CD138 expression is a molecular signature but not a developmental requirement for RORγt+ NKT17 cells

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Introduction

Invariant NKT (iNKT) cells are potent immunomodulatory cells that acquire effector function during their development in the thymus. IL-17-producing iNKT cells are commonly referred to as NKT17 cells, and they are unique among iNKT cells to express the heparan sulfate proteoglycan CD138 and the transcription factor RORγt. Whether and how CD138 and RORγt contribute to NKT17 cell differentiation, and whether there is an interplay between RORγt and CD138 expression to control iNKT lineage fate, remain mostly unknown. Here, we showed that CD138 expression was only associated with and not required for the differentiation and IL-17 production of NKT17 cells. Consequently, CD138-deficient mice still generated robust numbers of IL-17–producing RORγt+ NKT17 cells. Moreover, forced expression of RORγt significantly promoted the generation of thymic NKT17 cells, but did not induce CD138 expression on non-NKT17 cells. These results indicated that NKT17 cell generation and IL-17 production were driven by RORγt, employing mechanisms that were independent of CD138. Therefore, our study effectively dissociated CD138 expression from the RORγt-driven molecular pathway of NKT17 cell differentiation.
could play a similar role to CD122 in driving iNKT subset differentiation, with CD138 expression both associated with and required for NK1T7 cell generation. However, the developmental pathway of thymic NK1T7 cell generation remains incompletely mapped, and the molecular basis of NK1T7-specific expression of CD138 is unknown (16). As such, it is unclear whether CD138 expression is controlled by RORγt and whether RORγt itself could be a target of CD138 downstream signaling.

To address these questions, we performed a detailed analysis of CD138 expression during T cell development in the thymus. We identified mature CD4 and CD8 double-negative (DN) cells as the only thymocyte population to express CD138. Among CD138+ DN cells, nNKT cells comprised the vast majority of CD138 expressers (~90%). In agreement with previous findings (15), these CD138+ nNKT cells were exclusively of the NK1T7 lineage. Notably, CD138 expression was associated with but not required for the generation of NK1T7 cells because their development remained unimpaired in CD138-deficient (Sdc1−/−) BALB/c mice and because Sdc1−/− NK1T7 cells still produced copious amounts of IL-17. Moreover, the ectopic expression of RORγt in NKT1 and NKT2 cells failed to induce CD138 on these cells, thus dissociating CD138 expression from being a potential downstream target of RORγt. Collectively, these findings disentangle NK1T7-specific expression of CD138 from NK1T7 cell development and propose a model where CD138 expression is a consequence of but not a driving factor for NK1T7 cell differentiation.

Results

CD138 is expressed on a subset of thymic iNKT cells. To understand the role of CD138 in iNKT cell development, we first examined CD138 expression in total thymocytes of BALB/c mice. CD138 was absent on most thymocytes but present on a small subset of DN cells (Figure 1A). DN thymocytes comprise a heterogeneous population of immature and mature T cells (17, 18), and we found that CD138 expression among DN thymocytes was limited to a subpopulation of CD3hi mature T cells (Figure 1B, left). In addition to conventional αβ T cells, mature DN thymocytes also comprise γδ and nNKT cells (19, 20). Thus, we gated on CD138+C-D3hi mature DN cells and asked whether they would correspond to γδ and nNKT cells. Most CD138+CD3hi DN thymocytes were either γδ or nNKT cells (Figure 1B, right). Next, we asked whether all thymic γδ and nNKT cells would express CD138. However, this was not the case, as only a small fraction (around 5%) of thymic γδ T cells expressed CD138 (Figure 1C and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.148038DS16). Among nNKT cells, we found that approximately 20% of the cells were CD138+ (Figure 1D and Supplemental Figure 1B). To correlate CD138 expression with individual iNKT subsets, we next examined CD138 expression on NKT1, NKT2, and NK1T7 cells, which we identified by their distinct expression of the transcription factors T-bet, PLZF, and RORγt, respectively, as previously described (4, 7). Here, we found CD138 being exclusively expressed on RORγt+ NK1T7 cells (Figure 1E), which agrees with the seminal report by Hamad and colleagues who identified CD138 as an NK1T7 lineage–associated protein (15). Altogether, these results reaffirm CD138 as a surface marker that is uniquely expressed in the NK1T7 subset among iNKT cells.

