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Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of *i*NKT cells

You Jeong Lee[1], Keli L Holzapfel[1], Jinfang Zhu[2], Stephen C Jameson[1] & Kristin A Hogquist[1]

Invariant natural killer T cells (*i*NKT cells) can produce copious amounts of interleukin 4 (IL-4) early during infection. However, indirect evidence suggests they may produce this immunomodulatory cytokine in the steady state. Through intracellular staining for transcription factors, we have defined three subsets of *i*NKT cells (NKT1, NKT2 and NKT17) that produced distinct cytokines; these represented diverse lineages and not developmental stages, as previously thought. These subsets exhibited substantial interstrain variation in numbers. In several mouse strains, including BALB/c, NKT2 cells were abundant and were stimulated by self ligands to produce IL-4. In those strains, steady-state IL-4 conditioned CD8$^+$ T cells to become 'memory-like', increased serum concentrations of immunoglobulin E (IgE) and caused dendritic cells to produce chemokines. Thus, *i*NKT cell–derived IL-4 altered immunological properties under normal steady-state conditions.

It has become increasingly apparent that many gene-deficient and transgenic mice have a unique population of memory-like CD8$^+$ T cells in the thymus[1]. These cells have also been referred to as 'innate CD8$^+$ T cells' because they act like memory T cells and rapidly produce interferon-γ (IFN-γ), yet antigen recognition is not required for their differentiation[2]. Studies of the differentiation mechanism have revealed a common pathway whereby various genetic alterations all lead to increased numbers of αβ or γδ natural killer T cells (NKT cells) that express the transcription factor PLZF[3–6]. In all such models, interleukin 4 (IL-4), presumably produced by invariant NKT cells (*i*NKT cells) in the steady state, is required for CD8$^+$ T cells to express *Eomes* (which encodes the transcription factor eomesodermin (Eomes)) and adopt the characteristic features of memory CD8$^+$ T cells. Notably, not only those gene-deficient mice but also wild-type BALB/c mice have a distinct population of memory-like CD8$^+$ T cells that are dependent on *i*NKT cells and IL-4 (refs. 3,7). Even C57BL/6 (B6) mice have defined (although much smaller) populations of memory-like CD8$^+$ T cells[8]. These data all indicate that *i*NKT cells must have been activated to produce IL-4 continuously or at some point in their development.

In this study, we sought to identify the *i*NKT cells that produce IL-4 and give rise to memory-like CD8$^+$ T cells. Our data support a newly proposed classification of *i*NKT cells into NKT1, NKT2 and NKT17 lineages according to their function and transcription-factor expression[9], analogous to CD4$^+$ helper T cells. In several strains of mice, precursors of *i*NKT cells efficiently differentiated into NKT2 cells and had abundant secretion of IL-4 in the steady state. That IL-4 not only conditioned bystander CD8$^+$ T cells to become memory-like cells but also stimulated thymic dendritic cells (DCs) to secrete the chemokines CCL17 and CCL22 and stimulated peripheral B cells to produce immunoglobulin E (IgE).

RESULTS

IL-4 secretion by BALB/c *i*NKT cells in the steady state

The thymus of young BALB/c mice contains three to five times more *i*NKT cells than that of B6 mice, and these cells are hypothesized to secrete IL-4, which conditions bystander CD8$^+$ single-positive (SP) thymocytes to become memory-like CD8$^+$ T cells[3]. On the basis of that finding, we sought to further investigate the subset of *i*NKT cells that secrete IL-4 in the steady state. For this purpose, we used KN2 mice, in which the first two exons of the endogenous *Il4* gene are replaced with sequence encoding human CD2 and expression of human CD2 on the cell surface reports IL-4 secretion *in vivo*[10]. We used heterozygous mice with one wild-type allele and that marker on the other allele (so that IL-4 expression is maintained) in this study. We found that up to 40% of thymic *i*NKT cells from KN2 mice of the BALB/c strain (BALB/c-KN2 mice) expressed human CD2 on the cell surface, whereas *i*NKT cells from KN2 mice of the B6 strain (B6-KN2 mice) were largely negative for human CD2 (**Fig. 1a**), consistent with a published report[11]. Young BALB/c-KN2 mice had over tenfold more human CD2–positive cells in the thymus than did B6-KN2 mice (**Fig. 1b**). Peripheral *i*NKT cells also expressed human CD2 in BALB/c-KN2 mice, although not as abundantly as the thymus did (**Fig. 1a**). In contrast, hardly any conventional CD4$^+$ SP cells were positive for human

[1]The Department of Laboratory Medicine and Pathology, Center for Immunology, University of Minnesota, Minneapolis, Minnesota, USA. [2]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA. Correspondence should be addressed to K.A.H. (hogqu001@umn.edu).

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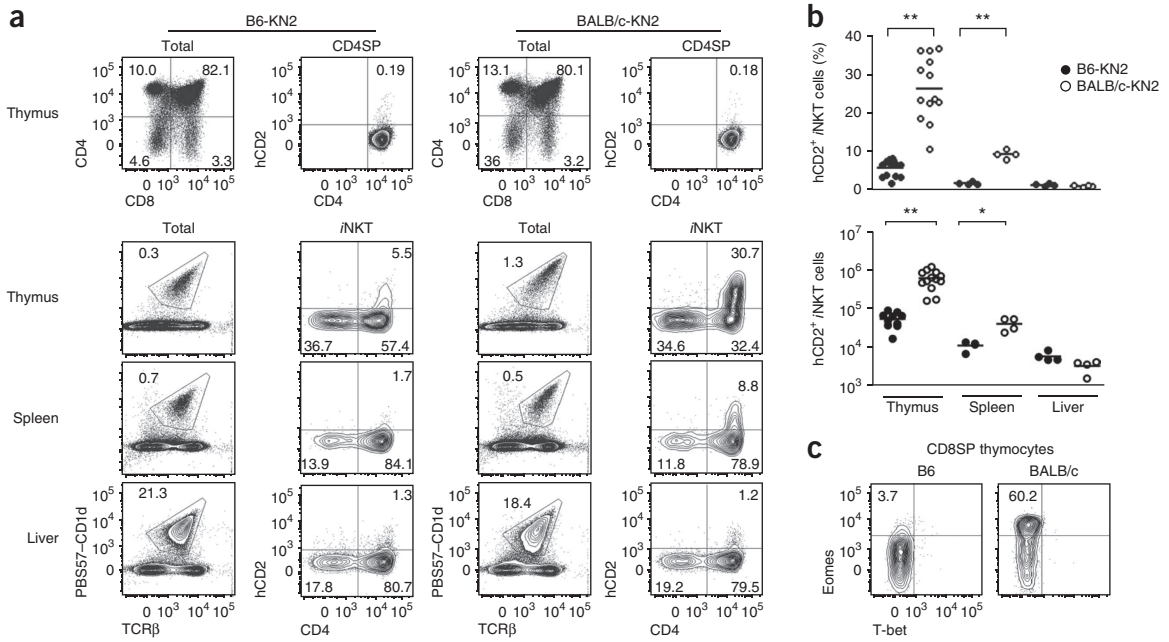

