Soluble γc cytokine receptor suppresses IL-15 signaling and impairs iNKT cell development in the thymus

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The soluble γc protein (sγc) is a naturally occurring splice isoform of the γc cytokine receptor that is produced by activated T cells and inhibits γc cytokine signaling. Here we show that sγc expression is also highly upregulated in immature CD4+CD8+ thymocytes but then downregulated in mature thymocytes. These results indicate a developmentally controlled mechanism for sγc expression and suggest a potential role for sγc in regulating T cell development in the thymus. Indeed, sγc overexpression resulted in significantly reduced thymocyte numbers and diminished expansion of immature thymocytes, concordant to its role in suppressing signaling by IL-7, a critical γc cytokine in early thymopoiesis. Notably, sγc overexpression also impaired generation of iNKT cells, resulting in reduced iNKT cell percentages and numbers in the thymus. iNKT cell development requires IL-15, and we found that sγc interfered with IL-15 signaling to suppress iNKT cell generation in the thymus. Thus, sγc represents a new mechanism to control cytokine availability during T cell development that constrains mature T cell production and specifically iNKT cell generation in the thymus.

Cytokines of the γc family play critical roles in T cell development in the thymus1,2. Among others, IL-7 is essential for thymopoiesis3, IL-2 is necessary for Foxp3+ Treg cell development4,5, and IL-15 is required for the development of invariant NKT (iNKT) cells in the thymus6,7. Notably, γc cytokine responsiveness is mostly acquired during or after initiation of lineage-specification during thymocyte development. As such, pre-selection CD4+CD8+ double-positive (DP) thymocytes are unresponsive to IL-78,9, and IL-7 responsiveness is acquired upon cessation of positive selection signals in post-selection CD4 or CD8 single-positive (SP) thymocytes9,10,11. DP thymocytes are also unresponsive to IL-2, and IL-2 responsiveness in CD4 thymocytes is only acquired by strong TCR engagements that also upregulate expression of the transcription factor Foxp312,13. We and others have previously proposed that such γc unresponsiveness in DP thymocytes is achieved through multiple redundant mechanisms9,10,14, and that prevention of pro-survival γc cytokine signaling is critical to ensure selection of self-peptide/MHC-specific, immunocompetent T cells10,15,16. The ability to respond to a specific γc cytokine depends on surface cytokine receptor expression. IL-7Rα expression is silenced in pre-selection DP thymocytes but induced upon TCR-mediated positive selection, which correlates with the inability of IL-7 signaling by DP cells17,18. Moreover, IL-2 receptor expression is absent in DP and most CD4SP thymocytes, but upregulated in Foxp3+ Treg precursor cells which depend on IL-2 for survival19. IL-2 receptor expression is also critical for generation of iNKT cells who utilize IL-2Rβ to be signaled by IL-15, a critical survival and differentiation cytokine for iNKT cells20,21. The failure to express cytokine receptors in a stage-specific manner is detrimental for thymocyte development and lineage differentiation22,23. Thus, understanding the molecular mechanisms that control expression of γc family cytokine receptors during differentiation of distinct thymocyte subsets is an important issue in T cell biology. Interestingly, and in contrast to the cytokine-proprietary receptors, the regulatory mechanism of γc expression has remained largely unmapped.

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γc is expressed on immature CD4, CD8 double-negative (DN) thymocytes for survival and proliferation, and γc expression is also upregulated upon positive selection to mediate lineage choice and effector cell differentiation19. Importantly, γc expression is downregulated on immature DP cells, presumably to suppress aberrant γc cytokine signaling that could provide pro-survival effects on pre-selection thymocytes19. However, the molecular pathway that suppresses γc expression on DP cells remains still veiled.

We have previously identified alternative splicing of γc pre-mRNA as a new mechanism to reduce surface γc protein expression24. The γc gene is encoded in 8 exons, and exon 6 encodes the entire transmembrane domain25. While the full-length γc protein is a transmembrane protein, the new splice isoform lacks exon 6 and thus the transmembrane region, making it a soluble secreted protein. Because the soluble form of γc (sγc) is generated at the expense of membrane γc protein expression, sγc expression inversely correlates with the amount of surface γc expression. Therefore, sγc expression represents a novel mechanism to suppress γc expression on cell surface.