To further understand the molecular mechanism that drives CD138 expression on NK1T7 cells, we next aimed to assess the role of RORγt in this process. Unfortunately, RORγt is a nonredundant requirement for iNKT cell generation in the thymus (21, 22), and RORγt-deficient (Rorc−/−) BALB/c mice are completely devoid of thymic iNKT cells (Figure 1F and Supplemental Figure 1C). Consequently, it was not possible to assess CD138 expression in iNKT cells of Rorc−/− mice, simply because iNKT cells fail to develop in the absence of RORγt. On the other hand, the generation of thymic γδ T cells did not depend on RORγt. In fact, γδ T cell development not only remained intact but resulted in significantly increased frequencies and numbers of γδ T cells in Rorc−/− BALB/c mice (Figure 1G and Supplemental Figure 1D). Thus, we could assess the expression of CD138 in thymic γδ T cells, and here we found a conspicuous lack of CD138-expressing γδ T cells when RORγt was absent (Figure 1H and Supplemental Figure 1E). Although these results indirectly support a RORγt requirement for CD138 expression, we consider it unlikely that RORγt expression is sufficient to induce CD138 expression on all T lineage cells. As such, we found that immature double-positive thymocytes, which comprise the main cell population in the thymus, expressed large amounts of RORγt but did not induce CD138 (Figure 1A) (23). These data indicated that CD138 expression is clearly associated with RORγt expression but that cellular factors other than RORγt also play roles in the induction of CD138 expression in T cells.

iNKT cell development in the absence of CD138. To further examine the role of CD138 in NK1T7 cell differentiation, we next assessed thymic iNKT cell development in BALB/c mice deficient for the gene Sdc1, which encodes CD138 (Sdc1−/−) (24). Neither the overall T cell development nor the generation of thymic
Figure 1. CD138 expression in thymocyte subpopulations. (A) CD138 expression was assessed on BALB/c thymocyte subsets, identified by their distinct CD4, CD8, and TCRβ expression. Results are representative of 3 independent experiments. (B) CD138 expression among DN thymocytes (left). CD138⁺CD3⁻ DN thymocytes are mostly CD1dTet⁺NiKT cells but also contain conventional αβ and γδ T cells (right). Results represent 3 independent experiments (total 6 BALB/c mice). (C) The dot plot and histogram show the identification and CD138 expression of thymic γδ T cells, respectively. Data represent 3 independent experiments (total 8 BALB/c mice). (D) The dot plot and histogram show the identification of NiKT cells and CD138 expression among thymic NiKT cells, respectively. Data summarize 4 independent experiments (total 9 BALB/c mice). (E) Individual NiKT subsets were identified by intranuclear transcription factor staining (dot plots), which were then assessed for CD138 expression (histograms). Numbers in dot plots indicate frequencies of each NiKT subset among CD24⁺CD1d Tet⁺ thymic NiKT cells. The results represent 3 independent experiments (total 4 BALB/c mice). (F) Dot plots show NiKT
nNKT cells were affected by the absence of CD138 (Figure 2A and Supplemental Figure 2). Moreover, the frequencies and numbers of nNKT cells in peripheral tissues, such as the liver and spleen, also remained unaffected in Sdc1−/− mice. These results indicated that CD138 is not a requirement for the thymic generation and the peripheral maintenance of nNKT cells (Supplemental Figure 3A and Supplemental Figure 4A). CD138 deficiency also did not alter the nNKT subset composition in the spleen and liver (Supplemental Figure 3B and Supplemental Figure 4B). However, we observed a modest but statistically significant increase in the frequencies and numbers of NK17 cells in Sdc1−/− thymocytes (Figure 2, B and C). Conversely, the frequency of thymic NK17 cells was significantly decreased in Sdc1−/− mice (Figure 2B). Unlike NK1 and NK17 cells, however, NK2 cells remained virtually unaffected in Sdc1−/− thymocytes, so that the frequency and number of thymic NK2 cells did not differ between Sdc1−/− and WT littermate mice (Figure 2, B and C). Altogether, CD138 deficiency did not impair but rather promoted the generation of NK17 cells, albeit at the expense of NK1 cells.

We next aimed to examine whether CD138-deficient NK17 cells would differ from CD138-sufficient NK17 cells regarding their phenotype and function. To this end, we assessed the abundance of the transcription factors PLZF and RORγt in Sdc1−/− NK17 and WT littermate NK17 cells but did not find any significant difference (Figure 2D). There were no differences in the expression of activation markers and cytokine receptors either (Supplemental Figure 5A). To determine whether NK17 cells in Sdc1−/− BALB/c mice are functionally competent, we next stimulated Sdc1−/− BALB/c thymocytes with PMA and ionomycin and assessed their IL-17 production. CD138 deficiency did not impair IL-17 production, and consistent with an increase in NK17 cell frequencies, we found that IL-17 expression was increased in PLZF+ thymocytes of Sdc1−/− BALB/c mice (Figure 2E). Collectively, these results showed that CD138 was not required for the generation or effector function of NK17 cells.