Figure 1 BALB/c *i*NKT cells produce IL-4 in the steady state. (**a**) Expression of human CD2 (hCD2) in conventional CD4+ SP (CD4SP) thymocytes (top row) and *i*NKT cells from thymus, spleen and liver that bind CD1d tetramer loaded with the α-galactosylceramide analog PBS57 (PBS57-CD1d) (bottom three rows), all from 7-week-old B6-KN2 and BALB/c-KN2 mice, assessed by flow cytometry. Numbers in quadrants or adjacent to outlined areas indicate percent cells in each throughout. (**b**) Frequency (top) and number (bottom) of human CD2–positive (hCD2+) *i*NKT cells in thymus, spleen and liver of 7- to 8-week-old B6-KN2 mice ($n = 4–10$) and BALB/c-KN2 mice ($n = 4–13$). Each symbol represents an individual mouse; small horizontal lines indicate the mean. $*P = 0.0095$ and $**P < 0.0001$ (unpaired two-tailed *t*-test). (**c**) Expression of Eomes and T-bet in B6 and BALB/c CD8+ SP (CD8SP) thymocytes, assessed by flow cytometry. Data are representative of at least five independent experiments.

CD2, either in the thymus (**Fig. 1a**) or periphery (data not shown) of either strain. These results indicated that *i*NKT cells were a major source of IL-4 *in vivo* in the steady state. As shown before[3], this greater abundance of IL-4 correlated with a greater frequency and number of Eomes+ memory-like CD8+ T cells in BALB/c mice (**Fig. 1c**).

Three transcription factors distinguish NKT cell subsets

To further characterize the IL-4-producing *i*NKT cells in BALB/c mice, we compared the developmental profiles of thymic *i*NKT cells in B6 and BALB/c mice. In the standard *i*NKT cell classification, expression of a combination of the markers CD24 (HSA), CD44 and NK1.1 is used to distinguish *i*NKT cells as stage 0, 1, 2 and 3 (ref. 12). However, NK1.1, which has been considered a marker of the terminal maturation of *i*NKT cells, is not expressed in BALB/c mice and does not correlate with functional capacity[13]. Therefore, instead of staining surface markers, we used intracellular staining of transcription factors, which are recognized equivalently in different mouse strains and are more closely linked to function. PLZF is a factor essential for the development and innate function of *i*NKT cells[14,15], and T-bet, GATA-3 and RORγt are transcription factors that regulate the differentiation of conventional CD4+ T cells into the T_H1, T_H2 and T_H17 lineages of helper T cells, respectively[16]. The combination of PLZF, T-bet and RORγt separated *i*NKT cells into three distinctive subsets and, analogous to the helper T cell lineage nomenclature, we designated these 'NKT1 cells', 'NKT2 cells' and 'NKT17 cells', respectively (**Fig. 2a** and **Supplementary Fig. 1a–d**). T_H2 cell–specific transcription factors, including GATA-3 and IRF4, had high expression in both NKT2 cells and NKT17 cells (**Supplementary Fig. 1a**). NKT1 cells had abundant expression of T-bet and low expression of GATA-3, consistent with a published report showing that all T cells, including T_H1 cells and *i*NKT cells, have variably low expression of GATA-3 (ref. 17). This classification roughly correlated with the conventional staging system in B6 mice, as NKT1 cells were predominantly stage 3 and NKT2 cells were stages 1 and 2 (**Fig. 2b**), although NKT17 cells cannot be distinguished from NKT2 cells by the conventional classification.

That method of defining *i*NKT cell subsets correlated precisely with cytokine production: thymic NKT1, NKT2 and NKT17 cells produced IFN-γ, IL-4 and IL-17, respectively (**Fig. 2c**), when stimulated with the phorbol ester PMA and ionomycin. NKT2 cells were abundant in BALB/c mice, whereas NKT1 cells were predominant in B6 mice (**Fig. 2d**). Numerically, 7- to 8-week-old BALB/c mice produced 7.5 times more NKT2 cells and 9 times more NKT17 cells than did B6 mice (**Fig. 2d**). However, the absolute number of NKT1 cells was not significantly different in these two strains (**Fig. 2d**). We similarly identified peripheral *i*NKT cells by this transcription factor–staining profile (**Supplementary Fig. 2a**). As in the thymus, NKT2 cells were predominant in the spleen of BALB/c mice, whereas NKT1 cells were predominant in B6 mice (**Supplementary Fig. 2a**,**b**). Liver *i*NKT cells were mostly NKT1 in both strains. NKT2 cells produced IL-4, but not IFN-γ, after strong agonistic stimulation *in vivo* with the synthetic lipid α-galactosylceramide, while peripheral NKT1 cells secreted IFN-γ and also IL-4 (albeit a low amount per cell; **Supplementary Fig. 2c**).

We also investigated other surface markers that can be used to discriminate these subsets. We found that CD122 (and NK1.1 in B6 mice) were reasonable markers of NKT1 cells in the thymus and, in the $CD122^{-}NK1.1^{-}$population, CD4 and CD27 could be used to differentiate NKT2 cells and NKT17 cells, as NKT2 cells expressed CD4 and CD27 but NKT17 cells had lower expression of both (**Supplementary Fig. 1b**). Thus, a combination of CD122 (or NK1.1 in B6 mice) together with CD4 or CD27 can be used to distinguish thymic *i*NKT cells, consistent with their transcription-factor profile. IL-17RB has been described as a surface marker that can be used