In the present study, we now identify DP thymocytes as a major source of sγc and we propose that alternative splicing into sγc could promote establishing the low level of surface γc on pre-selection DP thymocytes. Moreover, because sγc proteins suppress signaling of γc cytokines, such as IL-2 and IL-724, sγc production by DP thymocytes would create an overall suppressive milieu for γc cytokine signaling in the thymus. In fact, we found that sγc overexpression resulted in significantly diminished percentages and numbers of thymic iNKT cells, which are critically dependent on IL-15 signaling for their development and differentiation6,7,20,21. Specifically, increased sγc expression resulted in the loss of HSAhi mature iNKT cells, as it interfered with upregulation of anti-apoptotic Bcl-2 expression and induced increased cell death. Collectively, these data demonstrate a previously unappreciated role for sγc in downregulating surface γc expression and also in dampening γc cytokine signaling in thymocytes, which can inhibit the generation and differentiation of specific T cell subsets in the thymus.

Results

γc family cytokine receptor expression on thymocytes. Surface staining for γc family cytokine receptors revealed distinct and stage-specific expression of individual cytokine receptors (Fig. 1). Most γc family cytokine receptors were found on both CD4 and CD8 single positive (SP) thymocytes but absent on immature DP thymocytes. IL-4Rα, IL-21R and γc differed as they were also expressed on DP cells. Consequently, DP thymocytes would be unable to respond to IL-7, but they are equipped with IL-4 and IL-21 responsiveness. Importantly, while DP cells did express γc, the amount of surface γc was markedly lower compared to that on immature DN or mature SP thymocytes (Fig. 1). These results indicated and confirmed that γc expression is a developmentally controlled event that is specifically suppressed on pre-selection DP cells19. Reduced γc expression presumably helps avoiding signaling by pro-survival γc cytokines which could interfere with TCR-induced positive selection as previously suggested18.

To correlate γc expression with positive selection, next, we analyzed surface expression of γc and IL-7Rα on HSAhiTCRβlo pre-selection (gate I) and HSAloTCRβhi post-selection thymocytes (gate II) (Fig. 2a). Expression of IL-7Rα and γc was low on gate I immature DP thymocytes but upregulated on gate II mature SP cells, which illustrated developmental control of cytokine receptor expression in thymocytes. The molecular mechanism that downregulates γc expression on DP thymocytes is not known. However, we previously reported a post-transcriptional mechanism that can downregulate surface γc expression24. Specifically, we found that alternative splicing of γc transcripts produced a soluble form of γc (sγc) that was generated at the expense of membrane γc (mγc) protein expression24. Thus, increase in sγc expression conversely results in reduced surface γc expression. Interestingly, here we found that DP thymocytes expressed markedly higher levels of sγc transcripts than mature SP thymocytes (Fig. 2b right), and that increased sγc expression inversely correlated with decreased mγc protein expression in the same cells (Fig. 2b). These data suggest that alternative splicing of γc mRNA might contribute to downregulation of surface γc expression on DP thymocytes. Moreover, DP cells comprise up to 90% of total thymocytes so that they are a major source of sγc proteins, and thus render the thymus into an sγc-rich environment. However, if sγc plays a role in thymocyte differentiation is not known.

sγc overexpression impairs thymocyte development. To interrogate sγc’s effect on T cell development, we analyzed thymocytes in sγc transgenic mice (sγcTg)24. To generate sγcTg mice, a murine sγc cDNA was placed under the control of a human CD2 mini-cassette so that sγc is overexpressed in all T lineage cells. Increased sγc expression significantly reduced total thymocyte numbers, and we observed an inverse correlation of sγc expression and total thymocyte numbers in WT, sγc medium (M) and sγc high (H) expresser transgenics (Fig. 3a). All further experiments in this study were done with the sγc high expresser line. Assessing thymocyte profiles of sγcTg mice did not reveal any significant changes in TCRβhi mature T cell generation (Fig. 3b) or in CD4/CD8 lineage commitment (Fig. 3c left). However, we did find a significant increase in DN cell frequency (Fig. 3c right), suggesting a developmental defect in DN to DP cell transition, which would also explain the reduction in thymocyte numbers in sγcTg mice (Fig. 3d)26.