Phenotypic and functional analyses of thymic Sdc1−/− NK17 cells. To further examine the role of CD138 in NK17 cells, we wished to identify NK17 cells by surface markers other than CD138. Sdc1−/− NK17 cells lack CD138, so CD138 cannot be employed as a marker for NK17 cells. To this end, we stained thymic nNKT cells for CD4 and CD122, a combination of 2 surface molecules that was previously reported to discriminate the 3 nNKT subsets (25). Indeed, we found that NK17 cells, as identified by CD138 expression, were highly enriched in the CD4−CD122− DN population (Figure 3A). NK1 and NK2 cells, on the other hand, are CD138 negative, and these subsets were found in the CD4+ and CD122+ populations, but they were conspicuously absent among DN cells (Figure 3A). To test whether the DN subset would indeed correspond to NK17 cells, we next assessed surface CD138 expression on individual nNKT subsets. CD122+ cells corresponded to NK1 cells, whereas CD4+CD122− cells corresponded to NK2 cells, and neither of these subsets expressed CD138 (Figure 3B). DN cells, however, were highly enriched in CD138−/−NKT cells, indicating that they can be considered as NK17 cells. In agreement, we found that RORγt was highly expressed in DN but not in CD4+ or CD122−/−NKT cells (Supplemental Figure 5B). Altogether, the visualization of nNKT subsets by CD4 and CD122 permitted the identification of NK17 cells independent of CD138 expression.

Based on CD4 and CD122 staining, we next assessed intracellular perforin and granzyme A expression in thymic nNKT subsets of Sdc1−/− and WT littermate mice. Both perforin and granzyme A expression are linked with the cytotoxic function of nNKT cells (26), and we found them to be exclusively expressed in NK1 cells (Figure 3C). NK17 cells of Sdc1−/− and WT mice did not express these cytolytic molecules (Figure 3C). These results indicated that the lack of CD138 in Sdc1−/− NK17 cells did not result in their acquisition of NK1-like effector functions, and these data reaffirmed that NK17 cells retained their subset-specific characteristics independently of CD138.

Lastly, we asked whether CD138 plays a role in NK17 activation such that the lack of CD138 would alter the antigen responsiveness of NK17 cells. To address this question, we stimulated thymocytes of Sdc1−/− and WT mice with increasing amounts of α-GalCer and assessed the induction of CD69 and CD25, 2 prominent activation markers (27), on nNKT cells after overnight culture in vitro. α-GalCer stimulation induced a marked increase in CD25 and CD69 expression on nNKT cells of Sdc1−/− and WT mice (Supple-
Figure 2. iNKT cell development in CD138-deficient mice. (A) Identification of thymic iNKT cells in Sdc1−/− BALB/c mice. The dot plots are representative (left), and the iNKT frequency and number graphs show the summary (right) of 8 independent experiments with a total of 13 Sdc1−/− and 12 WT littermate BALB/c mice. Total thymocyte numbers are shown on top of the dot plots as mean ± SEM. Numbers in the box show frequencies of iNKT cells among total thymocytes. (B) iNKT subset distribution in Sdc1−/− BALB/c thymocytes. The frequencies of NKT1, NKT2, and NKT17 cells were determined by T-bet versus PLZF and RORγt versus PLZF expression. The dot plots are representative, and the graphs show the summary of 7 independent experiments with a total
of 12 Sdc1+/− and 11 WT littermate BALB/c mice. (C) Thymic NKT2 and NKT17 cell numbers were determined in Sdc1+/− BALB/c thymocytes. The results show the summary of 7 independent experiments with a total of 12 Sdc1+/− and 11 WT littermate BALB/c mice. (D) Phenotypic analysis of Sdc1+/− NKT17 cells. Thymic NKT17 in Sdc1+/− and WT littermate BALB/c mice was assessed for CD138, PLZF, and RORγt expression. Histograms represent 7 independent experiments with a total of 12 Sdc1+/− and 11 WT littermate BALB/c mice. (E) IL-17 production by PLZF+ innate cells in Sdc1+/− BALB/c thymocytes. Intracellular IL-17 was assessed among PLZF+ cells of freshly isolated Sdc1+/− BALB/c thymocytes upon PMA and ionomycin stimulation for 5 hours. Dot plots are representative, and the graph shows the summary of 3 independent experiments with a total of 4 Sdc1+/− and 4 WT littermate BALB/c mice. All data are presented as mean ± SEM. *P values were determined by unpaired 2-tailed Student’s t test. **P < 0.05, NS, not significant.

However, we failed to find any significant difference between Sdc1−/− and WT NKT17 cells (Figure 3D). Thus, the lack of CD138 did not affect the activation threshold or the antigen responsiveness of NKT17 cells. Collectively, we found that Sdc1−/− NKT17 cells did not significantly differ in their phenotype and effector molecule expression compared with CD138-expressing WT NKT17 cells.

