The cytokine receptor DR3 identifies and promotes the activation of thymic NKT17 cells

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Running title: DR3 is a functional marker of thymic NKT17 cells

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Abstract

Invariant natural killer T (*i*NKT) cells correspond to a population of thymus-generated T cells with innate-like characteristics and effector functions. Among the various *i*NKT subsets, NKT17 is the only subset that produces the proinflammatory cytokine IL-17. But, how NKT17 cells acquire this ability and what would selectively trigger their activation remain incompletely understood. Here, we identified the cytokine receptor DR3 being specifically expressed on thymic NKT17 cells and mostly absent on other thymic *i*NKT subsets. Moreover, DR3 ligation promoted the *in vivo* activation of thymic NKT17 cells and provided costimulatory effects upon agonistic α-GalCer stimulation. Thus, we identified a specific surface marker for thymic NKT17 cells that triggers their activation and augments their effector functions both *in vivo* and *in vitro*. These findings provide new insights for deciphering the role and function of murine NKT17 cells and for understanding the development and activation mechanisms of *i*NKT cells in general.

Keywords: CD138, IL-17, *i*NKT cells, RORγt, thymus.

Introduction

*i*NKT cells are thymus-derived effector T cells expressing a semi-invariant Vα14-Jα18 T cell receptor (TCR) that equips them with the ability to recognize glycolipids in the context of the non-classical MHC-I molecule, CD1d. Unlike conventional αβ T cells, *i*NKT cells possess the innate ability to express effector molecules and proinflammatory cytokines prior to antigen exposure. While *i*NKT cells are few in their number and limited in their TCR repertoire, they play critical roles in immunosurveillance, inflammation, and host defense [1,2]. There are several subsets of *i*NKT cells, among which three major populations, *i.e.,* NKT1, NKT2, and NKT17, have been identified [3]. In particular, NKT17 cells are noted for their ability to produce the proinflammatory cytokine IL-17 and for uniquely expressing the transcription factor RORγt [3]. NKT17 cells can be also identified by expression of the cell surface marker CD138 (Syndecan-1) [4]. However, the role of CD138 in NKT17 cell biology is not yet fully understood [4,5]. Because NKT17 cells are prominent producers of IL-17 in the thymus and in barrier tissues, such as the lung and skin [6,7], there is a keen interest in delineating the developmental requirements and activation mechanisms of NKT17 cells. Moreover, thymic *i*NKT cells are proposed to be mostly tissue-resident [8], so that the developmental pathways and functional qualities might differ between thymic and peripheral *i*NKT cells – including those of NKT17 cells –, and these issues also remain to be unraveled.

Here, we focused our efforts to characterize NKT17 cells in the thymus to identify effector molecules and markers that would identify and control the activity of this particular *i*NKT subset. As such, we scanned the expression of various cytokine receptors on thymic *i*NKT cells to assess the cytokine-driven circuitry of NKT17 cell differentiation, and we now report that the TNF receptor superfamily member Death Receptor-3 (DR3) [9] is highly and

specifically expressed on thymic NKT17 cells. Moreover, DR3 ligation by agonistic anti-DR3 antibodies exerted costimulatory function and promoted activation, proliferation, and cytokine production of thymic NKT17 cells, unveiling a new layer of control by a cytokine receptor in NKT17 cell biology.

Materials and Methods

Mice

BALB/cAnNCrl (BALB/c) and C57BL/6 mice were purchased from the Charles River Laboratories. CD138-deficient (*Sdc1*$^{-/-}$) mice and RORγt^{Tg} mice were previously described [5,10,11], and these animals were backcrossed in-house onto BALB/cAnNCrl background before analyses. *Foxp3*-DTR/EGFP mice (Cat #011003) and *Nr4a1*-GFP reporter mice (Cat #018974) were obtained from the Jackson Laboratory and crossed onto the BALB/cAnNCrl background [12,13]. Animal experiments were approved by the NCI Animal Care and Use Committee. All mice were cared for in accordance with the NIH guidelines.

Antibodies

Antibodies specific for the following antigens were used for staining: TCRβ (H57-597), CD4 (GK1.5), CD8α (53-6.7), Foxp3 (FJK-16s), T-bet (4B10), CD45 (30-F11), IFNγ (XMG1.2), IL-4 (11B11), CD24 (M1/69), CD138 (181-2), CD122 (TM-β1), DR3 (4C12), Galectin-9 (108A2), CD69 (H1.2F3), CD25 (PC61.5), IL-17A (eBio17B7), PLZF (9E12), and RORγt (Q31-378). Armenian Hamster IgG isotype Control Antibody (HTK888) was used as control for anti-DR3 staining. Rat IgG2a, κ Isotype Ctrl (RTK2758) was used as control for anti-Galectin-9 staining.

PBS-57-loaded mouse CD1d tetramers were obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA).

Enrichment of mature thymocytes

CD24-negative mature thymocytes were enriched by magnetic depletion of $CD24^+$ cells, as previously described [14]. In brief, total thymocytes were processed to single cell suspension in 10% FBS/HBSS (20×10^6 cells/ml) and incubated with rat anti-mouse CD24 antibodies (M1/69, Biolegend) (30 μg/100 $\times10^6$ cells) for 30 mins on ice. After washing off excess reagents, thymocytes were mixed with anti-rat IgG-conjugated BioMag beads (QIAgen) and incubated for 45 mins at 4℃on a MACSmix Tube Rotator (Miltenyi Biotec). Anti-CD24 antibody-bound cells were then magnetically removed, and non-binding cells were harvested for further experiments.

Flow cytometry

Fluorescence antibody-stained single-cell suspensions were analyzed using LSRFortessa or LSRII flow cytometers (BD Biosciences). For live cell analysis, dead cells were excluded by adding propidium iodide before running the samples on flow cytometers. For fixed cell staining and analysis, cells were stained with Ghost Dye Violet 510 (Tonbo) for exclusion of dead cells, followed by surface staining and fixation with Foxp3 fixation buffer for transcription factors (eBioscience) or intracellular fixation buffer for cytokines (eBioscience). Afterwards, cells were permeabilized using reagents from the Foxp3 intracellular kit according to the manufacturer's instructions (eBioscience). Excess reagents were removed by extensive washing in FACS buffer (0.5% BSA, 0.1% sodium azide in HBSS) before analysis.

Identification of i*NKT subsets by intracellular staining*

Thymic *i*NKT subsets were identified by staining for transcription factors as previously described [15]. In brief, thymocytes were stained with fluorescence-conjugated PBS-57-loaded mouse CD1d tetramers, followed by antibody staining for other surface markers for 40 minutes. After washing out excess reagents, cells were fixed in 150 µl of a 1:3 mixture of concentrate/diluent working solution of the Foxp3 Fixation Buffer and further diluted with 100 µl FACS buffer. After incubation for 20 minutes at room temperature, cells were washed twice with permeabilization buffer (eBioscience) before adding antibodies for transcription factor staining. After 1 hour of incubation at room temperature, cells were washed, resuspended in FACS buffer, and analyzed by flow cytometry.