To directly address this point, we examined surface CD44 and CD25 expression in lineage marker negative DN thymocytes and determined DN1–DN4 differentiation in sγcTg and WT thymocytes (Fig. 4a)27. Contrary to our expectation, however, we did not find any significant differences in DN1–4 subset frequencies between WT and sγcTg mice. We also did not find any significant difference in Ki-67 expression in individual DN subsets (Fig. 4b), suggesting that the proliferative potential of sγcTg DN cells did not differ from WT thymocytes. Finally, to examine the possibility that increased cell death of DN thymocytes would account for reduced cell numbers, we assessed caspase-3 activity and intracellular Bcl-2 contents in sγcTg and WT DN thymocytes (Fig. 4c). Decreased Bcl-2 expression is associated with increased susceptibility to apoptosis, and elevated caspase-3 activity is indicative of increased cell death28,29. However, we did not find any differences in their expression either between sγcTg and WT DN thymocytes (Fig. 4c).
Notably, DP thymocytes in sγcTg mice had been previously reported to contain increased percentages of CD25-positive cells24. Also, surface CD25 expression is diluted during the proliferative burst of DN to DP transition30. Thus, these results collectively suggested that reduced thymocyte numbers and increased DN cell percentages are results of reduced cell proliferation during DN to DP cell transition and not due to a developmental arrest at DN2/DN3 stage of T cell development.

**Thymic development of γδ T cells and Foxp3+ Treg cells in sγcTg mice.** To further assess the impact of increased sγc expression, next, we analyzed generation of individual thymic T cell subsets. We first assessed γδ T cell generation in the thymus and found it unaffected in sγcTg mice. Thymic γδ T cell numbers did not differ between WT and sγcTg mice, and because overall thymocyte numbers were decreased in sγcTg mice, this translated into increased percentages of γδ T cells in the thymus (Fig. 5a). Next, we examined generation of Foxp3+ T regulatory (Treg) cells in sγcTg thymocytes, and found a significant decrease in Foxp3+CD25+CD4SP Treg cell numbers (Fig. 5b). However, we did not find a decrease in Foxp3+CD25+ cell percentages among CD4SP thymocytes (Fig. 5b), which indicated that reduced Foxp3+ Treg cell number is due to an overall impairment in thymopoiesis and not because of a specific defect in thymic Treg cell generation.
s\(^\gamma_c\) overexpression impairs iNKT cell generation. iNKT cells are thymus-generated innate T lineage cells that depend on IL-15 for their development and differentiation\(^{7,31,32}\). iNKT cells can be identified by their TCR reactivity to lipid-loaded CD1d tetramers (CD1dTet)\(^{33}\), and here we found that both frequency and number of CD1dTet\(^+\) iNKT cells were significantly reduced in s\(^\gamma_c\)Tg thymocytes (Fig. 6a). Conventionally, iNKT cell development had been understood based on cell surface HSA (CD24), CD44 and NK1.1 expression\(^{34–36}\). The most immature CD1dTet\(^+\) iNKT cells express high levels of HSA and are defined as stage 0 iNKT cells. Upon further maturation, iNKT cells lose HSA expression but start expressing CD44 and then NK1.1, so that CD44\(^-\)NK1.1\(^-\) cells are stage 1, CD44\(^+\)NK1.1\(^-\) cells are stage 2, and CD44\(^+\)NK1.1\(^+\) cells are referred to as stage 3 iNKT cells\(^{35}\). Assessing WT and s\(^\gamma_c\)Tg thymic iNKT cells revealed no significant differences between WT and s\(^\gamma_c\)Tg mice when comparing in individual stages (Fig. 6b–d). However, there was a significant loss of s\(^\gamma_c\)Tg iNKT cells when comparing the combined frequency of mature iNKT cells, i.e. stage 1–3 (Fig. 6c). Because the frequency of immature stage 0 iNKT cells did not differ between s\(^\gamma_c\)Tg and WT control mice, these results suggest that s\(^\gamma_c\) overexpression did not target a specific developmental stage but rather induces an overall reduction of thymic iNKT cells.