CD138 deficiency does not affect innate CD8 T cell generation in the thymus. BALB/c mice contain a large fraction of NKT2 cells that serve as a major source of intrathymic IL-4 (4, 7, 28), which drives the generation of innate CD8 T cells in the thymus (4). Thus, BALB/c mice produce large numbers of innate-phenotype CD8 T cells (28, 29), and innate CD8 T cells produce copious amounts of IFN-γ to create a proinflammatory Th1 environment (30). Whether CD138 is involved in innate CD8 T cell generation is not known. However, we considered it important to assess this possibility because CD138 alters the thymic iNKT subset composition. To this end, we next analyzed thymocyte development in Sdc1−/− and WT littermate BALB/c mice. The CD4 versus CD8 thymocyte profile and the frequency of TCRβhi CD8 single-positive (CD8SP) thymocytes remained unaltered in Sdc1−/− mice, suggesting that the generation of conventional and innate CD8 T cells was comparable to that of CD138-sufficient WT littermate mice (Figure 4A). Indeed, the frequency and number of CD44hiCD122+ and CD44hiCXCR3+ CD8SP cells that corresponded to innate CD8 T cells did not significantly differ between Sdc1−/− and WT littermate BALB/c mice (Figure 4B). To further confirm that CD138 deficiency did not impair the effector function of Sdc1−/− innate CD8 T cells, we also stimulated Sdc1−/− and WT littermate BALB/c thymocytes with PMA and ionomycin and assessed IFN-γ production in CD8SP thymocytes. As expected, CD8SP thymocytes from Sdc1−/− BALB/c mice produced similar amounts of IFN-γ as WT littermate BALB/c CD8SP cells (Figure 4C), further demonstrating that CD138 is not required for the development or effector function of innate CD8 T cells.

Forced expression of RORγt alters the subset composition of thymic iNKT cells. RORγt is the transcription factor that specifies NKT17 lineage differentiation in iNKT cells (22). Because CD138 is exclusively expressed on RORγt+ NKT17 cells (15), we next asked whether forced expression of RORγt was sufficient to induce CD138 expression on iNKT cells. To this end, we examined thymic iNKT cells of RORγt-transgenic (RORγtTg) and WT littermate BALB/c mice for surface CD138 expression. RORγtTg mice have been previously described (23), and they express the murine RORγt cDNA under the control of the proximal Lck promoter. Accordingly, all thymocytes, including thymic iNKT cells, are forced to express RORγt (Supplemental Figure 6A). Although RORγt overexpression did not significantly alter the frequency of thymic iNKT cells (Figure 5A), strikingly, the frequency of CD138+ cells was dramatically increased among iNKT cells (Figure 5A). In agreement with the effect on iNKT cells, the forced expression of RORγt also dramatically increased the frequency of CD138+ cells among thymic γδ T cells (Supplemental Figure 6B). Thus, the abundance of RORγt correlated with the frequency of CD138+ T cells and presumably drove their generation in the thymus.

To determine whether the increase in CD138+ iNKT cell frequency is associated with an increase in NKT17 cells, we next assessed the iNKT subset composition in RORγtTg and WT littermate BALB/c thymocytes (Figure 5B and Supplemental Figure 6C). Intracellular staining for RORγt and PLZF showed that the frequency of NKT17 cells, which correspond to PLZF+RORγt+ iNKT cells, was indeed dramatically increased in RORγtTg thymocytes (Figure 5B). Notably, the increase in NKT17 cell frequency and cell number was concomitant with the loss of PLZF+ NKT2 cells and T-bet+ NKT1 cells (Figure 5B and Supplemental Figure 6C). These results document that forced expression of RORγt altered the thymic iNKT subset composition and further indicate that RORγt expression was sufficient to impose NKT17 lineage fate on developing iNKT cells.

We next aimed to assess whether CD138 expression is a direct target of RORγt. CD138 expression could have been upregulated by increased RORγt activity, but also as a consequence of NKT17 lineage differentiation, independently of RORγt. In this regard, we wished to examine whether the forced expression of RORγt would be sufficient to induce the ectopic expression of CD138 in NKT1 cells because NKT1 cells express neither RORγt nor CD138 (15). We confirmed that NKT1 cells of RORγtTg mice coexpressed RORγt with T-bet (Figure 6).
Figure 3. Functional and phenotypical characterization of Sdc1−/− iNKT cells. (A) Identification of thymic NKT17 cells based on CD122 and CD4 expression. CD138+ and CD138− iNKT cells were assessed for surface CD122 and CD4 expression. Data are representative of 5 independent experiments with a total of 7 BALB/c mice. (B) iNKT subset classification based on CD122 and CD4 expression. Thymic iNKT cells of Sdc1−/− and WT littermate BALB/c thymocytes were assessed for CD122 and CD4 expression, visualizing the 3 subsets of NKT1 (CD122+), NKT2 (CD122−CD4+), and NKT17 (CD122−CD4−) cells (left). CD138 expression was assessed in the indicated iNKT subsets of Sdc1−/− and WT littermate BALB/c thymocytes. Data are representative of 2 independent experiments.
Independent experiments. All data are presented as mean ± SEM. NS, not significant.

Values were determined by unpaired 2-tailed Student's t test. Surface CD69 and CD25 expression was assessed on NKT17 cells upon overnight in vitro stimulation of Sdc1–/– and WT littermate BALB/c thymocytes with the indicated amounts of α-GalCer. Contour plots are representative and bar graph shows the summary of 3 independent experiments. All data are presented as mean ± SEM. P values were determined by unpaired 2-tailed Student's t test. NS, not significant.