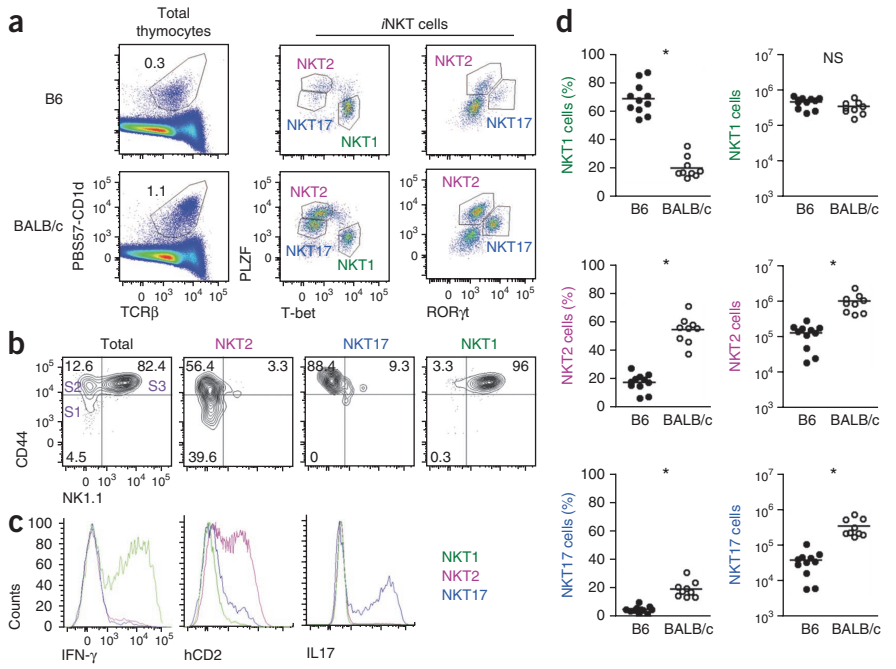

Figure 2 PLZF, RORγt and T-bet differentiate NKT1, NKT2 and NKT17 cells. (**a**) Intracellular PLZF, T-bet and RORγt in thymic *i*NKT cells from 7-week-old B6 and BALB/c mice, identifying NKT1, NKT2 and NKT17 cells (outlined areas in plots). (**b**) Expression of CD44 and NK1.1 on each *i*NKT subset defined in **a**; S1, S2 and S3 (far left) indicate the conventional stages. (**c**) Intracellular cytokines and human CD2 in BALB/c-KN2 thymic *i*NKT cells stimulated with PMA and ionomycin. (**d**) Frequency (left) and number (right) of each *i*NKT cell subset in the thymus of 7- to 8-week-old B6 mice ($n = 11$) and BALB/c mice ($n = 9$). Each symbol represents an individual mouse; small horizontal lines indicate the mean. NS, not significant ($P = 0.06$ in **d**); $^*P < 0.001$ (unpaired two-tailed *t*-test). Data are representative of at least ten (**a**) or three (**b**) or eight (**c**) independent experiments, or are pooled from three independent experiments (**d**).

to distinguish NKT cell subsets[18]. NKT2 cells were IL-17RB$^+$CD4$^+$ and NKT1 cells were IL-17RB$^-$CD4$^+$ or IL-17RB$^-$CD4$^-$ (**Supplementary Fig. 1d**). However, NKT17 cells were IL-17RB$^+$CD4$^-$ in B6 mice but IL-17RB$^-$CD4$^-$ in BALB/c mice (**Supplementary Fig. 1d**). We have summarized the phenotypic and functional properties of *i*NKT cell subsets (**Table 1**).

IL-4-producing NKT2 cells do not give rise to NKT1 cells

The conventional classification of *i*NKT cells defined in B6 mice would indicate a linear differentiation model in which CD44^{lo}NK1.1$^-$ (stage 1) cells upregulate CD44 expression (stage 2) before finally expressing NK1.1 (stage 3), and stage 1 cells produce mainly IL-4, while stage 3 cells produce both IFN-γ and IL-4 (ref. 12). However, we found here that *i*NKT cells with those markers were heterogeneous and expressed distinct transcription factors, more suggestive of a model in which multiple lineages differentiate from a common progenitor[9]. Thus, we sought to address whether NKT2 cells, which were in the CD44^{hi}NK1.1$^-$ (stage 2) gate, gave rise to NK1.1$^+$ NKT1 cells (stage 3) or not. It has been established that NK1.1$^-$CD44^{hi} cells can give rise to NK1.1$^+$ cells[19,20]. However, published work suggests that the fraction of NK1.1$^-$ cells that express IL-17RB do not give rise to NK1.1$^+$ cells[18]. We investigated expression of IL-17RB in BALB/c-KN2 and B6-KN2 mice and found that expression of human CD2 was confined to an IL-17RB$^+$ population (**Supplementary Fig. 1c**), which suggested that the NKT2 cells that secreted IL-4 in the steady state were not developmental intermediates that would give rise to NKT1 cells. To more rigorously assess the precursor-progenitor relationship, we sorted human CD2–positive and human CD2–negative NKT2 cells from B6-KN2 and BALB/c-KN2 mice that also have a green fluorescent protein (GFP) reporter for T-bet[21] (T-bet–GFP), labeled them with a violet cell tracer and injected them intrathymically into congenic hosts (**Fig. 3**). Four days later, a substantial fraction of human CD2–negative cells had converted into NKT1 cells that expressed T-bet–GFP in thymus and spleen, whereas human CD2–positive NKT2 cells did not. This result indicated that NKT2 cells were not precursors of NKT1 cells. Also these data showed that NK1.1$^-$ *i*NKT cell populations were heterogeneous and included terminally differentiated IL-4-producing cells (human CD2–positive NKT2 cells), as well as progenitors of NKT1 cells.

NKT2 cells produce IL-4 in the steady state

To determine which NKT subset produced IL-4 in the steady state, we examined expression of human CD2 in KN2 mice directly *ex vivo*. We observed *ex vivo* expression of human CD2 exclusively in NKT2 cells (**Fig. 4a**). In addition to the greater abundance of NKT2 cells in BALB/c mice than in B6 mice (**Fig. 2d**), the mean fluorescence intensity of human CD2 was higher in BALB/c NKT2 cells than in B6 NKT2 cells (**Fig. 4a**). This suggested not only that did more cells with the potential to produce IL-4 exist in the steady state in BALB/c mice but also that more of them were actively producing IL-4 *in vivo*.

Since both NK1.1$^-$ NKT2 and NK1.1$^+$ NKT1 have the potential to produce IL-4 (ref. 20) (**Supplementary Fig. 2c**), we sought to definitively determine if steady-state IL-4 was produced by NKT2 cells or NKT1 cells. Thus, we analyzed T-bet-deficient (*Tbx21*$^{-/-}$) mice, as these mice have a substantial deficiency in NK1.1$^+$ *i*NKT cells[22]. Although that report suggested that T-bet deficiency results in a lower total number of *i*NKT cells[22], we did not observe a deficiency in total *i*NKT cells in young (6-week-old) *Tbx21*$^{-/-}$ mice (**Fig. 4b**). Instead, while *Tbx21*$^{-/-}$ mice had significantly fewer NKT1 cells than wild-type mice had, *Tbx21*$^{-/-}$ mice had a compensatory higher frequency and number of NKT2 and NKT17 cells (**Fig. 4c**). The difference between our results and that published report[22] may be related

Table 1 Transcription factors differentiate *i*NKT cells into the NKT1, NKT2 and NKT17 lineages

	PLZF	T-bet	RORγt	GATA-3	IRF4	Main cytokine	Surface markers	Conventional classification
NKT2	High	–	–	+	+	IL-4	CD4$^+$CD27$^+$CD122$^-$	Stages 1 and 2
NKT17	Intermediate	–	+	+	+	IL-17	CD4$^-$CD27$^-$CD122$^-$	Stage 2
NKT1	Low	+	–	+ or –	–	IFN-γ	CD4$^+$ or CD4$^-$ CD27$^+$CD122$^+$	Stage 3

Characteristics of the three NKT cell subsets, identified by intracellular staining for transcription factors, cytokine production, surface expression of markers, relative to that of to the conventional stage classification defined by expression of CD44 and NK1.1.

