three Mcl-1^{Tg}, four Bim^{KO}, and three Noxa^{KO} mice. (E) Surface γ c abundance on Mcl-1-deficient TCR β ^{low} DP thymocytes was assessed by gating on human CD4 reporter protein (hCD4)-positive cells in Mcl-1^{fl/fl} CD2-Cre mice. Data are means \pm

SEM of three independent experiments with five WT, four ROR γ ^{KO}, and three *Mcl1*^{fl/fl}*CD2*-Cre mice. (F) Forward scatter (FSC) signals were determined in thymocytes of the indicated mice as a measure of cell size. Data are means \pm SEM of four independent experiments with four mice for each genotype.

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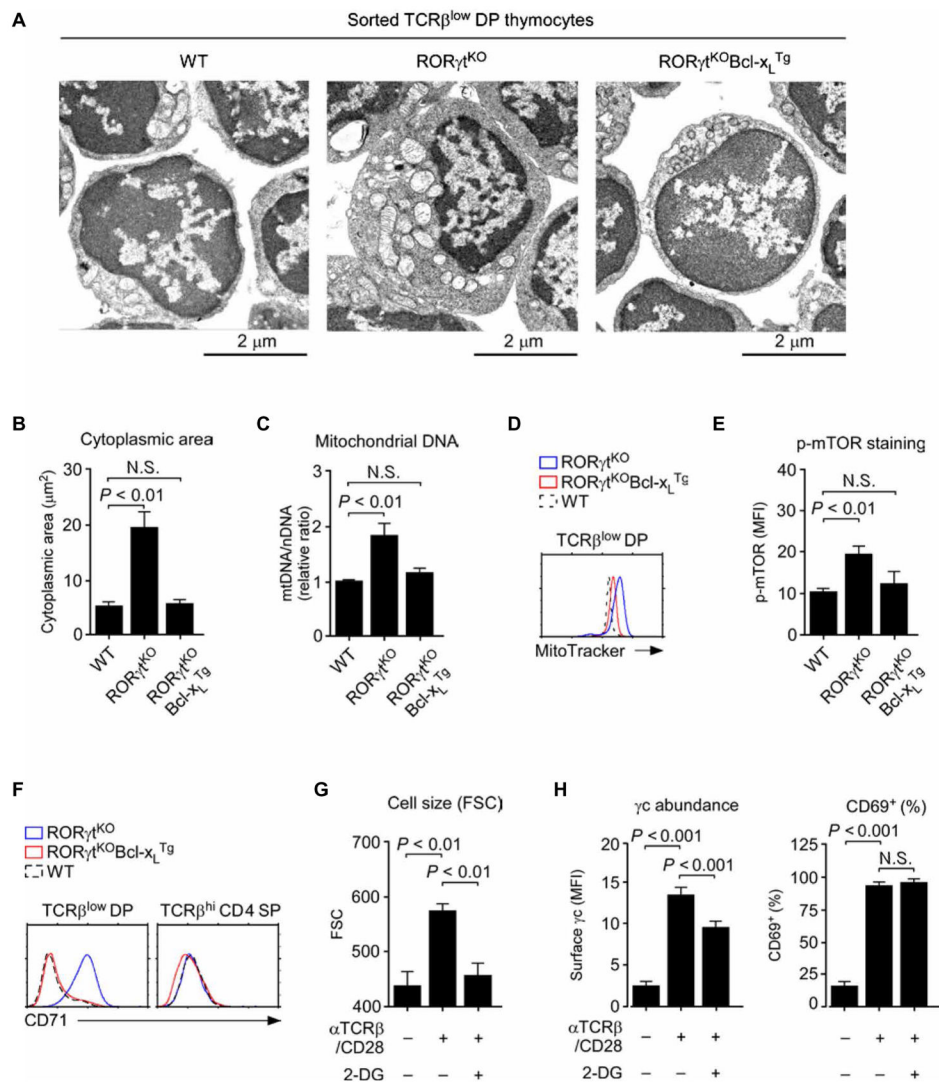


Fig. 5. $\text{ROR}\gamma^{\text{t}}$ is required to suppress metabolic activity in immature DP thymocytes. (A) Electron microscopy of sorted $\text{TCR}\beta^{\text{low}}$ DP thymocytes from the indicated mice. Images are representative of at least five analyses per genotype. (B) Cytoplasmic areas were determined from electron microscopy images of $\text{TCR}\beta^{\text{low}}$ DP thymocytes from the indicated mice using the National Institutes of Health (NIH) ImageJ software. Data are means \pm SEM of five to nine cells for each genotype. (C) Mitochondrial content was determined as the ratio of mtDNA to nDNA upon total DNA extraction and PCR analysis of sorted $\text{TCR}\beta^{\text{low}}$ DP thymocytes from the indicated mice. Data are means \pm SEM of two independent experiments. (D) Histogram shows representative MitoTracker Green staining of $\text{TCR}\beta^{\text{low}}$ DP thymocytes from the indicated mice. Data are representative of four independent experiments. (E) Analysis of the amount of intracellular phosphorylated mammalian target of rapamycin (p-mTOR) in $\text{TCR}\beta^{\text{low}}$ DP thymocytes from the indicated mice. Data are means \pm SEM of four independent experiments. (F) Flow cytometric analysis of cell surface CD71 abundance on $\text{TCR}\beta^{\text{low}}$ DP (left) and mature $\text{TCR}\beta^{\text{hi}}$ CD4 SP (right) thymocytes from the indicated mice. Histograms are representative of three independent experiments.