EdU incorporation assay of thymic i*NKT subsets*

To assess *in vivo* cell proliferation, we employed EdU incorporation assays followed by Click-iT chemistry-based detection (Click-iT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit, Thermo Fisher). In brief, mice were i.p. injected with 1 mg of EdU diluted in 200 µL of PBS, and analyzed 16 hours after injection for intranuclear EdU incorporation in thymocytes and thymic *i*NKT cells.

Anti-DR3 agonistic antibody and α-GalCer injection

For *in vivo* anti-DR3 ligation, mice were injected i.p. with either 10 µg anti-DR3 antibody (4C12, Biolegend) or 10 µg Armenian Hamster IgG control antibody (HTK888, Biolegend). Thymus and spleen were harvested at the indicated time points for further analysis. For *in vivo* α-GalCer injection, mice were i.p. injected with either 2 µg α-GalCer or vehicle (0.5% Tween-

20) in the presence or absence of 10 μg anti-DR3 antibody (4C12, Biolegend). 4 days after injection, thymus and spleen were harvested for further analysis.

In vitro stimulation of thymic *i*NKT cells

Single cell suspension of freshly isolated thymocytes were plated into 24-well plates at 2×10^6 cells/ml with 100 ng/ml of α-GalCer in the presence or absence of anti-DR3 antibody (2 μg/ml) or with anti-DR3 antibody alone (10 μg/ml) [16]. Cells were cultured overnight at 37℃ in a 7.5% CO_2 incubator before analysis by flow cytometry.

Statistics

Data are shown as the mean ± SEM. Two-tailed Student's *t*-test was used to calculate P values. *P* values of less than 0.05 were considered significant, where *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$; NS, not significant. Statistical data were analyzed using the GraphPad Prism 8 software.

Results

The cytokine receptor DR3 is highly expressed on thymic NKT17 cells

We embarked on this study to uncover new regulatory mechanisms and effector functions that are specifically associated with individual *i*NKT subsets, and particularly with NKT17 cells. While CD138 is a specific marker for NKT17 cells, CD138 is largely dispensable for their generation and effector function [4,5]. Thus, functional markers that are specific to NKT17 cells are yet to be identified. Because cytokines play critical roles in the generation and survival of *i*NKT cells [1,2,17], we screened a panel of cytokine receptors for their *i*NKT subset-specific expression, and here we identified the TNF receptor superfamily member 25 (TNFRSF25), also known as DR3 [18], being highly expressed on thymic NKT17 cells (**Fig. 1a and 1b**). DR3 was further found on $Foxp3^+$ Treg cells as previously described [19], but also expressed on some mature thymocytes whose identities remain to be unraveled (**Supplementary Fig. 1**). Among thymic *i*NKT cells, DR3 expression correlated with CD138 expression in both BALB/c and C57BL/6 mice (**Fig. 1a, 1b, and Supplementary Fig. 2a**), indicating that DR3 expression is specific to thymic NKT17 cells independently of the genetic background. In fact, DR3 versus CD138 staining permitted the clear identification of NKT17 cells (**Supplementary Fig. 2b**), which we found not to differ in their TCR Vβ usage compared to other thymic *i*NKT cells (**Supplementary Fig. 2b and 2c**), but were phenotypically distinct regarding Egr2 and CCR6 (**Supplementary Fig. 3**) as well as perforin and granzyme A expression (**Supplementary Fig. 4**), among others. Because DR3 was coexpressed with CD138, we next assessed whether DR3 would require CD138 for its expression. However, this was not the case because DR3 remained abundantly expressed on NKT17 cells of CD138-deficient ($Sdc1^{-/-}$) BALB/c mice (**Fig. 1c**). Altogether, these results put forward DR3 as a new surface marker for thymic NKT17 cells.

While NKT17 cells continued to express large amounts of DR3 in peripheral tissues, such as in the lymph node (LN) and lung (**Supplementary Fig. 5**), we found that other *i*NKT cells in the periphery, *i.e.* CD138-negative non-NKT17 cells, also expressed substantial amounts of DR3 (**Supplementary Fig. 6a and 6b**). The promiscuous DR3 expression in peripheral *i*NKT cells contrasted to the phenotype of non-NKT17 cells in the thymus, which were virtually void of DR3. In this regard, we found that the phenotype and function of thymic *i*NKT cells markedly differed from those of peripheral *i*NKT cells, correlating with the thymic NKT17-specific expression of DR3. In accordance, NKT17 cells in the LN and lung expressed substantially lower amounts of RORγt and CD138 compared to thymic NKT17 cells (**Fig. 1d**), and produced significantly reduced amounts of IL-17 on a per cell basis (**Fig. 1e**). Consequently, thymic NKT17 cells represent a distinct tissue-specific subset of *i*NKT cells, whereby DR3 is a highly selective marker for thymic NKT17 cells but less so for peripheral NKT17 cells.

To further understand the molecular mechanisms that induce DR3 on thymic NKT17 cells, we next assessed the role of RORγt, the master transcription factor of NKT17 cells [6]. Notably, the forced expression of RORγt promoted the generation of thymic NKT17 cells (**Fig. 1f**) [11], and dramatically increased both the frequency and number of DR3-expressing *i*NKT cells, as demonstrated in RORγt-transgenic ($ROR\gamma t^{Tg}$) BALB/c mice (**Fig. 1g and Supplementary Fig. 6c**). Importantly, the ectopic expression of RORγt was sufficient to upregulate DR3 expression also in other thymic *i*NKT subsets, *e.g.* CD138-negative non-NKT17 cells (**Fig. 1h**), as well as in conventional CD4 single positive (SP) and CD8SP thymocytes (**Supplementary Fig. 6d**). These results suggested that DR3 expression would be controlled downstream of RORγt, and in support of this notion, RORγt ChIP-PCR identified direct binding of RORγt in the 5' regulatory region of the *Tnfrsf25* gene, which encodes DR3 (**Supplementary**

Fig. 7). Altogether, the acquisition of a DR3$^+$ phenotype parallels the differentiation of RORγt$^+$ NKT17 thymic *i*NKT cells, presumably through a direct regulatory pathway of DR3 expression by RORγt.

DR3 is a functional marker of thymic NKT17 cells

Among conventional αβ T cells, DR3 is highly expressed on Foxp3$^+$ Treg cells and provides costimulatory function. As such, stimulation with the DR3 ligand TNF-like ligand 1A (TL1A) or ligation with agonistic anti-DR3 antibodies triggers the activation of Foxp3$^+$ Treg cells [19,20]. Thus, we asked whether DR3 ligation would also activate thymic NKT17 cells. To this end, we injected BALB/c mice with anti-DR3 antibodies and assessed their effect on thymic *i*NKT cells at 1 week after injection. Of note, we utilized BALB/c mice that were engineered to express *Foxp3*-GFP reporter proteins (*Foxp3*-DTR/EGFP mice) [21] to verify the *in vivo* effects of anti-DR3 injection by monitoring Foxp3$^+$ Treg cells. Assessing GFP-expressing CD4 T cells confirmed that DR3 ligation indeed induced a substantial expansion of Foxp3$^+$ Treg cells (**Fig. 2a**). In contrast, the frequency and number of thymic CD138$^+$ *i*NKT cells, which we considered as NKT17 cells, did not increase. Instead, DR3 ligation resulted in a dramatic loss of CD138$^+$ *i*NKT cells (**Fig. 2b**). Thus, DR3 activation appeared to be detrimental rather than stimulatory for thymic NKT17 cells.