Discussion

The molecular mechanism that drives the trifurcation of developing NKT cells into distinct NKT effector subsets remains incompletely understood. Because CD138 is absent on immature NKT cells but exclusively expressed on mature NKT17 cells (15), here we examined the requirement for CD138 in...
Figure 4. Thymocyte development in CD138-deficient mice. (A) T cell development in the thymus of Sdc1<sup>−/−</sup> BALB/c mice. Mature thymocytes were identified by high levels of TCR<sub>β</sub> expression and then further assessed for CD4 and CD8 coreceptor expression. Histograms and contour plots (left) are representative, and the graph showing the frequency of CD8 T cells (right) is a summary of 6 independent experiments with a total of 10 Sdc1<sup>−/−</sup> and 10 WT littermate BALB/c mice. (B) Innate-type marker expression and cell numbers of CD8SP thymocytes of Sdc1<sup>−/−</sup> BALB/c mice. CD44 versus CXCR3 (top) and CD44 versus CD122 (bottom) expression profiles, and the frequencies and numbers of innate-type cells were assessed in TCR<sub>β</sub><sup>hi</sup> CD8SP thymocytes of
NKT17 cell generation. We also asked whether CD138 expression is a target of RORγt, a transcription factor that specifies NKT17 lineage fate (10, 16). Our results confirmed CD138 as a highly selective marker associated with NKT17 cells (15), but we also report that CD138 was not required for the phenotypic or functional maturation of IL-17–producing iNKT cells. Moreover, the forced expression of RORγt was sufficient to impose NKT17 subset fate on thymic iNKT cells but without a significant increase in total iNKT cell numbers. These results document RORγt as a positive regulator of NKT17 cell generation that promotes NKT17 cell differentiation during thymic iNKT cell differentiation. Curiously, not all iNKT cells in such RORγt+ thymocytes had upregulated CD138 expression, indicating that factors other than RORγt also contribute to the NKT17-specific expression of CD138. Thus, CD138 might accompany NKT17 cell differentiation and might be associated with RORγt expression, but CD138 expression itself is presumably not a direct molecular target of RORγt.

Among the various iNKT subsets, NKT17 cells have attracted much interest because they are considered the major intrathymic αβ T cells that produce IL-17 (16). IFN-γ is the signature cytokine of NKT1 cells, but IFN-γ is also expressed by innate CD8 T cells (30). IL-4 is highly expressed by NKT2 cells, but it can also be produced by CD44hi memory-phenotype CD4SP cells (32, 33). Intrathymic IL-17, however, is primarily produced by NKT17 cells, thus establishing a unique role for NKT17 cells among thymic αβ T cells. Along these lines, the developmental program of NKT17 cells also contains some unique features (16). For example, the runt family transcription factor Runx1 plays a nonredundant role specifically in NKT17 cell generation (34), and the transcriptional repressor NKAP exclusively promotes NKT17 cell generation, as illustrated in the dramatically diminished numbers of NKT17 cells in NKAP-deficient mice (35). As previously reported and reaffirmed in our study, NKT17 cells are the only expressers of CD138 among thymic iNKT cells (15). Despite its selective expression on NKT17 cells, however, CD138 was not required to specify NKT17 subset fate or to produce IL-17. Thus, the biological significance of CD138 expression on NKT17 cells remains unclear.

CD138 is a cell-surface heparan sulfate proteoglycan that is primarily expressed on epithelial cells but is also found on immune cells (36). Among others, CD138 is expressed on end-differentiated plasma cells and on a subset of IL-17–producing γδ T (γδ 17) cells (37, 38). CD138 is also highly expressed on myeloma cells such that it is not only used as a biomarker but also considered as a target for treatment of multiple myeloma (39). Functionally, CD138 expression has been proposed to promote the survival and homeostasis of mature plasma cells as well as peripheral γδ 17 cells (37, 38). Notably, such a prosurvival effect was associated with increased proliferation in γδT17 cells but in a manner that is T cell intrinsic and independent of CD138 expression on nonhematopoietic cells (37). Indeed, CD138 binds antiapoptotic factors, such as APRIL (40), and it can interact with cytokines, chemokines, and growth factors, which promote the survival of CD138-expressing cells (41). While a cell-intrinsic effect of CD138 is evidently a major pathway to promote the survival of CD138+ immune cells, CD138 also exerts its effects in a cell-extrinsic fashion. As such, the extracellular domain of CD138 can be cleaved from the cell surface and shed into the environment, a process that is controlled by the small GTPase Rab5 and mediated by surface secretases (36, 42). Because soluble CD138 retains its biologically active heparan sulfate chains, secreted CD138 proteins can bind to and modulate the activity of soluble factors, including cytokines and growth factors. Along these lines, the increased abundance of soluble CD138 has often been associated with inflammation and leukocyte migration, which could either mitigate or exacerbate immune responses (41, 43). However, interpreting the biological implication of increased soluble CD138 expression is not always straightforward because CD138 interacts with multiple molecules in a context-dependent manner.