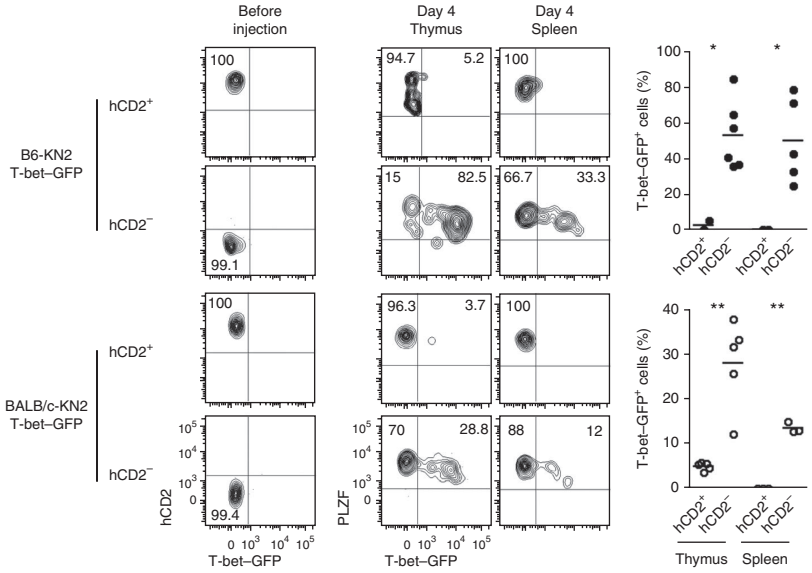

Figure 3 IL-4-producing NKT2 cells do not give rise to NKT1 cells. T-bet–GFP expression (far right) in donor cells from the thymus and spleen of congenic host mice given intrathymic injection of violet cell tracer–labeled B6-KN2 and BALB/c-KN2 T-bet^{GFP} thymocytes (enriched for *i*NKT cells by depletion of CD8$^+$ and CD24$^+$ cells and sorted as NKT2 cells positive or negative for human CD2), analyzed 4 d after transfer. Each symbol (right) represents an individual mouse; small horizontal lines indicate the mean. *, $P < 0.01$; **, $P < 0.001$ (unpaired two-tailed *t*-test). Data are pooled from three independent experiments with two to six recipient mice (right).

to the age of the mice, as in older mice (10–14 weeks of age), the overall frequency and total number of *i*NKT cells was lower in *Tbx21*$^{-/-}$ mice than in wild-type mice, due to the much lower abundance of NKT1 cells but not of NKT2 cells or NKT17 cells (**Supplementary Fig. 3a**,**b**). Furthermore, that result was a cell-intrinsic effect, as in mixed–bone marrow chimeras, *Tbx21*$^{-/-}$ bone marrow cells generated more NKT2 cells and NKT17 cells and fewer NKT1 cells than did wild-type bone marrow cells regardless of donor cell frequencies (**Supplementary Fig. 3c**,**d**). Collectively, these findings suggested that T-bet directed the dominance of NKT1 cells in B6 mice and that T-bet deficiency redirected cell fates to facilitate the development of NKT2 cells and NKT17 cells.

To obtain evidence of developmental exposure to IL-4, we examined CD8$^+$ SP thymocytes. Up to 30% of CD8$^+$ SP thymocytes in *Tbx21*$^{-/-}$ B6 mice had abundant expression of Eomes, the hallmark of the development of IL-4-dependent memory-like CD8$^+$ T cells (**Fig. 4d** and **Supplementary Fig. 3e**). The source of IL-4 in T-bet-deficient mice was *i*NKT cells, as mice doubly deficient in T-bet and the antigen-presenting molecule CD1d did not generate any Eomes$^+$ CD8$^+$ SP thymocytes (**Supplementary Fig. 3f**,**g**). This analysis of T-bet-deficient mice demonstrated that steady-state IL-4 did not require NK1.1$^+$ NKT1 cells; instead, it was enhanced by a compensatory greater abundance of NKT2 cells in such mutant mice.

Since mice deficient in the transcription factor KLF2 have a greater abundance of *i*NKT cells and memory-like CD8$^+$ T cells[3], we examined *i*NKT cell subsets in such mice. We found that KLF2 deficiency resulted in tenfold more NKT2 cells in B6 mice than in their wild-type B6 counterparts, but did not result in more NKT1 cells or NKT17 cells (**Supplementary Fig. 4**). KLF2-deficient mice had substantially more memory-like CD8$^+$ T cells than did their wild-type counterparts (**Supplementary Fig. 4**), as reported before[23]; this provided further evidence that NKT2 cells were the source of IL-4 produced in the steady state. This genetic evidence, together with the data obtained by intrathymic injection, supported a lineage-diversification model of *i*NKT cells in which a common progenitor gives rise to the NKT1, NKT2 and NKT17 lineages, instead of the linear conversion of IL-4-producing *i*NKT cells into IFN-γ-secreting cells (**Supplementary Fig. 5**).

Diversification of *i*NKT cell lineages in various inbred strains

Having confirmed that *i*NKT lineage diversification happened differently in B6 mice and BALB/c mice (**Fig. 2d**), we sought to determine which pattern was representative among inbred mouse strains. We compared six different commonly used inbred strains of mice (**Fig. 5**). Mice of a similar age showed variability in the frequency (**Fig. 5a**,**b**) and number (data not shown) of total thymic *i*NKT cells. Using intracellular straining of transcription factors, we determined the relative proportion of NKT1, NKT2 and NKT17 cells in each. B6 mice were at one end of the spectrum, with more NKT1 cells and fewer NKT2 cells, whereas BALB/c were at the other end of the spectrum. The six strains showed a general inverse relationship in the proportion of NKT1 cells versus NKT2 cells (**Fig. 5b**). Notably, three strains (CBA, DBA/2 and BALB/c) had a substantial population of memory-like CD8$^+$ thymocytes that expressed Eomes (**Fig. 5a**,**c**). Indeed, there was a positive correlation between strains with a high frequency of NKT2 cells and those with Eomes-expressing memory-like CD8$^+$ thymocytes (**Fig. 5d**; $R^2 = 0.49$). Because IL-4 production by NKT2 cells was important for the development of memory-like CD8$^+$ thymocytes in BALB/c mice, we sorted NKT2 cells from all strains and measured *Il4* expression by RT-PCR. The abundance of IL-4 mRNA in NKT2 cells correlated even more very tightly with the number of memory-like CD8$^+$ thymocytes in the inbred strains of mice (**Fig. 5d**; $R^2 = 0.83$), which suggested the applicability of a universal mechanism of IL-4-dependent induction of memory-like CD8$^+$ thymocytes regardless of genetic background.