Because we identified NKT17 cells based on their CD138 expression (**Fig. 2b**) [5], we could not exclude the possibility that NKT17 frequency only "appeared" to be reduced because of CD138 downregulation. Shedding of the CD138 ectodomain is a well-documented process that results in the loss of surface CD138 [22]. If such was the case, DR3 ligation could have triggered CD138 downregulation without altering the composition of the thymic *i*NKT cells. To

test this possibility, we examined the thymic *i*NKT subset composition based on transcription factor expression. Strikingly, the frequency and number of NKT17 cells, when identified as PLZF^{int}RORγt^{+} *i*NKT cells [15], remained unaltered by *in vivo* DR3 ligation (**Fig. 2c**). These results revealed that DR3 ligation induces the shedding of CD138, but that the frequency and number of thymic NKT17 cells remain unaffected by DR3 stimulation alone.

Because the process of cell fixation and permeabilization complicates the accurate assessment of surface protein expression, we considered it important to identify unmanipulated NKT17 cells using cell surface markers other than CD138. Hence, we employed CD4 and CD122 expression to discriminate individual *i*NKT subsets [23]. CD122 is highly expressed on NKT1 cells [3,24], while NKT2 cells are CD122-negative but CD4-positive (CD122^{-}CD4^{+}). Most NKT17 cells, on the other hand, are negative for both CD4 and CD122 [23]. Indeed, CD122/CD4 double-negative (DN) *i*NKT cells were RORγt^{+} and expressed high levels of both DR3 and CD138, confirming them as NKT17 cells (**Fig. 2d**). Therefore, the combined use of CD122 and CD4 permitted us to identify NKT17 cells by cell surface staining, without using CD138. Using this staining strategy, we found that DR3 was highly expressed on DN *i*NKT cells of CD138-deficient *Sdc1*$^{-/-}$ mice (**Fig. 2e**), and not on other thymic *i*NKT subsets (**Fig. 2f**), reaffirming CD122 and CD4 DN *i*NKT cells as NKT17 cells. These results indicated that DR3 expression is a *bona fide* marker for thymic NKT17 cells, independently of CD138.

DR3 ligation selectively acts on thymic NKT17 cells

Equipped with this toolkit that identifies NKT17 cells by surface staining, we next assessed the effect of DR3 ligation on thymic NKT17 cells. One week after agonistic anti-DR3 antibody injection into BALB/c mice, we found that NKT17 cells had upregulated expression of the

activation marker CD69 [25] but downregulated DR3 and CD138, with minimal or no upregulation of CD69 on NKT1 and NKT2 cells (**Fig. 3a and Supplementary Fig. 8a**). Also, anti-DR3-induced CD69 expression did not require CD138, because *Sdc1*$^{-/-}$ NKT17 cells were still activated by DR3 stimulation (**Fig. 3b, left**) and their cell numbers did not differ to *Sdc1*$^{+/+}$ NKT17 cells (**Fig. 3b, right**). Previously, DR3 signaling was reported to require the coexpression of galectin-9 [26], and we found that NKT17 cells were incidentally the only thymic *i*NKT subset that expressed both DR3 and galectin-9 (**Supplementary Fig. 8b**). Because the endogenous ligand for DR3, *i.e.* TL1A, is also highly expressed in thymic medullary epithelial cells (**Supplementary Fig. 8c and 8d**), thymic NKT17 cells are uniquely equipped and located to be responsive to DR3 signaling.

To examine the effector function of DR3-activated thymic *i*NKT cells, we next injected BALB/c mice for two consecutive days with anti-DR3 antibodies, confirmed *i*NKT cell activation by CD69 upregulation (**Supplementary Fig. 9a**), and then assessed the signature cytokine expression for each *i*NKT subset. Surprisingly, IL-17 expression remained unaffected in these acutely anti-DR3-activated mice (**Supplementary Fig. 9b**). These results indicated that DR3 ligation alone fails to fully activate and induce effector function in thymic NKT17 cells. In this regard, it was interesting to find that anti-DR3 injection into *Nr4a1*-GFP mice, which report TCR signaling strength [13], significantly upregulated *Nr4a1* reporter expression on thymic NKT17 cells (**Fig. 3c**). We further mapped how DR3 activation could augment TCR signaling using pharmacological inhibitors, and identified the p38 MAPK pathway being involved in this process (**Supplementary Fig. 10**). Altogether, these results suggested that, firstly, DR3 ligation intersects with and presumably augments TCR signaling by enhancing the p38 MAPK pathway,

but, secondly, the DR3-mediated amplification of tonic TCR signals alone is insufficient to fully activate thymic NKT17 cells.

To examine if DR3 ligation could augment *i*NKT cell activation in the context of agonistic α-GalCer administration [27], we next determined the effect of DR3 ligation using *in vitro* assays. Here, we treated BALB/c thymocytes with α-GalCer and determined the CD69 and CD25 expression on *i*NKT cells after overnight costimulation in the presence or absence of anti-DR3 antibodies. As expected, α-GalCer stimulation induced CD69 and CD25 expression on NKT17 cells, but the addition of anti-DR3 antibodies further augmented the potency of α-GalCer, revealing a costimulatory effect of DR3 on TCR-stimulated *i*NKT cells (**Fig. 3d and Supplementary Fig. 11a**). Anti-DR3 also boosted IL-17 but not IL-4 production of α-GalCer-stimulated *i*NKT cells, affirming a costimulatory effect of DR3 that is specific to NKT17 cells (**Fig. 3e and Supplementary Fig. 11b**). The same effects of anti-DR3 antibodies were observed when thymic *i*NKT cells were stimulated with the natural ligand, TL1A, instead of agonistic anti-DR3 antibodies, such that TL1A activation induced downregulation of DR3 and CD138 while potently upregulated CD69 and CD25 on NKT17 cells (**Supplementary Fig. 12a)**. In addition, TL1A costimulation in the presence of α-GalCer promoted IL-17A expression. (**Supplementary Fig. 12b, 12c and 12d**). Thus, DR3 potentially represents a functionally relevant costimulatory molecule for NKT17 cell activation, in contrast to CD28, which is highly expressed on but does not play a significant costimulatory role in mature *i*NKT cells (**Supplementary Fig. 13**) [28], and to 4-1BB, which is not expressed on thymic *i*NKT cells (**Supplementary Fig. 13a**) [29].