It is not clear whether, and if so how, CD138 would affect the biology of NKT17 cells because the generation of NKT17 cells is not adversely affected in mice that are deficient in CD138. Moreover, it also remains unclear to us why the lack of CD138, which is specifically expressed on NKT17 cells, would promote, albeit modestly, the differentiation of NKT17 cells. Because the NKT1 cell frequency is decreased in the absence of CD138, it is tempting to postulate that NKT1 and NKT17 cells, but not NKT2 cells, branch out from a common precursor by alternative lineage choice. Indeed, such a model was recently suggested.
Figure 5. NKT subset differentiation in RORγtTg BALB/c mice. (A) CD138 expression on thymic NKT cells of RORγtTg and WT littermate BALB/c mice. The dot plots identify and show the frequency of thymic NKT cells (top), and the histograms show CD138 expression among NKT cells (bottom). Graphs (right) show the frequency of NKT cells among total thymocytes and the frequency of CD138+ cells among thymic NKT cells. Data show summary of 2 independent experiments with a total of 7 RORγtTg and 6 WT littermate BALB/c mice. (B) Thymic NKT subset composition of RORγtTg and WT littermate BALB/c mice. The dot plots show the frequencies of each NKT subset identified by PLZF versus T-bet and PLZF versus RORγt staining (left). The graphs show the frequencies of NKT1, NKT2, and NKT17 cells among thymic mature NKT cells (right). Data summarize 4 independent experiments with a total of 16 RORγtTg and 9 WT littermate BALB/c mice. (C) RORγt and CD138 expression in T-bet+ NKT1 cells of RORγtTg and WT littermate BALB/c mice. The histograms show RORγt and CD138 expression in thymic NKT1 cells (left). The graphs show the MFI of RORγt and CD138 expression in thymic NKT1 cells of the indicated mice (right). Data are the summary of 4 independent experiments with a total of 16 RORγtTg and 9 WT littermate BALB/c mice. All data are presented as mean ± SEM. P values were determined by unpaired 2-tailed Student’s t test. **P < 0.01; ***P < 0.001; NS, not significant.
Figure 6. Lack of innate CD8 T cells in RORγtTg BALB/c thymocytes. (A) T cell development in the thymus of RORγtTg BALB/c mice. Mature thymocytes were identified by high levels of TCRβ expression and then further assessed for CD4 and CD8 coreceptor expression. Histograms and contour plots (left) are representative, and the graph showing the frequency of CD8SP T cells (right) is a summary of 3 independent experiments with a total of 9 RORγtTg and 6 WT littermate BALB/c mice. (B) Innate-type marker expression and cell numbers of CD8SP thymocytes of RORγtTg and WT littermate BALB/c mice. CD44 versus CXCR3 and CD44 versus CD122 expression profiles and the frequencies and numbers of innate-type cells were assessed in TCRβhi CD8SP thymocytes of RORγtTg and WT littermate BALB/c mice. The contour plots represent and the graphs summarize 2 independent experiments with a total of 6 RORγtTg and 6 WT littermate BALB/c mice. (C) Intranuclear staining for Eomes in mature CD8SP thymocytes of RORγtTg and WT littermate BALB/c mice. The histogram is...
based on single-cell RNA-Seq analysis (44). Accordingly, NKT2 cells represent a developmental branching point for NKT1 and NKT17 cells, and CD138 could potentially influence subset differentiation at this point by suppressing NKT17 but promoting NKT1 cell generation. Consequently, CD138 expression could be a homeostatic tool to self-limit the expansion of NKT17 cells by promoting NKT1 lineage choice and act as a negative regulatory feedback signal that controls the size of the NKT17 cell pool in the thymus. How such a CD138-mediated mechanism can be understood in the context of the current models of NKT subset differentiation will need further study.

Currently, 2 distinct but not mutually exclusive models are proposed to explain thymic NKT cell differentiation. The linear differentiation model posits that NKT cell effector functions are acquired along a well-explored pathway of thymic differentiation that is defined by CD44 and NK1.1 expression (45). The lineage-diversification model (4), on the other hand, proposes that a common NKT precursor gives rise to 3 distinct NKT subsets. According to the lineage diversification model, there is no precursor-progeny relationship between the subsets, and their specific effector functions depend on the NKT subset identity. Regardless of the model, however, it is important to know what cellular signals drive the acquisition of specific effector functions at a specific developmental stage or for a particular subset of NKT cells. Much progress has been made in this area with the help of genetic mouse models (4). Specifically, the preferential loss or increase of a particular NKT subset in different mouse strains has helped to assess the molecular machinery that drives NKT lineage fate (46). C57BL/6 mice, for example, contain mostly NKT1 cells, whereas BALB/c mice produce greater frequencies of NKT2 and NKT17 cells (4, 46). The transcription factor KLF13 was found to increase NKT2 cell differentiation in BALB/c mice (28), but it remains unclear why BALB/c mice would express larger amounts of KLF13 and what signals in BALB/c mice would increase KLF13 expression. The increased frequency of NKT17 cells in Sdc1<sup>−/−</sup> BALB/c mice now suggests that CD138 is another factor that influences NKT subset-specific differentiation. However, a comprehensive model that integrates all these different factors for NKT subset specification is currently not available. Nonetheless, it is evident that the expression of signature transcription factors is the main driver of NKT subset differentiation, and the forced expression of RORγt, as shown in this study, is sufficient to promote NKT17 fate onto developing thymic NKT cells.