Stimulation of IL-4-producing NKT2 cells by self ligands

In helper T cells, IL-4 production is stimulated via the antigen receptor[16]. However, it has been shown that in various innate lymphoid cells, IL-4 can be stimulated by cytokines such as IL-25 and IL-33 (ref. 24). To understand what type of signals were stimulating IL-4 in NKT2 cells, we studied Nur77^{GFP} mice, which have transgenic expression of a Nur77-GFP reporter that is driven by signaling via the antigen receptor and is independent of cytokine or inflammatory signals[25]. In these mice, expression of Nur77-GFP was rapidly upregulated in peripheral *i*NKT cells after injection with α-galactosylceramide or with the putative self lipids β-glucosylceramide (**Fig. 6a**) or isoglobotrihexosylceramide (data not shown). Through the use of Nur77^{GFP} mice, we sought to compare the perception of signaling via the T cell antigen receptor (TCR) by NKT2 cells with that of other subsets, hypothesizing that a difference in the presentation of self lipid antigen might regulate IL-4 secretion in the steady state. We fully backcrossed B6 Nur77^{GFP} mice onto the BALB/c background (to generate BALB/c Nur77^{GFP} mice) and compared the GFP expression of *i*NKT cells in B6 mice with that of BALB/c mice. Notably,

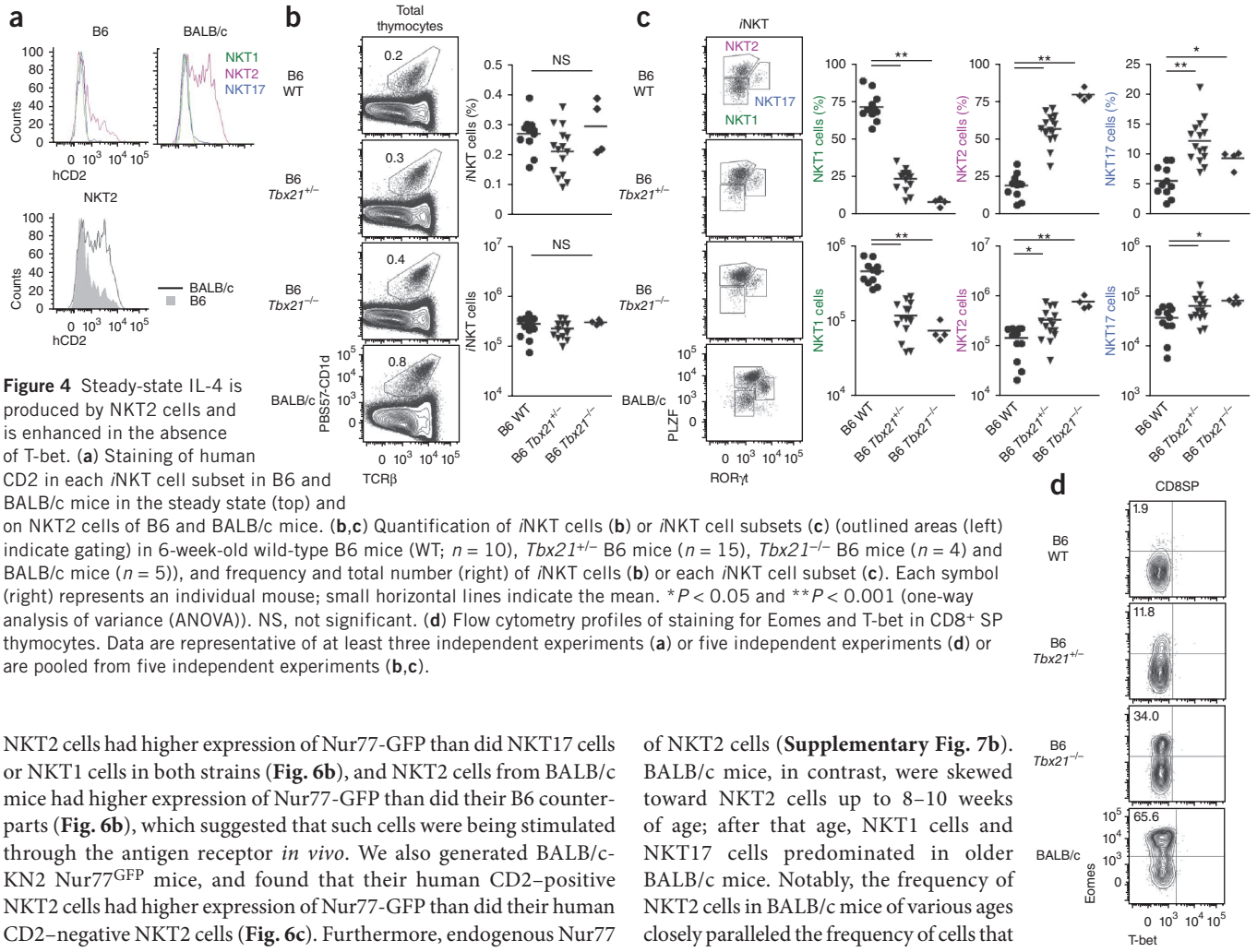

Figure 4 Steady-state IL-4 is produced by NKT2 cells and is enhanced in the absence of T-bet. (**a**) Staining of human CD2 in each *i*NKT cell subset in B6 and BALB/c mice in the steady state (top) and on NKT2 cells of B6 and BALB/c mice. (**b**,**c**) Quantification of *i*NKT cells (**b**) or *i*NKT cell subsets (**c**) (outlined areas (left) indicate gating) in 6-week-old wild-type B6 mice (WT; $n = 10$), *Tbx21*$^{+/-}$ B6 mice ($n = 15$), *Tbx21*$^{-/-}$ B6 mice ($n = 4$) and BALB/c mice ($n = 5$)), and frequency and total number (right) of *i*NKT cells (**b**) or each *i*NKT cell subset (**c**). Each symbol (right) represents an individual mouse; small horizontal lines indicate the mean. $^{*}P < 0.05$ and $^{**}P < 0.001$ (one-way analysis of variance (ANOVA)). NS, not significant. (**d**) Flow cytometry profiles of staining for Eomes and T-bet in CD8^{+} SP thymocytes. Data are representative of at least three independent experiments (**a**) or five independent experiments (**d**) or are pooled from five independent experiments (**b**,**c**).