DR3 ligation potentiates TCR signaling in thymic NKT17 cells

Prompted by the *in vitro* effects of DR3 costimulation, we next asked whether the *in vivo* coadministration of agonistic anti-DR3 antibodies would boost thymic *i*NKT cell activation by α-GalCer. To this end, we injected BALB/c mice with α-GalCer in the presence or absence of anti-DR3 antibodies and assessed the frequency of IL-17-producing thymic *i*NKT cells after 4 days. Unlike peripheral *i*NKT cells which can be activated within hours of α-GalCer administration [27,30], thymic *i*NKT cells needed a prolonged time (days) to respond to α-GalCer injection so that we employed a 4-days after α-GalCer injection scheme, which we found to be optimal for their analysis. Curiously, α-GalCer injection alone failed to increase IL-17 expression of thymic *i*NKT cells (**Fig. 4a**), and such was also the case for IL-4 (**Supplementary Fig. 14**). These results suggested that either the amount of injected α-GalCer was insufficient to activate thymic *i*NKT cells or that TCR engagement by α-GalCer would require costimulatory signals for activation. The coinjection of anti-DR3 with α-GalCer strongly supported the latter case, as there was a marked increase in IL-17-producing *i*NKT cells in α-GalCer/anti-DR3 coinjected mice (**Fig. 4a**). As expected, such an effect was specific to the NKT17 subset because IL-4 expression remained unaltered (**Supplementary Fig. 14**). Importantly, the increase in IL-17 production was not due to an increased propensity of IL-17 production by α-GalCer/anti-DR3 stimulated RORγt$^+$ cells, because we did not find increased frequencies of IL-17-producing cells among RORγt$^+$ *i*NKT cells (**Supplementary Fig. 15**).

To directly demonstrate that the increase in IL-17 production was due to an increase of NKT17 cells, we assessed the thymic *i*NKT subset composition of α-GalCer/anti-DR3-injected mice. Here we found a significant increase in the frequency and number (1.92 fold) of NKT17 cells compared to α-GalCer alone injected mice (**Fig. 4b and Supplementary Fig. 16**). Mechanistically, such an increase in NKT17 cells was driven by a dramatic increase in cell

proliferation as demonstrated in EdU incorporation assays (**Fig. 4c**). Specifically, the coadminstration of α-GalCer and anti-DR3, but not of α-GalCer alone, induced vigorous proliferation of thymic NKT17 cells. Collectively, these results indicated that DR3 acts as costimulatory molecule for TCR-signaled *i*NKT cells, and that such an effect is limited to the NKT17 subset among thymic *i*NKT cells because of the highly restricted expression of DR3 in that particular subset.

Discussion

*i*NKT cells in mice are highly restricted in their TCR repertoire as they all express the compulsory invariant Vα14-Jα18 TCRα, which, on one hand, furnishes them with the ability to bind CD1d molecules, but, on the other hand, also constrains their antigen specicities to a limited set of glycolipids [31]. As a corollary, all *i*NKT cells are equipped to respond to the classical *i*NKT cell antigen α-GalCer [1], and this shared antigen reactivity is observed in all mature *i*NKT cells, regardless of their effector function and subset identity. However, such common antigen reactivity raises the question of how a targeted immune response can be elicited if there is no subset-specific reactivity in *i*NKT cell activation. This conundrum has been attempted to be explained by identifying distinct subpopulations of *i*NKT cells expressing diverse TCRβ chains. As such, *i*NKT cells with different TCR Vβ usages would react differently to disparate glycolipid antigens, while they would be still activated by the canonical α-GalCer [32]. Nonetheless, it remains unclear whether these diverse glycolipid specificities are actually associated with distinct *i*NKT effector functions, and there is currently no evidence that certain TCR Vβ sequences would correlate with a particular *i*NKT subset identity. In fact, here, we also did not find any preferential TCR Vβ usage in $DR3^+$ NKT17 cells compared to other *i*NKT cells. Alternatively, it has been established that the abundance of surface TCR molecules differs between *i*NKT subsets, such that NKT2 cells express the largest amount of TCR followed by NKT17 and then NKT1 cells, which express the lowest abundance of TCR [15,33]. Such difference in TCR expression would translate into distinct TCR avidities so that NKT2 cells would be the most responsive while NKT1 cells would be the least antigen-responsive *i*NKT subset, establishing some kind of subset specificity in *i*NKT immune response. But, while such a scenario might establish a hierarchy in subset reactivity, it would not explain how a specific

*i*NKT subset, in particular *i*NKT cells with low avidity TCRs, can be selectively targeted for activation.

In this regard, the identification of DR3 being selectively expressed on the NKT17 subset provides a new perspective on how subset-specific activation can be achieved. Because DR3 is costimulatory to TCR signaling, the coactivation of DR3 with TCR stimulation will sensitize NKT17 cells to even low amounts of glycolipids that other *i*NKT subsets may not be cognizant of. Moreover, as we demonstrated in this study, DR3 ligation potentiates TCR signaling, further amplifying the immune response specifically in thymic NKT17 cells. Thus, we consider the subset-specific expression of a costimulatory molecule, such as DR3, as a mechanism to target and limit the activation to a select *i*NKT subset in the context of a preimposed antigen specificity that is common to all *i*NKT cells.

Whether costimulatory molecules, other than the cytokine receptor DR3, are also expressed in a subset-specific manner is currently unclear to us. IL-17Rβ, the receptor for IL-25, was previously reported to be absent in NKT1 cells [34], but its shared expression with NKT17 and NKT2 cells renders this marker promiscuous in its potential function. Expression of the IL-15 receptor IL-2Rβ is specific to the NKT1 subset [3], but we recently demonstrated that IL-15 is only necessary for the generation of NKT1 cells in the thymus and largely dispensable for the maintenance and effector function of NKT1 cells in the periphery [17]. Therefore, the role of IL-2Rβ as an NKT1-specific marker and its disparate significance in the thymus versus peripheral tissues also need further clarification. Along these lines, here, we need to stress that the costimulatory role of DR3 might also differ between the thymus and peripheral tissues, as we found DR3 expression being highly specific to NKT17 cells in the thymus but rather indiscriminate on peripheral *i*NKT cells. Because we demonstrated that DR3 expression is

controlled downstream of RORγt, we are also uncertain how to interpret that peripheral NKT1 and NKT2 cells, which lack RORγt, can have induced DR3. Because Foxp3$^+$ Treg cells also express DR3 in the absence of RORγt [19], evidently, alternative regulatory mechanisms of DR3 expression should exist. The molecular pathway of DR3 regulation and its role in the activation and effector function of peripheral *i*NKT cells are important issues that we aim to address in our follow-up studies.

While we showed that DR3 ligation acts as a co-stimulator of TCR-signaled NKT17 cells, we also wish to point out that our study is limited in its scope as the precise mechanism how DR3 would potentiate NKT17 cell activation is not fully addressed. DR3 signaling can trigger multiple downstream pathways, including the activation of NF-kB, MAPK, and JNK but also pro-apoptotic caspases, so that DR3 ligation was found to trigger cell death or increased survival and proliferation [9]. In thymic NKT17 cells, DR3 ligation bolsters TCR signaling so that activation markers such as CD69 and CD25 are further upregulated and cell proliferation is increased. Because DR3 ligation itself was insufficient to promote IL-17 production and only acted in concert with agonistic TCR signaling to synergistically drive the activation and cytokine expression of NKT17 cells, we postulate that the DR3 signaling intersects with the TCR signaling pathway to augment TCR-induced activation. But, this remains to be experimentally assessed.