iNKT cells can be also categorized into discrete subsets based on their function and transcription factor expression\(^{37}\). PLZF\(^b\) T-bet\(^+\) cells correspond to IFN-\(\gamma\)-producing NKT1, PLZF\(^b\)ROR-\(\gamma\)- cells are IL-4-producing
NKT2, and PLZFintRORγt+ are IL-17-producing NKT17 cells38. In C57BL/6 (B6) WT mice, the majority of thymic iNKT cells are NKT1 cells with only few NKT2 and NKT17 cells. Such iNKT cell distribution is not developmentally fixed, and changes with mouse strains as illustrated by significantly increased NKT2 and NKT17 cell percentages in BALB/c mice (Fig. 6e)37. We found that sγcTg mice, which were maintained on a B6 background, showed identical distribution of NKT subsets to control WT B6 cells (Fig. 6e). Additionally, when dividing iNKT cells into two major subsets of CD4+ and DNiNKT cells32, we also did not find any difference between sγcTg and WT mice (Fig. 6f). Collectively, these results demonstrate that sγc overexpression is detrimental for thymic iNKT cell generation, and that sγc affected iNKT cell frequency and number without targeting a specific iNKT subset or specific developmental stage.

sγc interferes with IL-15 signaling in iNKT cells. To further understand the molecular basis of iNKT cell loss in sγcTg mice, next we examined whether increased sγc expression is a cell intrinsic requirement to suppress iNKT cell generation. We generated bone marrow (BM) chimeras where WT origin donor cells were used to reconstitute thymus development in RAG-deficient host mice, either alone or mixed at an unequal ratio (1:2) with sγcTg origin bone marrow cells. When analyzing the frequency of WT donor origin (CD45.1) iNKT cells, we found that WT origin BM cells gave rise to significantly reduced frequencies of iNKT cells, if they developed in a mixed thymic environment with sγcTg origin thymocytes. Thus, sγcTg origin BM cells impaired the generation of iNKT cells not only for sγcTg but also for WT iNKT cells (Fig. 7a). These results indicate that sγc's effect to suppress iNKT cell development is mediated by a cell extrinsic mechanism.
iNKT cell development in the thymus depends on IL-15, and defect in iNKT cell generation in sγcTg mice could be a direct consequence of impaired IL-15 signaling. Thus, we assessed expression of surface γc and IL-2Rβ which are the signaling units of a functional IL-15 receptor. We did not find any significant difference in γc and IL-2Rβ expression between WT and sγcTg iNKT cells, which indicated that sγcTg did not impair iNKT cell generation because of defects in cytokine receptor expression.

IL-15 signaling is considered critical for iNKT cells because it induces expression of anti-apoptotic proteins. Bcl-2 is a pro-survival factor downstream of IL-15 signaling, and we found that IL-15-induced Bcl-2 expression was profoundly impaired in the presence of sγc proteins. Recombinant sγc proteins were produced in 293 T cells, and we confirmed successful formation of disulfide-linked sγc homo-dimers which represent the bioactive form of sγc protein.

Discussion
Generation of soluble γc cytokine receptors through alternative pre-mRNA splicing results in two distinct but interlaced events: production of sγc proteins and diminished surface γc protein expression. Both events are detrimental for γc cytokine signaling. Notably, the effect of alternative splicing is limited to sγc producing cells.
...thesemselvesthussecretionofsgcproteinscaninfluencethefunctionofothercellsintrans.Thus,thephysiologicalroleofsγcproteinscanbewide-ranginganddiverse.Wehaveassessedtheeffectofsγcexpressiononthymicdevelopment,andalshowthatincreasedsγcproductionresultsinimpairedthymopoiesis,whichisaprocessdependentonIL-7signaling1,andalsoindiminishedNKTcellgeneration,whichisaneventdependentonIL-15signaling7,21,39. Generation of IL-2-dependent Foxp3+ Treg cells or IL-7-dependent CD8SP thymocytes5,10,41, on the other hand, were not affected. These results propose a hierarchy in γc cytokine responsiveness of post-selection thymocytes, with IL-15 being highly susceptible to increased concentrations of inhibitory sγc proteins, and IL-2 and IL-7 signaling more resistant to sγc-mediated inhibition. Collectively, this study reports a new role for sγc in suppressing IL-15 signaling, and it demonstrates that sγc can affect concentration and differentiation of mature T cell subsets in the thymus.