NKT2 cells had higher expression of Nur77-GFP than did NKT17 cells or NKT1 cells in both strains (**Fig. 6b**), and NKT2 cells from BALB/c mice had higher expression of Nur77-GFP than did their B6 counterparts (**Fig. 6b**), which suggested that such cells were being stimulated through the antigen receptor *in vivo*. We also generated BALB/c-KN2 Nur77^{GFP} mice, and found that their human CD2–positive NKT2 cells had higher expression of Nur77-GFP than did their human CD2–negative NKT2 cells (**Fig. 6c**). Furthermore, endogenous Nur77 expression correlated well with IL-4 expression in sorted NKT2 cells from various inbred strains (**Fig. 6d**), consistent with the idea that stimulation of *i*NKT cells through the TCR drives IL-4 production.

To further determine if signaling via the antigen receptor in the steady state was important for IL-4 expression by NKT2 cells, we intrathymically transferred thymic KN2 *i*NKT cells into wild-type or CD1d-deficient hosts (**Supplementary Fig. 6a**). Consistent with the data obtained with Nur77^{GFP} mice, we observed a lower mean fluorescence intensity for human CD2 on NKT2 cells (**Fig. 6e**), as well as a lower frequency of human CD2–positive NKT2 cells, in CD1d-deficient hosts than in wild-type hosts 6 d after transfer (**Supplementary Fig. 6a**). We also found a significantly altered frequency of cells expressing β-chain variable region 2 ($V_{\beta}2$) and β-chain variable region 7 ($V_{\beta}7$) of the TCR in the NKT2 population, relative to that of the NKT1 or NKT17 population, in both B6 mice and BALB/c mice (**Fig. 6f** and **Supplementary Fig. 6b**), which indicated a distinct TCR repertoire for NKT2 cells. Collectively, these results indicated an association of IL-4 production with TCR stimulation.

If self or commensal lipid ligands were driving the activation and/or differentiation of NKT2 cells in the steady state, it is possible that this might vary with age. Thus, we examined *i*NKT cell subsets and IL-4 production in B6 mice and BALB/c mice of various ages. At all ages, BALB/c mice produced more intrathymic *i*NKT cells than did B6 mice (**Supplementary Fig. 7a**). Consistent with published reports[20], B6 mice were skewed toward NKT1 cells at all ages, with the exception of very young (3-week-old) mice, which had a modest proportion (30–35%) of NKT2 cells (**Supplementary Fig. 7b**). BALB/c mice, in contrast, were skewed toward NKT2 cells up to 8–10 weeks of age; after that age, NKT1 cells and NKT17 cells predominated in older BALB/c mice. Notably, the frequency of NKT2 cells in BALB/c mice of various ages closely paralleled the frequency of cells that produced IL-4 (positive for human CD2) and the development of memory-like CD8^{+} T cells (**Supplementary Fig. 7c**). Although we do not know which molecular factors drive such age-dependent differences, this does suggest the potential for environmental influences on *i*NKT cell differentiation.

Steady-state IL-4 influences CD8^{+} T cells, B cells and thymic DCs

We speculated that if *i*NKT cells provide sufficient IL-4 for the generation of memory-like CD8^{+} T cells in some strains of mice, it might have other systemic effects. IL-4 stimulates B cells to secrete IgE[26], and BALB/c mice have higher concentrations of IgE in serum than do B6 mice[27]. We confirmed that BALB/c mice had more than ten times higher concentrations of IgE in serum than did their age-matched B6 counterparts (**Fig. 7a**). That difference was attenuated in CD1d-deficient BALB/c mice, which suggested that at least some of the IL-4 that stimulated B cells to secrete IgE was generated by *i*NKT cells. *Tbx21*$^{+/-}$ B6 mice had significantly higher concentrations of IgE in serum than did wild-type B6 mice, which was further compatible with increased production of IL-4 by NKT2 cells.

It has also been shown that IL-4 can cause DCs to secrete the 'T_H2' chemokines CCL17 and CCL22 (ref. 28). On the basis of that finding, we investigated the expression of CCL17 and CCL22 in thymic DCs. We purified the two main thymic DC subtypes, CD8α^{+} and SIRPα^{+}, and analyzed CCL17 and CCL22 by RT-PCR. Those chemokines, which were produced mainly by the SIRPα^{+} DCs, as reported before[29], had more abundant expression in BALB/c mice than in B6 mice (**Fig. 7b**).