Collectively, our study identified the cytokine receptor DR3 as a new costimulatory molecule that is specifically expressed on NKT17 cells among thymic *i*NKT cells and thus, augments their activation. In this regard, DR3 represents a class of immunomodulatory molecules whose expression and function are linked to a specific *i*NKT subset. These results open new avenues for elucidating how different *i*NKT subsets that express the same invariant

TCR and respond to the same agonistic glycolipid, *i.e.,* α-GalCer, can elicit subset-specific immune responses *in vivo*.

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Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

Approval of animal experiments was granted by the NCI Animal Care and Use Committee under the animal protocol number EIB-076 and ASP-421. All mice were cared for in accordance with the Public Health Service policy on human care and use of laboratory animals and NIH guidelines.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability

Not applicable.

Author contributions

SL and NL designed and performed the experiments, analyzed the data, and contributed to the writing of the manuscript. CL and AC performed experiments, analyzed the data, and commented on the manuscript. EW and FM provided reagents, experimental expertise, and edited the manuscript. JP conceived the project, analyzed the data, and wrote the manuscript.

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Figure legend

Figure 1. Cytokine receptor DR3 is highly expressed on thymic NKT17 cells

a. *i*NKT subsets were identified in BALB/c thymocytes by intracellular staining for RORγt and PLZF and then assessed for subset-specific expression of DR3 and CD138. The data are representative of 2 independent experiments with a total of 4 BALB/c mice.

b. Quantification of DR3 and CD138 expression (ΔMFI) in *i*NKT subsets. The data show the summary of 2 independent experiments with a total of at least 4 BALB/c mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

c. DR3 and CD138 expression on thymic NKT17 cells of CD138-deficient (*Sdc1*$^{-/-}$) and littermate control (LMC) BALB/c mice. The data are representative of 2 independent experiments with a total 4 *Sdc1*$^{-/-}$ and 4 LMC BALB/c mice.

d. Quantification of intracellular RORγt and surface CD138 expression (ΔMFI) in the thymus, LN, and lung NKT17 cells of BALB/c mice. The bar graphs are summary of at least 4 independent experiments. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

e. Thymocytes and splenocytes of BALB/c mice were treated with PMA, ionomycin, and BFA for 3 hours before assessing IL-17A expression in *i*NKT cells. Contour plots are representative and bar graph is the summary of 3 independent experiments with a total of 4 BALB/c mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

f. *i*NKT subset composition were assessed in thymic *i*NKT cells of RORγt^{Tg} and LMC BALB/c mice. Contour plots are representative of 3 independent experiments with a total of 4 RORγt^{Tg} and 4 LMC BALB/c mice.

g. Thymic *i*NKT cells of RORγt^{Tg} and LMC BALB/c thymocytes were assessed for surface DR3 and CD138 expression. The contour plot is representative (top), and the bar graphs (bottom) show a summary from 3 independent experiments with a total of 6 RORγt^{Tg} and 6 LMC mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

h. RORγt and DR3 expression on non-NKT17 thymic *i*NKT cells of RORγt^{Tg} and LMC BALB/c thymocytes. Histograms are representative (top), and the bar graphs (bottom) show summary of 3 independent experiments with at least 3 RORγt^{Tg} and 3 WT mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

Figure 2. *In vivo* effects of DR3 ligation on Foxp3$^+$ Treg and CD138$^+$ NKT17 cells

a. Contour plots show *Foxp3*-GFP versus CD25 expression of splenic CD4$^+$ T cells (left), and bar graphs (right) show the frequencies and cell numbers of splenic CD25$^+$Foxp3$^+$ Treg cells, after 1 week of injection with anti-DR3 or isotype control antibodies into BALB/c *Foxp3*-GFP reporter mice. The results are summarized from 4 independent experiments with a total of 4 mice injected with anti-DR3 and 4 mice injected with isotype control. Statistical significance was determined by paired two-tailed Student's *t*-tests.

b. Identification and enumeration of CD138$^+$ thymic *i*NKT cells of BALB/c *Foxp3*-GFP reporter mice one week after injection of anti-DR3 or isotype control antibody (Ctrl IgG). The contour plots (left) are representative, and the bar graphs (right) are a summary of data from 11 independent experiments with a total of 11 mice for each group. Statistical significance was determined by paired two-tailed Student's *t*-tests.

c. Identification and enumeration of thymic RORγt$^+$ NKT17 cells upon anti-DR3 injection. BALB/c mice were i.p. injected with anti-DR3 or control antibodies and analyzed after 1 week

for their thymic *i*NKT subset composition. Contour plots (left) are representative and bar graphs (right) show summary of 3 independent experiments with a total of 7 BALB/c mice for each group. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

d. Subsets were identified in total thymic *i*NKT cells by CD4 versus CD122 expression (contour plot). The expression of NKT17-specific signature molecules was quantified for each *i*NKT subset (bar graphs). The contour plot is representative (left), and the bar graphs (right) are summary of data from 3 independent experiments with a total of 3 BALB/c mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

e. CD138 and DR3 expression on $CD4^{-}CD122^{-}$ thymic *i*NKT cells of CD138-deficient ($Sdc1^{-/-}$) and LMC BALB/c mice. The histograms are representative of 3 independent experiments with a total of 5 $Sdc1^{-/-}$ and 5 LMC BALB/c mice.

f. DR3 expression on thymic *i*NKT subsets identified by the disparate expression of CD4 and CD122 in CD138-deficient ($Sdc1^{-/-}$) BALB/c mice. The bar graph shows summary of data from 3 independent experiments with a total of 5 $Sdc1^{-/-}$ BALB/c mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

Figure 3. DR3 is a specific and functional marker of thymic NKT17 cells

a. Activation marker expression on thymic NKT17 cells of BALB/c *Foxp3*-GFP reporter mice after 1 week of anti-DR3 antibody injection. The contour plots (top) are representative, and the bar graphs (bottom) are summary of data from 7 independent experiments with each at least 5 mice for anti-DR3 or isotype control antibody injection. Statistical significance was determined by paired two-tailed Student's *t*-tests.

b. CD69 expression (left) and cell numbers (right) of thymic NKT17 cells from *Sdc1*$^{-/-}$ and LMC BALB/c mice one week after injection with anti-DR3 or isotype control antibodies (Ctrl IgG). The bar graphs show summary from 4 independent experiments with a total of at least 3 mice for each group. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

c. CD69 and *Nr4a1*-GFP expression on CD138^{+} thymic NKT17 cells of *Nr4a1*-GFP reporter mice after overnight injection with anti-DR3 or isotype control antibodies (Ctrl IgG). Results show a summary of three independent experiments with a total of at least 3 mice for each group. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

d. Frequencies of CD69^{+}CD25^{+} activated thymic NKT17 cells upon overnight *in vitro* stimulation with α-GalCer (100 ng/ml) in the presence or absence of anti-DR3 (2 μg/ml). The bar graph is summary from 4 independent experiments with a total of 10 BALB/c mice for each group. Statistical significance was determined by paired two-tailed Student's *t*-tests.

e. IL-17A production of thymic *i*NKT cells upon α-GalCer and anti-DR3 stimulation. Freshly isolated BALB/c thymocytes were stimulated overnight with α-GalCer in the presence or absence of anti-DR3, and treated the next day with BFA without PMA and ionomycin for 3 hours before assessing their cytokine expression. Contour plots are representative (left), and bar graphs (right) show summary of 3 experiments with a total of 8 mice for each group. Statistical significance was determined by paired two-tailed Student's *t*-tests.