Because sγc is highly expressed by DP thymocytes and because DP thymocytes comprise the vast majority (~85%) of thymocytes42, these results further suggest a role for DP thymocytes as a major source of sγc protein that dampens γc cytokine signaling in the thymus. Consequently, secretion of sγc proteins represents a new function for DP cells, and it suggests that DP thymocytes play an active role in thymic T cell differentiation by modulating γc cytokine signaling. Conventionally, DP thymocytes have been considered as only a transient developmental stage that is short-lived and that serves no other purpose than providing a pool of random TCR repertoire to be positively selected by the thymic self-peptide/MHC complexes43,44. In fact, DP thymocytes do not produce cytokines, and they are not considered to participate in T cell selection or maturation. Moreover, DP thymocytes are metabolically inactive and do not consume nutrients or compete for pro-survival factorst41. Along these lines, termination of IL-7Rα expression on DP thymocytes has been suggested to prevent DP cells from consuming IL-7 which would interfere with IL-7-dependent proliferation of DN thymocytes5,10,41. Thus, DP thymocytes are thought to be a developmentally inert population that do not affect or control differentiation or selection of T cells in the thymus. On the other hand, there is an increasing body of evidence that shows DP thymocytes actively participating in T cell development in a cell extrinsic fashion. Such an idea is illustrated by the requirement for DP thymocytes to promote γδ T cell signature gene expression in immature DN thymocytes45, and also by a requirement for SLAM-SLAM homotypic interactions among DP thymocytes for positive selection of NKT cells46,47. In the current study, we report a new mechanism of how DP cells affect thymic T cell differentiation, which is through the secretion of inhibitory sγc proteins. We think that sγc is the first of a class of soluble factors that are expressed by DP cells to interfere with thymic development. sγc differs from other factors expressed by DP thymocytes, such as lymphotoxin and SLAM46,47, because it is not expressed in a membrane-bound form and does not require cell-cell contact. Collectively, DP thymocytes are a major source of sγc proteins, and sγc sets the threshold for γc cytokine signaling and tunes γc cytokine responsiveness during T cell development in the thymus.

The inhibitory mechanism of sγc proteins has been previously described24. In brief, sγc proteins form homo-dimers that bind with high affinity to unliganded cytokine receptors, such as IL-7Rα and IL-2Rα. Direct binding of sγc to IL-7Rα or IL-2Rα sequesters these receptors and can prevent them from binding to membrane γc proteins, which is necessary for cytokine signaling. Because IL-2 and IL-15 share the same IL-2Rγc complex for ligand binding and signaling1, by implication, sγc binding to IL-2Rγc should interfere with both IL-2 and IL-15 signaling. Interestingly, during sγcTg T cell development, we found that IL-15 but not IL-2-dependent events were impaired.