(G) FSC signals were used to determine cell size for WT CD4 SP thymocytes stimulated with plate-bound antibodies against TCR and CD28 (α TCR β /CD28; each at 1 μ g/ml) in the presence or absence of 10 mM 2-deoxy-D-glucose (2-DG). Data are means \pm SEM of three independent experiments. (H) Cell surface γ c (left) and CD69 (right) abundance was determined on WT CD4 SP thymocytes stimulated with plate-bound antibodies against TCR and CD28 (each at 1 μ g/ml) in the presence or absence of 10 mM 2-DG. Data are means \pm SEM of three independent experiments.

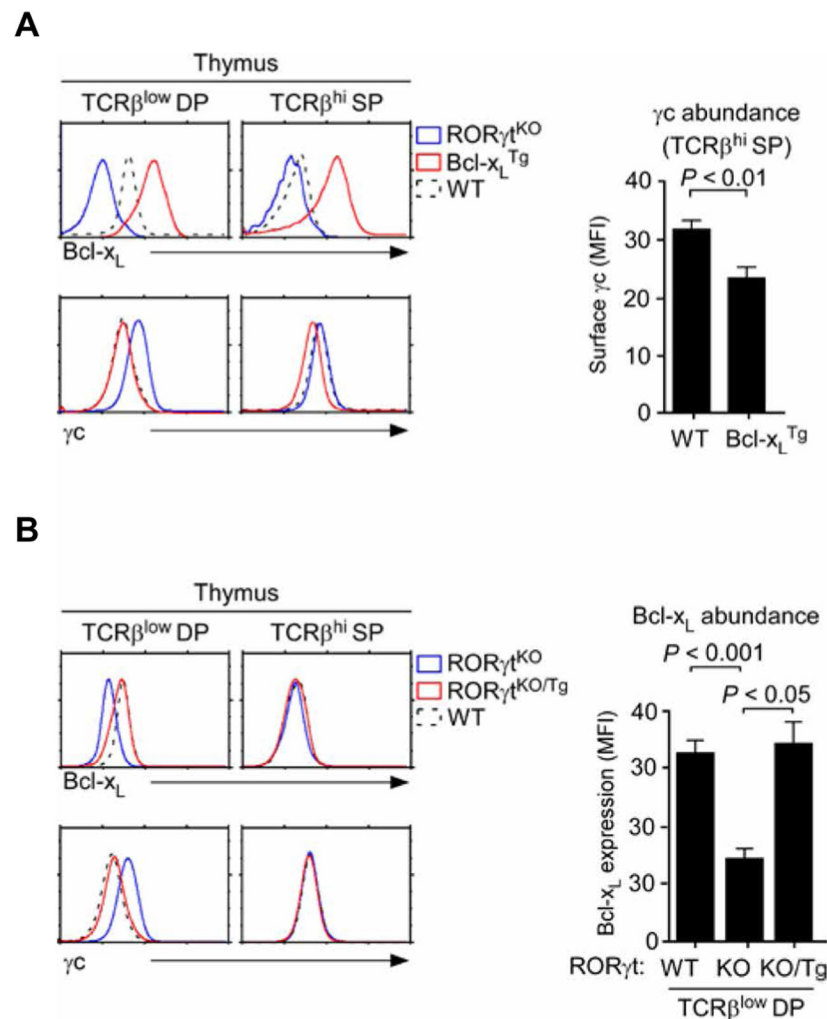


Fig. 6. Enforced Bcl-x_L expression reduces γC abundance.

(A) Intracellular Bcl-x_L and cell surface abundance of γC on thymocytes from ROR $\gamma\text{t}^{\text{KO}}$, Bcl-x_L^{Tg}, and WT mice were determined by flow cytometry. Left: Histograms are representative of three independent experiments. Right: Bar graphs are means \pm SEM of three independent experiments with a total of three mice for each genotype. (B) Intracellular Bcl-x_L and cell surface abundance of γC on thymocytes from ROR $\gamma\text{t}^{\text{KO}}$, ROR $\gamma\text{t}^{\text{KO/Tg}}$, and WT mice were determined by flow cytometry. Left: Histograms are representative of three independent experiments. Right: Bar graphs are means \pm SEM of three independent experiments with a total of three mice for each genotype.