Figure 4. DR3 ligation augments the activation of NKT17 cells *in vivo*

a. IL-17A production of thymic *i*NKT cells upon *in vivo* α-GalCer and anti-DR3 stimulation. BALB/c mice were analyzed 4 days after i.p. injection with α-GalCer in the presence or absence of anti-DR3. The thymocytes were treated with PMA, ionomycin, and BFA for 3 hours before

assessing their cytokine expression. Contour plots are representative (left), and bar graphs (right) show summary of 5 experiments with a total of at least 7 mice for each group. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

b. Subset composition of thymic *i*NKT cells of BALB/c mice 4 days after injection with α-GalCer and/or anti-DR3 antibodies (left). Bar graph (right) shows the frequency of thymic NKT17 cells for each condition. Results are representative of 6 independent experiments with a total of at least 7 BALB/c mice. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

c. EdU incorporation of thymic *i*NKT cells upon *in vivo* α-GalCer and anti-DR3 stimulation. BALB/c mice were analyzed 4 days after i.p. injection with α-GalCer in the presence or absence of anti-DR3 and 16 hours after EdU i.p. injection. Contour plots are representative (left), and bar graph (right) shows the summary of 5 experiments with a total of 7 mice for each group. Statistical significance was determined by unpaired two-tailed Student's *t*-tests.