Figure 5. Thymic γδ T cell and Foxp3+ Treg cell generation are unaffected in sγcTg mice. (a) Frequency and number of γδ T cells were determined from WT and sγcTg thymocytes. Data are the mean and SEM of 11 sγcTg and WT control thymocytes. (b) Frequency and number of Foxp3+ Treg cells were determined from WT and sγcTg mice. Data are the mean and SEM of 11 sγcTg and WT control thymocytes.
Figure 6. Impaired generation of thymic iNKT cells in sγcTg mice. (a) Frequency and number of iNKT cells in WT and sγcTg thymocytes. Data show summary (mean ± SEM) from 13 sγcTg and 9 WT control thymocytes. (b) iNKT cell stages in WT and sγcTg thymic iNKT cells. CD1dTet⁺ HSA⁺ mature iNKT cells (top) were assessed for CD44 and NK1.1 expression (bottom). Results are representative of 13 sγcTg and 9 WT control mice in 4 independent experiments. (c) Frequencies of immature Stage 0 (ST0) and mature stage 1–3 (ST1–3) iNKT cells in WT and sγcTg mice. Data show mean and SEM of 13 sγcTg and 9 WT control thymocytes. (d) Frequencies of distinct iNKT cell stages in WT and sγcTg thymocytes. Data show mean and SEM of 13 sγcTg and 9 WT control thymocytes. (e) Transcription factor expression in thymic iNKT cells from WT (C57BL/6), sγcTg, and BALB/c mice as assessed by intracellular staining for PLZF versus RORγt. Numbers indicate percentages of PLZF⁻RORγt⁻ (NKT1) cells, PLZF⁺RORγt⁻ (NKT2) cells and PLZF⁺RORγt⁺ (NKT17) cells among CD1dTet⁺ iNKT cells that expressed PLZF. (f) CD4⁺ versus DN iNKT cell ratio in WT and sγcTg thymocytes. Data show mean and SEM of 3 independent experiments with each 3 WT and 4 sγcTg mice.
iNKT cell development was significantly blunted but Foxp3^+ Treg cell generation remained intact. These results suggested distinct susceptibility of IL-2 versus IL-15 signaling to sγc-mediated inhibition. Why IL-15 signaling would be more perceptive to sγc blockade than IL-2 signaling is not clear. As a potential explanation, we considered the

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**Figure 7. Recombinant sγc proteins suppress IL-15 signaling.** (a) iNKT cells in thymus of bone marrow chimeric mice. Bone marrow of WT (CD45.1) and sγcTg (CD45.2) mice were transferred into irradiated RAG-deficient mice, and thymocytes were analyzed 8 weeks after reconstitution. iNKT cell generation was assessed in WT-origin donor cells in single WT (single BM) or unequally-mixed (1:2 ratio of WT versus sγcTg, mixed BM) bone marrow chimeric mice. Bar graphs show percentages of iNKT cells among CD45.1^+ or CD45.2^+ thymocytes in single BM and mixed BM mice. Data are representative and summary of 3 independent experiments. (b) Surface IL-2Rβ and γc expression on mature HSA^+CD1dTet^+ gated WT and sγcTg iNKT cells. Results are representative of 6 sγcTg and 4 WT control mice from 2 independent experiments. (c) Expression of recombinant sγc proteins. Culture supernatant of sγc expressing 293 T cells were immunoprecipitated (IP) and immunoblotted (IB) for sγc proteins using anti-γc ectodomain antibodies (α-γc-ED). Immunoprecipitates were resolved by SDS-PAGE under reducing (+ DTT) or non-reducing conditions. (d) Thymic iNKT cell survival upon 3-day in vitro IL-15 stimulation in the presence or absence of recombinant sγc proteins. Cell viability was determined by Annexin V staining. Histograms are representative of 3 independent experiments (left). Bar graphs show mean and SEM of 3 independent experiments (right). (e) Intracellular Bcl-2 expression in thymic iNKT cells stimulated for 3 days with IL-15 in the presence or absence of recombinant sγc proteins. Histograms show representative results from three independent experiments (left). Bar graphs show mean and SEM of 3 independent experiments (right).
fact that IL-15 signaling requires IL-15 trans-presentation by IL-15Rαγc, and that nNKT cell development depends on IL-15Rα-mediated IL-15 trans-presentation by thymic stromal cells. Formation of a quaternary complex of IL-2Rαβγc hetero-dimers on one cell with an IL-15/IL-15Rα on another cell could be more susceptible to steric hindrance by sγc proteins than the assembly of a functional IL-2Rαβγc signaling complex on the same cell. Altogether, the current results demonstrate an interference of sγc with IL-15-dependent steps during T cell development, and confirm the in vivo significance of sγc proteins in thymocyte differentiation.