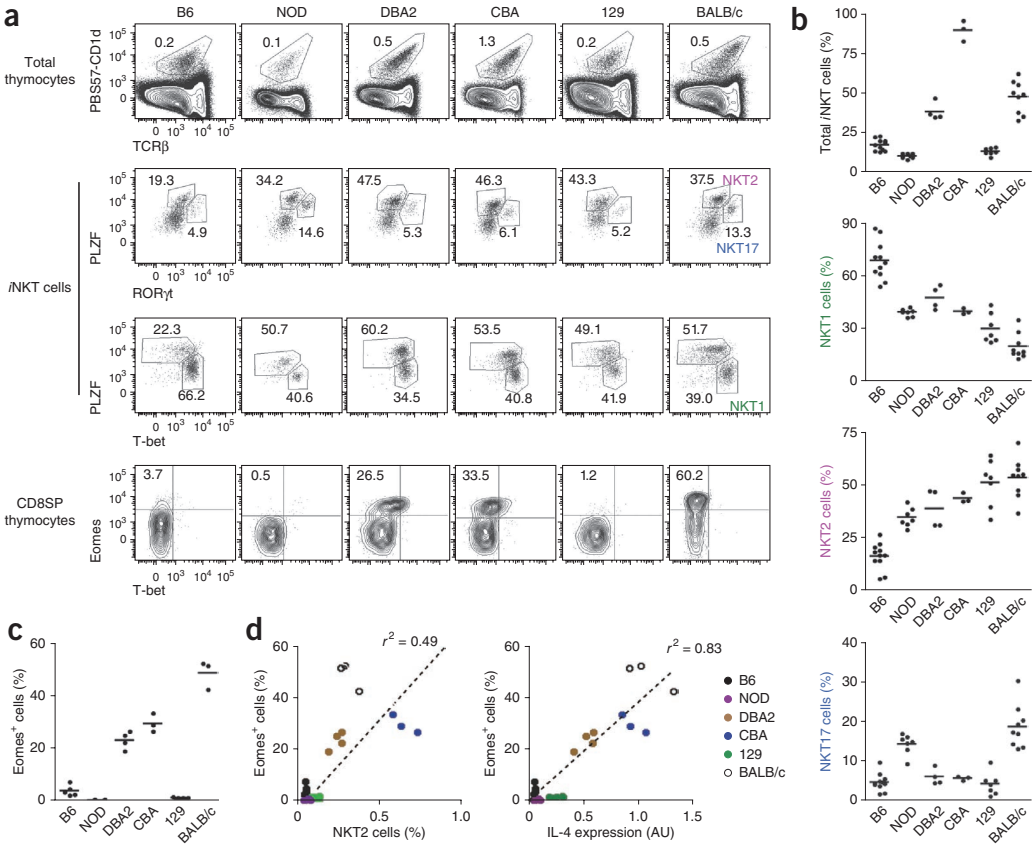

Figure 5 Interstrain comparison of *i*NKT cell subsets; production of IL-4 by NKT2 cells is associated with memory-like CD8$^+$ T cells. (**a**) Flow cytometry profiles of total thymocytes, thymic *i*NKT cells and CD8$^+$ SP thymocytes in 7- to 8-week-old mice of various strains (above plots). (**b**) Frequency of *i*NKT cells among total thymocytes (top) and of each subset (NKT1, NKT2 and NKT17) among total thymic *i*NKT cells (middle and bottom) in mice of the strains in **a**. (**c**) Frequency of thymic CD8$^+$ SP thymocytes that express Eomes, detected by intracellular staining. (**d**) Correlation of Eomes expression in CD8$^+$ SP thymocytes with the frequency of NKT2 cells among total thymocytes (left) or abundance of IL-4 mRNA in NKT2 cells (right); mRNA presented in arbitrary units relative to the abundance in sorted NKT2 cells, normalized to that of GAPDH and multiplied by the frequency of NKT2 cells among total thymocytes. Linear regression was used to calculate goodness of fit (r^2). Each symbol (**b**–**d**) represents an individual mouse; small horizontal lines indicate the mean. Data are pooled from seven independent experiments with five to eleven B6 mice, three to nine BALB/c mice, seven 129 mice, seven NOD mice, four DBA2 mice and three CBA mice.

Notably, DCs from *Cd1d*$^{-/-}$ BALB/c mice had lower expression of CCL17 and CCL22 than did their wild-type BALB/c counterparts, similar to that of their B6 counterparts, and IL-4 deficiency abrogated the expression of CCL17 and CCL22 expression entirely. Collectively, these results showed that IL-4 produced by *i*NKT cells not only influenced the generation of memory CD8$^+$ T cells but also

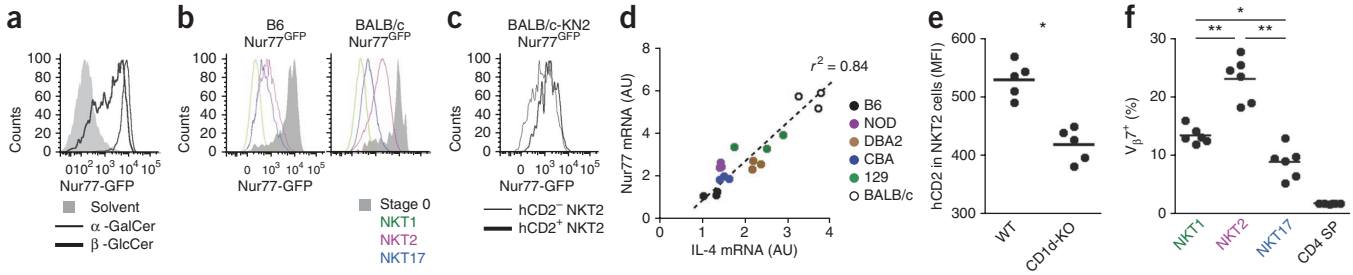

Figure 6 IL-4-producing *i*NKT cells are stimulated by self ligands. (**a**) Expression of Nur77-GFP by splenic *i*NKT cells from B6 Nur77^{GFP} mice given injection of bone marrow–derived DCs pulsed with solvent, α-galactosylceramide (α-GalCer) or β-glucosylceramide (β-GlcCer), analyzed 16 h after DC transfer. (**b**) Expression of Nur77-GFP by *i*NKT cells at stage 0 (CD24^{hi} cells) or in each *i*NKT cell subset from B6 and BALB/c Nur77^{GFP} mice. (**c**) Expression of Nur77-GFP in human CD2–positive and human CD2–negative NKT2 cells from BALB/c-KN2 Nur77^{GFP} mice. (**d**) Gene expression of Nur77 mRNA versus that of IL-4 mRNA in sorted NKT2 cells, presented in arbitrary units normalized to that of GAPDH (r^2, as in **Fig. 5b**). (**e**) Expression of human CD2 in NKT2 cells obtained from BALB/c wild-type host mice (WT; $n = 5$) or BALB/c *Cd1d*$^{-/-}$ host mice (CD1d-KO; $n = 5$) 6 d after injection of thymic *i*NKT cells enriched from BALB/c-KN2 mice. MFI, mean fluorescence intensity. $^*P = 0.0004$, wild-type versus *Cd1d*$^{-/-}$ (unpaired two-tailed *t*-test). (**f**) Use of the $V_\beta7$ TCR by NKT1, NKT2 and NKT17 cells among total thymic *i*NKT cells from B6 mice ($n = 6$). $^*P < 0.05$ and $^{**}P < 0.001$ (one-way ANOVA). Each symbol (**d**–**f**) represents an individual mouse; small horizontal lines indicate the mean. Data are representative of two independent experiments with four mice per group (**a**), three independent experiments (**b**,**c**), or two independent experiments with three mice per group (**d**–**f**).

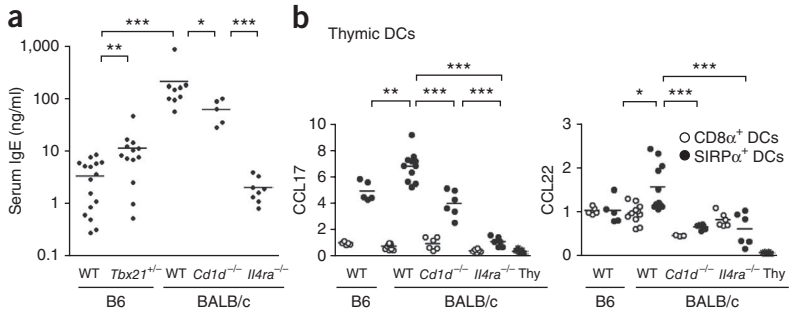

Figure 7 Steady-state IL-4 produced by *i*NKT cells influences B cells and thymic DCs. (**a**) Enzyme-linked immunosorbent assay of IgE in serum from wild-type B6 mice ($n = 16$), *Tbx21*$^{+/-}$ B6 mice ($n = 13$), wild-type BALB/c mice ($n = 9$), *Cd1d*$^{-/-}$ BALB/c mice ($n = 5$) and BALB/c mice deficient in the IL-4 receptor α-chain (*Il4ra*$^{-/-}$; $n = 8$). (**b**) Real-time RT-PCR analysis of the expression of CCL17 and CCL22 in sorted thymic CD8α$^+$ or SIRPα$^+$ DCs from mice of various strains (below plot) and in thymocytes (Thy) from wild-type BALB/c mice (far right); results are normalized to those of GAPDH. Each symbol represents an individual mouse; small horizontal lines indicate the mean. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (unpaired two-tailed *t*-test (**a**) or one-way ANOVA (**b**)). Data are pooled from three independent experiments (**a**) or four independent experiments with six to twelve mice per group (**b**).

influenced B cells and thymic DCs and possibly contributed to the T_H2 dominance in BALB/c mice.