Figure

Figure 1

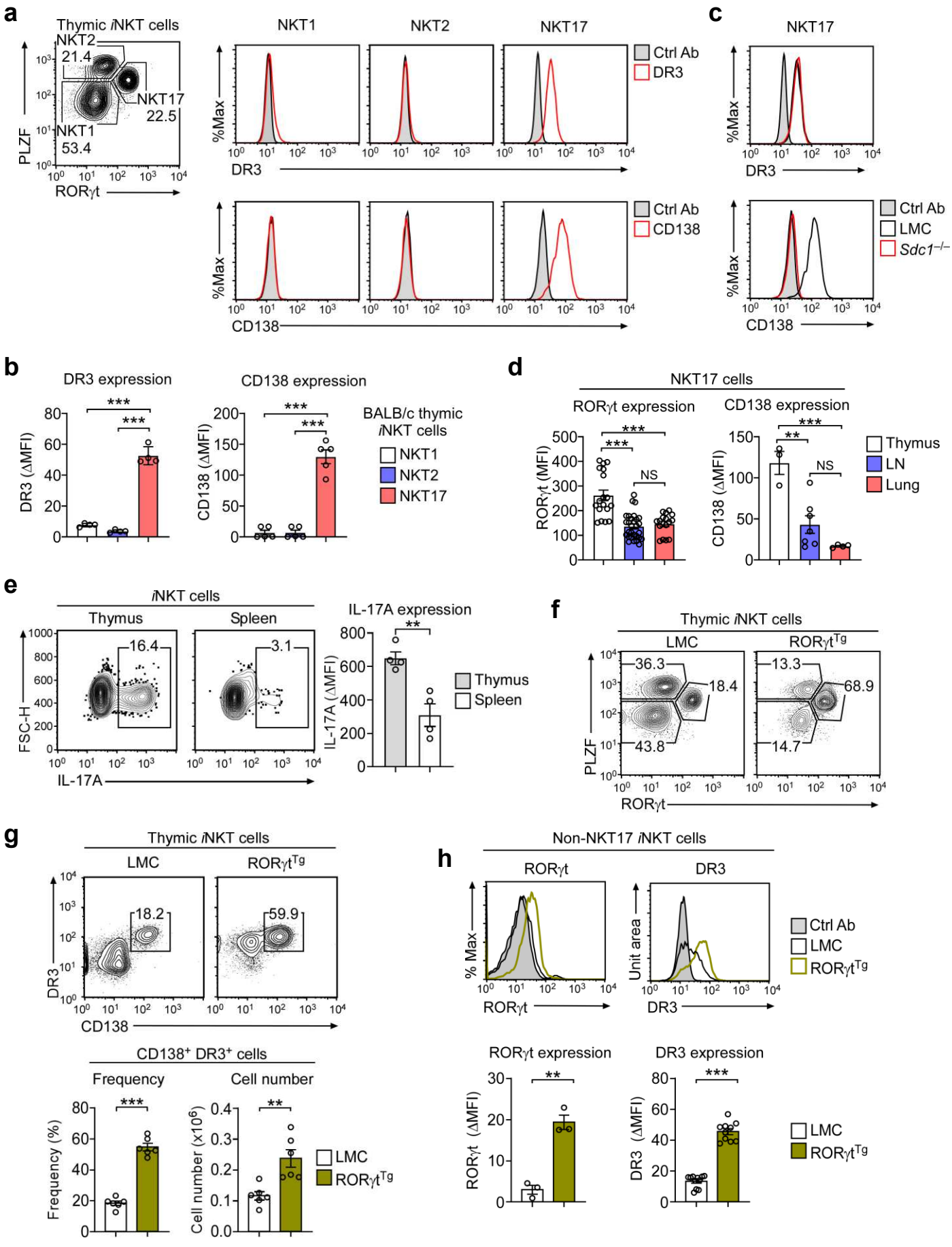


Figure 2

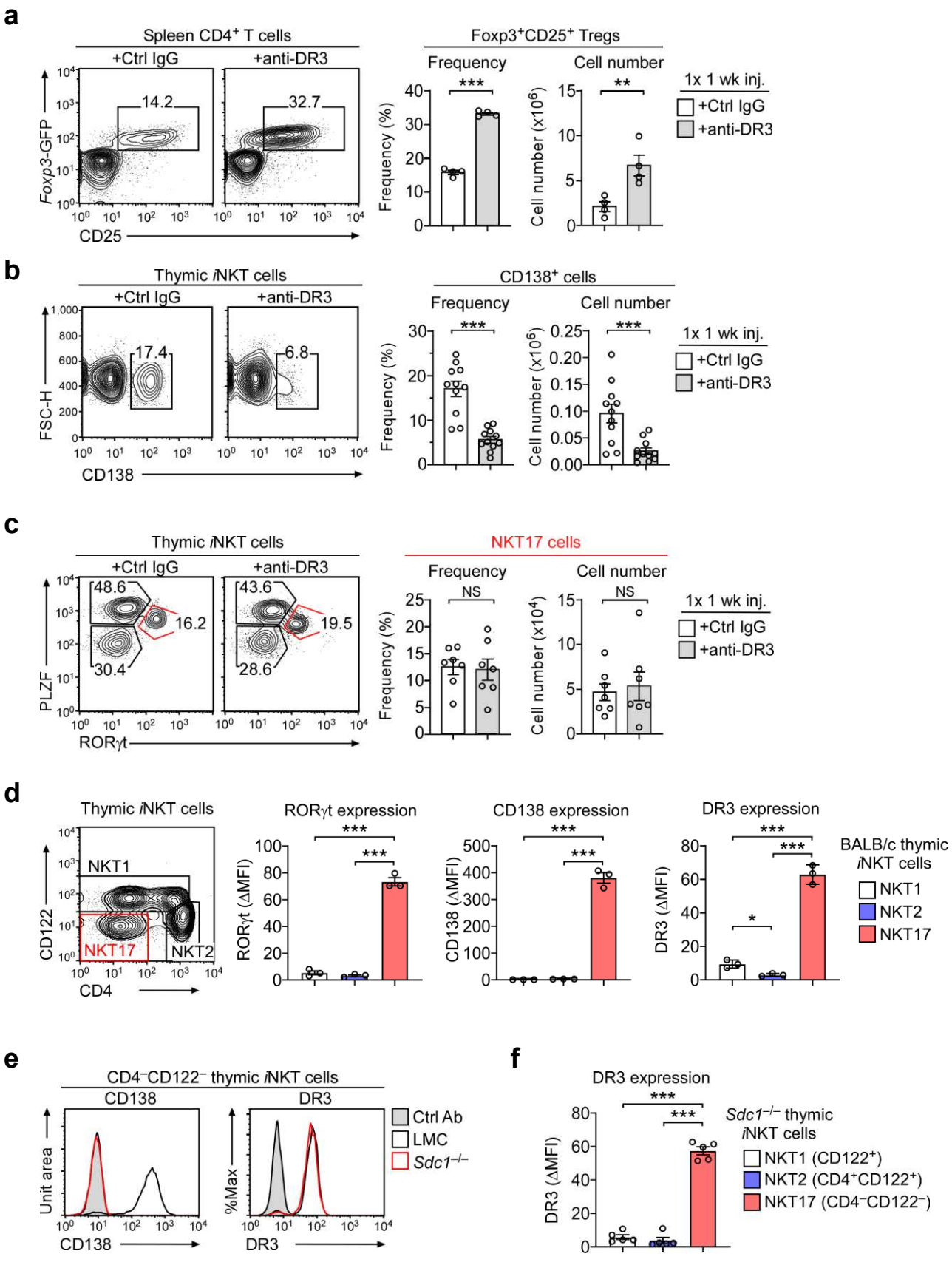

a
Spleen CD4+ T cells
+Ctrl IgG
+anti-DR3
14.2
32.7
Foxp3-GFP
CD25
Foxp3+CD25+ Tregs
Frequency
Cell number
***
**
Frequency (%)
Cell number (x10^6)
1x 1 wk inj.
+Ctrl IgG
+anti-DR3
b
Thymic iNKT cells
+Ctrl IgG
+anti-DR3
17.4
6.8
FSC-H
CD138
CD138+ cells
Frequency
Cell number
***
***
Frequency (%)
Cell number (x10^6)
1x 1 wk inj.
+Ctrl IgG
+anti-DR3
c
Thymic iNKT cells
+Ctrl IgG
+anti-DR3
48.6
16.2
30.4
43.6
19.5
28.6
PLZF
RORγt
NKT17 cells
Frequency
Cell number
NS
NS
Frequency (%)
Cell number (x10^4)
1x 1 wk inj.
+Ctrl IgG
+anti-DR3
d
Thymic iNKT cells
NKT1
NKT17
NKT2
CD122
CD4
RORγt expression
CD138 expression
DR3 expression
***
***
RORγt (ΔMFI)
CD138 (ΔMFI)
DR3 (ΔMFI)
*
BALB/c thymic iNKT cells
NKT1
NKT2
NKT17
e
CD4−CD122− thymic iNKT cells
CD138
DR3
Unit area
%Max
CD138
DR3
Ctrl Ab
LMC
Sdc1−/−
f
DR3 expression
***
***
DR3 (ΔMFI)
Sdc1−/− thymic iNKT cells
NKT1 (CD122+)
NKT2 (CD4+CD122+)
NKT17 (CD4−CD122−)