Altogether, the current study untangles the expression of CD138 from the differentiation of NKT17 cells and demonstrates that NKT17 subset generation is driven by mechanisms independent of CD138. Thus, CD138 is certainly a marker of RORγt expression in NKT cells but it is not a requirement for RORγt induction or IL-17 expression. Notably, immature double-positive thymocytes lack CD138 expression but they express large amounts of RORγt (23, 47). These results further indicate that RORγt expression itself is not sufficient to drive CD138 and/or IL-17 expression. In fact, the cellular and developmental context of RORγt expression is critical to impose effector function and drive subset specification during T cell development. Identification of these factors is the aim of our future studies.

**Methods**

**Mice.** BALB/c and C57BL/6 (B6) mice of both sexes were obtained from Charles River Laboratories and analyzed between 6 and 12 weeks of age. CD138-deficient mice (Sdc1<sup>−/−</sup>) were previously described (24) and maintained on a BALB/cAnNCrl background (Charles River Laboratories) at the NIH. RORγt<sup>−/−</sup> mice were generated in-house (23) and either maintained on a C57BL/6 background or backcrossed to BALB/cAnNCrl mice. RORγt-deficient mice (Rorc<sup>−/−</sup>) were obtained from The Jackson Laboratory (47) and backcrossed to BALB/cAnNCrl mice.

**Flow cytometry.** Single-cell suspensions were prepared from the thymus of the experimental mice and stained with fluorescence-conjugated antibodies as previously described (48). After staining, cells were analyzed using LSRFortessa, LSRFortessa X-20, or LSR II flow cytometers (BD Biosciences) and software designed in-house at the Experimental Immunology Branch, National Cancer Institute. Live cells were identified using forward-scatter exclusion of dead cells stained with propidium iodide. For intracellular staining, cells were first stained with Ghost Dye Violet 510 (Tonbo Biosciences) for dead-cell exclu-
Figure 7. iNKT subset differentiation in RORγtTg C57BL/6 mice. (A) CD138 expression on thymic iNKT cells of RORγtTg and WT littermate C57BL/6 mice. The dot plots identify and show the frequency of thymic iNKT cells (top), and the histograms show CD138 expression among iNKT cells of C57BL/6 mice (bottom). The graphs show the frequency of iNKT cells among total thymocytes (left) and the frequency of CD138+ cells among thymic iNKT cells (right). Data summarize 6 independent experiments with a total of 12 RORγtTg and 13 WT littermate C57BL/6 mice. (B) Thymic iNKT subset composition of RORγtTg and WT littermate C57BL/6 mice. The dot plots show the frequencies of each iNKT subset identified by PLZF versus RORγt and PLZF versus T-bet staining (left). The graphs show the frequencies of NKT1, NKT2, and NKT17 cells among thymic mature iNKT cells (right). Data summarize 3 independent experiments with a total of 5 RORγtTg and 7 WT littermate C57BL/6 mice. (C) RORγt and CD138 expression in T-bet+ NKT1 cells of RORγtTg and WT littermate C57BL/6 mice. Histograms show RORγt and CD138 expression in thymic NKT1 cells (left). The graphs show the MFI of RORγt and CD138 expression in thymic NKT1 cells of the indicated mice (right). Data summarize 2 independent experiments with a total of 4 RORγTg and 5 WT littermate C57BL/6 mice. All data are presented as mean ± SEM. P values were determined by unpaired 2-tailed Student’s t test. **P < 0.01; ***P < 0.001; NS, not significant.
sions, followed by surface staining and fixing with intracellular fixation buffer (eBioscience) or Foxp3 fixation buffer (eBioscience). After fixation, cells were permeabilized using the Foxp3 intracellular staining kit according to the manufacturer’s instructions (Thermo Fisher eBioscience). The following antibodies were used for staining: TCRβ (eBioscience, clone H57-597), CD4 (Tonbo Biosciences, clone GK1.5), CD8 (Tonbo Biosciences, clone 53-67), CD24 (Biolegend, clone M1/69), CD138 (BD Bioscience, clone 281-2), TCRγδ (Biolegend, clone GL3), CD122 (eBioscience, clone TM-β1), CXCR3 (eBioscience, clone CXCR3-173), CD44 (Tonbo Biosciences, clone IM7), CD69 (Biolegend, clone H1.2F3), CD5 (eBioscience, clone 53-7-3), IL-7RA (eBioscience, clone A7R34), CD132 (BD Bioscience, clone 4G3), CD25 (eBioscience, clone PC61.5), IL-17 (eBioscience, clone eBio17B7), IFN-γ (Biolegend, clone XMG1.2), PLZF (Biolegend, clone 9E12), RORγt (BD Bioscience, clone Q31-378), T-bet (eBioscience, clone eBio4B10), Eomes (eBioscience, clone Dan1mag), perforin (Biolegend, clone A16009A), and granzyme A (eBioscience, clone GzA-3G8.5). CD1d tetramers loaded with PBS-57 and unloaded controls were obtained from the NIH tetramer facility (Emory University, Atlanta, GA).