The roles of sγc cytokines in thymocyte development are well appreciated. Positive selection and lineage choice are two distinct events. While TCR signaling controls positive selection, NKT cells which is dependent on c-Myc. What cellular signals expansion upon differentiation into stage 1? Following positive selection, IL-7 signaling induces Runx3 expression and imposes CD8 lineage choice, whereas IL-2 signaling is necessary to upregulate Foxp3 and promote Treg cell differentiation in CD4SP cells. For nNKT cells, IL-15 is a critical maturation and differentiation signal, and the absence of IL-15 results in paucity of NKT cells in both the thymus and peripheral tissues. Thus, the reduced thymic nNKT cell numbers in sγcTg is in line with impaired IL-15 signaling by sγc and the requirement for IL-15 in nNKT cell generation.

Importantly, thymic nNKT cells comprise a functionally and phenotypically heterogeneous population that contains distinct subsets of nNKT cells with differing degree of IL-15 dependency. NKT1 cells, which correspond largely to stage 3 nNKT cells, express high levels of T-bet which in turn is critical for their maturation, survival and effector function. Both NKT1 lineage choice and T-bet upregulation depend on IL-15 signaling, so that impaired IL-15 signaling mostly affects NKT1 cells. NKT17 cells, on the other hand, depend exclusively on IL-7, but not IL-15, for their survival and homeostasis. Thus, it was curious that sγc overexpression not only reduced number and frequency of IL-15-dependent NKT1 cells, but also of NKT17 and even NKT2 cells. However, these results can be reconciled when taking into account that sγc does only not inhibit IL-15 signaling, but also signaling by IL-2, IL-7, and presumably other sγc cytokines. Accordingly, sγc would not only block generation of IL-15-dependent NKT1 cells, but could also impair IL-7-dependent NKT17 cell development in the thymus. Because NKT2 cells were also reduced by sγc overexpression, this scenario further suggests a role of sγc signaling in NKT2 lineage differentiation too.

Finally, the current results do not exclude the possibility that sγcTg could have interfered with cell proliferation to diminish thymic nNKT cell numbers. Positively selected stage 0 nNKT cells undergo massive (~100 fold) expansion upon differentiation into stage 1 nNKT cells which is dependent on c-Myc. What cellular signals drive the proliferation is not clear, and we cannot formally discard the possibility that IL-15 could be involved in c-Myc-dependent proliferation during stage 0/1 transition. Whether this is indeed the case still remains to be tested. In sum, the inhibitory effect of sγc on IL-15 signaling in vivo and the impaired generation of thymic nNKT cells in sγcTg mice put forward a model of cytokine regulatory mechanism that requires integration of a role of sγc in controlling sγc cytokine signaling.

Materials and Methods

Mice. C57BL/6 (CD45.2), CD45.1 congenic mice, and RAG−/− mice were obtained from Charles River, Wilmington, MA, and from the Orient Bio, Korea. Soluble sγc-transgenic mice were described and maintained in our colony. Animal experiments were approved by the Pusan National University Institutional Animal Care and Use Committee (PNU-2014-0620) and the NCI Animal Care and Use Committee. All mice were cared for in accordance with Pusan National University School of Medicine and NIH guidelines.

Flow cytometry. Single cell suspensions were prepared from the thymus of indicated mice. Data were acquired using LSR Fortessa or LSRII flow cytometers (BD Biosciences) and analyzed using FlowJo. Live cells were gated by forward scatter exclusion of dead cells stained with propidium iodide. The following antibodies were used for staining: TCRβ (H57-597), HSA (30-F1), IL-7Rα (A7R34), NK1.1 (PK136), IL-2Rα (PC61.5), IL-2Rβ (TM-β1), IL-4Rα (M1), Foxp3 (FJK-16b), RORγt (AKFJS-9) and isotype control antibodies, all from eBioscience; TCRαβ (GL3), CD44 (IM7), γc (4G3), CD4 (GK1.5 and RM4.5), and CD8α (53-6-7) from BD Biosciences; IL-9Rα (RM9A4), Bcl-2 (BCL10/C4), PLZF (9E12), and IL-2Rα (4A9) from BioLegend. Fluorochrome-conjugated CD1d tetramers loaded with PBS-567 and unloaded controls were obtained from the NIH tetramer facility (Emory University, Atlanta, GA). Intranuclear Foxp3, PLZF, and RORγt proteins were detected using a Foxp3 staining kit according to the manufacturer’s instructions (eBioscience). Active caspase-3 induction was determined using the CaspGLOW™ fluorescein active caspase-3 staining kit (eBioscience).