Figure 3

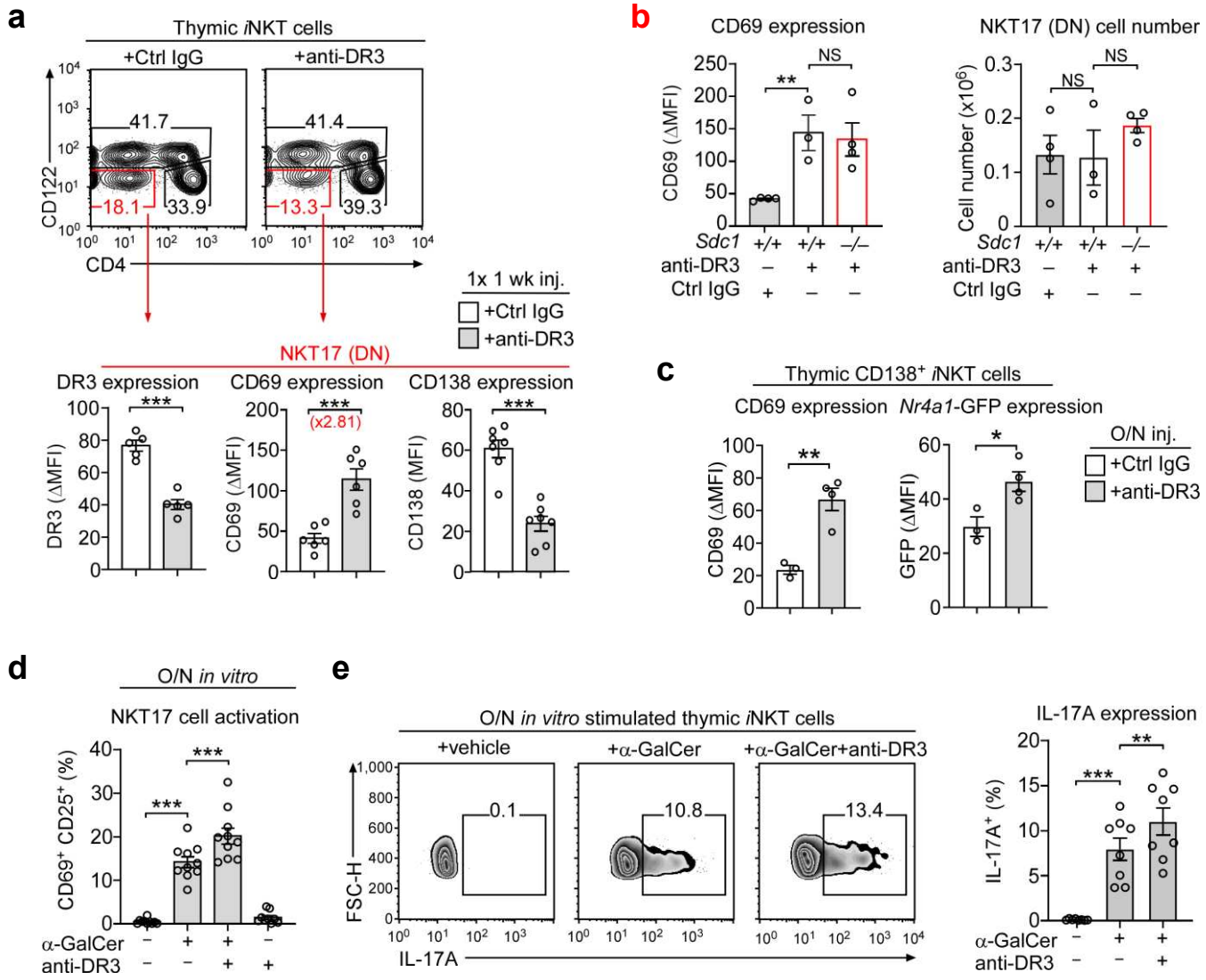
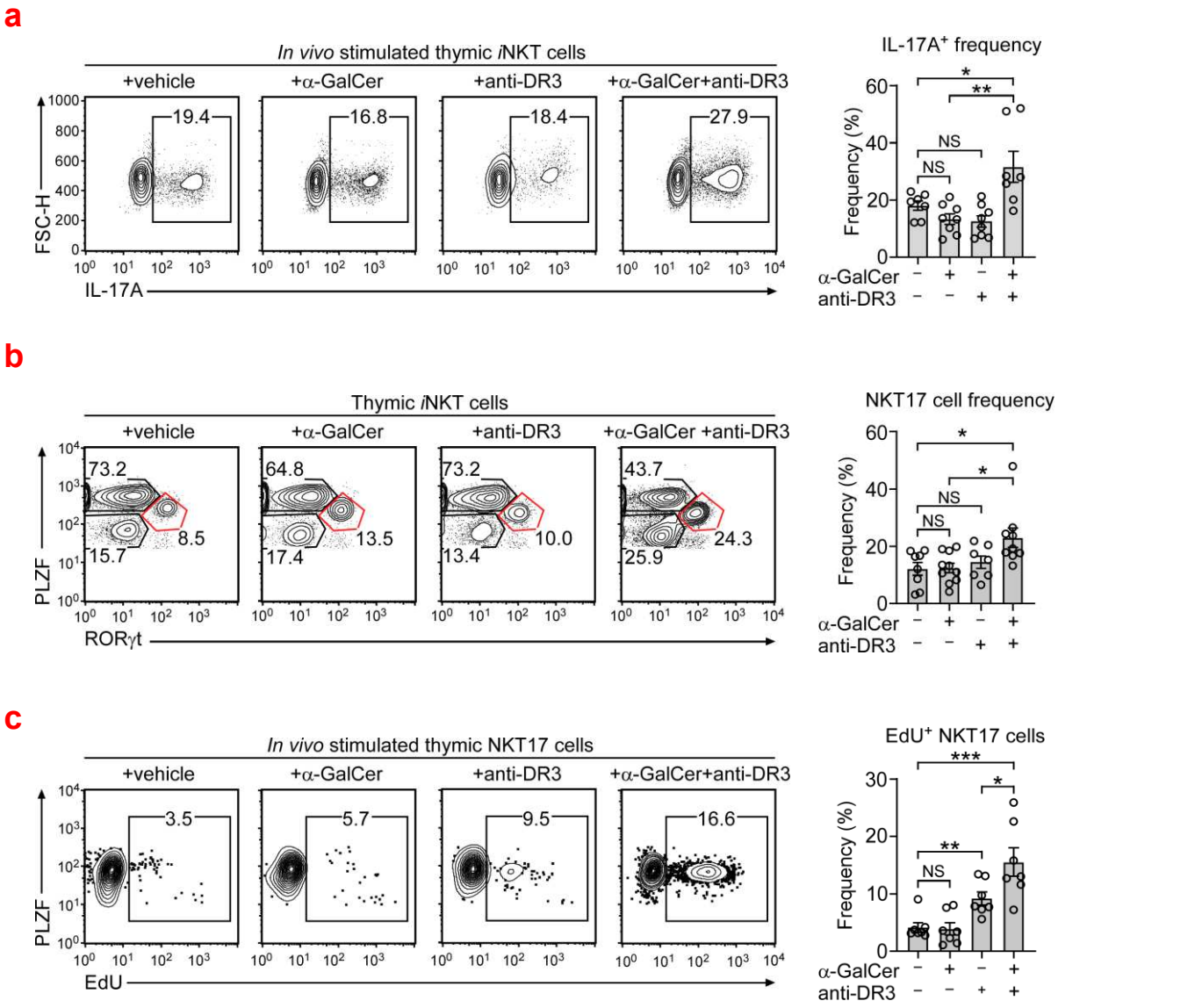

a
Thymic iNKT cells
+Ctrl IgG
+anti-DR3
41.7
41.4
18.1
33.9
13.3
39.3
CD122
CD4
1x 1 wk inj.
+Ctrl IgG
+anti-DR3
NKT17 (DN)
DR3 expression
CD69 expression
CD138 expression
DR3 (ΔMFI)
CD69 (ΔMFI)
CD138 (MFI)
***
(x2.81)
b
CD69 expression
NKT17 (DN) cell number
CD69 (ΔMFI)
Cell number (x10^6)
NS
**
Sdc1
anti-DR3
Ctrl IgG
c
Thymic CD138+ iNKT cells
CD69 expression
Nr4a1-GFP expression
GFP (ΔMFI)
*
O/N inj.
d
O/N in vitro
NKT17 cell activation
CD69+ CD25+ (%)
α-GalCer
anti-DR3
e
O/N in vitro stimulated thymic iNKT cells
+vehicle
+α-GalCer
+α-GalCer+anti-DR3
FSC-H
IL-17A
0.1
10.8
13.4
IL-17A expression
IL-17A+ (%)

Figure 4


a
In vivo stimulated thymic iNKT cells
+vehicle
+α-GalCer
+anti-DR3
+α-GalCer+anti-DR3
19.4
16.8
18.4
27.9
FSC-H
IL-17A
IL-17A+ frequency
Frequency (%)
NS
NS
*
**
α-GalCer
anti-DR3
b
Thymic iNKT cells
73.2
8.5
15.7
64.8
13.5
17.4
73.2
10.0
13.4
43.7
24.3
25.9
PLZF
RORγt
NKT17 cell frequency
c
In vivo stimulated thymic NKT17 cells
3.5
5.7
9.5
16.6
EdU
EdU+ NKT17 cells
***