Lymphocyte isolation. Lymphocytes were processed into single-cell suspensions from the thymus, spleen, and liver, as previously described (7). For enrichment of liver lymphocytes, livers of the indicated mice were gently pressed through 70 μm cell strainers (BD Biosciences), and the tissue suspensions were washed 2 times in ice-cold PBS. Cell pellets were resuspended in 40% Percoll and layered on top of 70% Percoll. After centrifugation at room temperature for 25 minutes at 1135 g, the cells in the interphase were harvested, washed with medium, and used for iNKT cell analysis. For iNKT cell enrichment from splenocytes, B cells were depleted using anti–mouse IgG magnetic beads. In brief, splenocytes were resuspended in HBSS supplemented with 10% FCS, and then mixed with anti–mouse IgG-conjugated BioMag beads (QIAGEN). After incubation for 40 minutes on a MACSmix Tube Rotator (Miltenyi Biotec) at 4°C, the beads were magnetically removed, and the nonbinding cells were harvested for further analysis.

iNKT cell subset analysis by intracellular staining. iNKT cells were first identified by PBS-57–loaded mouse CD1d tetramers followed by staining for surface markers, as previously described (48). For each staining, 5 million cells were fixed in 150 μL of a 1:3 mixture of concentrate/diluent working solution of Foxp3 transcription factor staining buffer (eBioscience) plus 100 μL FACS buffer (0.5% BSA, 0.1% sodium azide in HBSS), after which they were incubated at room temperature for 20 minutes. Cells were washed twice with 1× permeabilization buffer (eBioscience) before adding antibodies for transcription factors, such as PLZF, RORγt, and T-bet. After 1 hour of room temperature incubation, the cells were washed and analyzed by flow cytometry.

Detection of intracellular cytokine production. Freshly isolated thymocytes were stimulated with PMA (25 ng/mL) and ionomycin (1 μM) (both from Sigma-Aldrich) for a total of 5 hours. Brefeldin A (eBioscience) was added for the last 4 hours of incubation. Stimulation was terminated by washing cells in ice-cold FACs buffer. For dead-cell exclusion, stimulated cells were stained with Ghost Dye Violet 510 (Tonbo Biosciences) for 25 minutes at 4°C, and excess reagents were washed out with FACs buffer. Surface staining was performed before the cells were fixed and permeabilized using the Foxp3 transcription factor staining buffer kit according to the manufacturer’s instructions (eBioscience). Cells were incubated at room temperature for 1 hour after adding the anti-cytokine antibodies, i.e., IL-17 and IFN-γ. After incubation, stained cells were washed and analyzed by flow cytometry.

In vitro stimulation of iNKT cells with α-GalCer. Thymocytes were processed into single-cell suspension (2 × 10⁶ cells/mL) in RPMI-1640 media supplemented with 10% FCS and plated into 24-well plates with different concentrations of α-GalCer (30, 100, and 300 ng/mL). Cells were incubated overnight at 37°C in a 7.5% CO₂ incubator before staining and analysis by flow cytometry. The α-GalCer (KRN7000, Funakoshi) solution was prepared as previously described (49).

Intracellular staining of thymic iNKT cells. For intracellular staining and analysis of thymocytes, dead cells were excluded by Ghost Dye Violet 510 (Tonbo Biosciences) staining. Cells were then stained with PBS-57–loaded mouse CD1d tetramers followed by staining for surface makers. Cells were fixed with IC fixation buffer (eBioscience) and permeabilized using Foxp3 transcription factor staining buffer kit (eBioscience) according to the manufacturer’s instructions. Cells were incubated for 1 hour at room temperature after adding the antibodies, such as anti-perforin and anti-granzyme A. After incubation, stained cells were washed and analyzed by flow cytometry.

Statistics. Results are shown as mean ± SEM. A 2-tailed Student’s t test was used to calculate P values. P values of less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 8 software.
Study approval. Animal experiments were approved by the National Cancer Institute Animal Care and Use Committee. All mice were cared for in accordance with NIH guidelines.

Author contributions
SL, JK, and AC designed and performed the experiments, analyzed the data, and contributed to the writing of the manuscript. PWP provided reagents, reviewed the data, and commented on the manuscript. JHP conceived the project, analyzed the data, and wrote the manuscript.

Acknowledgments
We thank Joo-Young Park (Seoul National University) for the critical review and discussion of this manuscript. This study was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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