Quantitative Real-Time PCR. Total RNA was isolated from sorted thymocytes with the RNeasy Mini kit (Qiagen). RNA was reverse transcribed into cDNA by oligo (dT) priming with the QuantiTect Reverse transcription kit (Qiagen). Quantitative RT-PCR (qRT-PCR) was performed with an ABI PRISM 7900HT Sequence Detection System and the QuantiTect SYBR Green detection system (Qiagen). Primers sequences are as follows.

Expression of recombinant soluble sγc protein. Recombinant sγc proteins were produced by transient transfection of 293T human embryonic kidney cells with a mammalian expression vector pEGFP-N1 (Clontech) encoding a murine sγc DNA. Cells were transfected with Lipofectamine™ 2000 (Invitrogen). Culture supernatant containing sγc proteins was collected 3 days after transfection and analyzed by Western blot for recovery and purity. Concentration of sγc protein was measured by ELISA as previously described.
In vitro stimulation with recombinant IL-15. Thymocytes were incubated in vitro with 20 ng/ml recombinant human IL-15 (Peprotech) in the presence or absence of recombinant sIL-15 (500 ng/ml). Thymocytes were harvested 3 days after incubation, and stained for intracellular Bcl-2 expression. Annexin V staining was performed according to the manufacturer’s instructions (BD Biosciences).

DN thymocyte subsets analysis. For DN1-DN4 thymocyte analysis, whole thymocytes were first incubated with the following biotinylated antibodies; anti- TCRβ, -CD8, -GL3, -DX5, -MAC1, and -GR1, followed by FITC-conjugated streptavidin. FITC-signal negative thymocytes were considered as lineage marker negative cells (Lin−) and assessed for CD44 and CD25 expression using APC-conjugated anti-CD44 and PE-conjugated anti-CD25 antibodies (all from BD Biosciences). Intracellular Ki-67 staining of DN subsets was performed after fixation and permeabilization (Foxp3 transcription factor staining buffer set, eBioscience) of surface-stained thymocytes using anti-Ki-67 antibodies (eBioscience).

Bone marrow chimeras. Radiation bone marrow chimeras were constructed by reconstituting lethally irradiated (600 Rad) RAG−/− host mice with a total of 15 × 10^6 T-cell-depleted bone marrow (BM) cells either from WT (CD45.1) or sIL-15Tg (CD45.2). For unequal bone marrow reconstitution, T cell-depleted BM cells from WT and sIL-15Tg mice were mixed at 1:2 ratio (WT:sIL-15Tg), and 15 × 10^6 mixed BM cells were injected into irradiated RAG−/− host mice. Chimeric mice were analyzed 8 weeks after reconstitution. Thymocytes from both BM chimeric mice were gated on CD45.1 or CD45.2 to distinguish WT and sIL-15Tg donor cells.

Statistical analysis. Data are shown as mean ± SEM. Statistical differences were analyzed by unpaired two-tailed Student’s t-test. P values of less than 0.05 were considered significant. *p < 0.05, **p < 0.01, ***p < 0.001. All statistical analysis was performed using GraphPad Prism.

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Author Contributions

J.H.P. and C.H. conceived and designed the study. J.Y.P., Y.J., E.K., M.A.L. and C.H. performed experiments and analyzed data. J.Y.P., Y.K.P., S.H.P., J.H.P. and C.H. analyzed and interpreted the results. J.H.P. and C.H. wrote the manuscript. J.H.P. and C.H. conceived and designed the study. J.Y.P., Y.J., E.K., M.A.L. and C.H. performed experiments and analyzed data. J.Y.P., Y.K.P., S.H.P., J.H.P. and C.H. analyzed and interpreted the results. J.H.P. and C.H. wrote the manuscript. J.H.P. and C.H. conceived and designed the study. J.Y.P., Y.J., E.K., M.A.L. and C.H. performed experiments and analyzed data. J.Y.P., Y.K.P., S.H.P., J.H.P. and C.H. analyzed and interpreted the results. J.H.P. and C.H. wrote the manuscript.

Additional Information

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