DISCUSSION

Until recently, the NKT cell field had embraced a sequential lineage developmental model[20] in which 'developmental intermediates' produce T_H2-type cytokines and 'mature' NK1.1$^+$ *i*NKT cells produce T_H1 cytokines. However, on the basis of our findings of the expression of distinct transcription factors in *i*NKT cell subsets, we considered an alternative 'lineage diversification' model for *i*NKT cells[9], analogous to the differentiation of effector helper T cells[16] and innate lymphoid cells[30]. In support of such a model, we observed that *i*NKT cell subsets existed in different ratios in different inbred strains of mice, that NKT2 cells and NKT1 cells lacked a precursor-progeny relationship and that T-bet deficiency blocked the development of NKT1 cells while increasing the abundance of NKT17 cells and NKT2 cells. In a fashion complementary to T-bet deficiency, GATA-3 deficiency is reported to block differentiation of the NK1.1$^-$CD4$^+$ (NKT2) cell or NK1.1$^-$CD4$^-$ (NKT17) cell lineage while allowing NK1.1$^+$ (NKT1) cells to mature[31]. Such data support a model in which the interplay of T-bet, GATA-3 and RORγt regulates the differentiation of NKT1 cells, NKT2 cells and NKT17 cells from a PLZF^{hi} common progenitor, rather than a linear maturation process. Given the analogous model of the differentiation of helper T cells[16], we also sought an *i*NKT cell subset that expressed the transcriptional repressor Bcl-6, which, together with the chemokine receptor CXCR5 and the costimulatory molecule PD-1, is a hallmark of follicular helper T cells. Cells with that phenotype have been described among peripheral *i*NKT cells from immunized mice[32]. However, we did not find substantial numbers of such cells in the thymus of young adult B6 mice or BALB/c mice in the steady state (data not shown).

We found here that in several strains of mice, NKT2 cells produced an IL-4-rich environment and resulted in the development of large numbers of innate CD8$^+$ T cells, among other IL-4-mediated effects. Furthermore, our data suggested that NKT2 cells continually perceived stimulation through their TCR. Many self lipids have been proposed as endogenous ligands for *i*NKT cells[33–35], and it is possible that distinct lipids could be presented by distinct thymic antigen-presenting cells. The finding that mice with an inability to produce ether lipids have a 'preferential' defect in NK1.1$^+$ *i*NKT (NKT1) cells[34] supports the idea that distinct endogenous lipids influence the positive selection and differentiation of *i*NKT cells. We observed differences in the $V_β$ repertoire of NKT2 cells and that of NKT1 cells or NKT17 cells, which further supported the idea that distinct *i*NKT cell subsets may be stimulated by different TCR ligands.

Nonetheless, certain cytokines can also cause cells to produce IL-4. A published report has shown that CD4$^+$ NK1.1$^-$ *i*NKT cells (similar to our NKT2 cells) express IL-17RB, a receptor for IL-25 (ref. 36). IL-25 is a cytokine produced by stromal cells that can promote the T_H2 immune response[24]. Indeed, we found human CD2–positive (IL-4-producing) cells in the IL-17RB$^+$ population both in B6 mice and in BALB/c mice. IL-17RB$^+$ *i*NKT cells produce IL-4 when exposed to exogenous IL-25, and deficiency in the receptor for IL-25 partially prevents the development of NK1.1$^-$ *i*NKT cells[18]. However, B6 NKT2 cells produced less IL-4 than did their BALB/c counterparts despite similar expression of IL-17RB by both, and B6 NKT17 cells also expressed IL-17RB, which indicated that IL-17RB was not sufficient to drive IL-4 secretion. It remains to be determined if a combination of TCR stimulation provided by self ligands and stromal cells secreting IL-25 might determine either the frequency of NKT2 cells or their ability to secrete IL-4 in BALB/c mice.

The relative abundance of NKT2 cells and NKT17 cells in BALB/c mice raises questions about whether skewing of NKT cell subsets is due to defective generation of NKT1 cells or enhanced generation of NKT2 cells and NKT17 cells. The absolute number of NKT1 cells in the thymus was not different in B6 mice versus BALB/c mice, whereas the number of NKT2 cells and NKT17 cells was higher in BALB/c mice, which suggested that it may be the NKT2 and NKT17 lineages that are regulated differently in BALB/c mice versus B6 mice. BALB/c mice that lack the transcription factor KLF13 do not have memory-like CD8$^+$ T cells[7]. That suggests that KLF13 may be important for the differentiation of NKT2 cells. Nonetheless, the amount of KLF13 itself does not differ in B6 mice versus BALB/c mice[7], so the contributing genetic differences of these two strains remain undefined. Among six different inbred strains, we generally observed a reciprocal relationship between NKT1 cells and NKT2 cells, but not necessarily between NKT1 and NKT17 cells. Notably, B6 mice were much more highly 'NKT1 cell skewed' than were other strains. As NK1.1 was expressed only on NKT1 cells, NK1.1 expression identified a substantial fraction of *i*NKT cells in B6 mice, but this was not the case in other inbred strains.

Our studies also identified a substantial age dependence for the skewing of *i*NKT cell subsets, with B6 mice having 35% NKT2 cells at 3 weeks of age, which dropped to less than 5% by adulthood, while BALB/c mice had up to 50% NKT2 cells, which also dropped to less than 5%, but not until 18 weeks of age. We do not understand the factors that caused this. One possibility, suggested by a published study, relates to Lin28b, which regulates the Let7 family of microRNAs and directs fetal-like lymphopoiesis[37]. While we found modestly prolonged expression of Lin28b in the thymus of neonatal BALB/c mice

relative to its expression in that of B6 mice (data not shown), BALB/c chimeras generated with fetal precursor cells with high expression of Lin28b generated approximately the same ratio of NKT1, NKT2 and NKT17 lineages as did chimeras generated with adult precursor cells with low expression of Lin28b (data not shown), which ruled out the possibility that NKT cell subsets were skewed by intrinsic differences in fetal and adult progenitor cells. Another possibility is that the thymic microenvironment of BALB/c mice supports the differentiation of precursors of *i*NKT cells into the NKT2 and NKT17 lineages, possibly owing to differences in lipid antigen, the expression of signaling lymphocytic-activation molecule on antigen-presenting cells or the production of inductive cytokines; some of these factors may differ with age.

B6 mice and BALB/c mice are the prototypes of strains with dominating T_H1 responses and T_H2 responses, respectively. In a mouse model of human asthma, ovalbumin-specific T_H2 responses induce airway hyper-responsiveness and lung inflammation in BALB/c mice[38]. As T-bet-deficient B6 mice become susceptible to the induction of airway hyper-responsiveness[39] and BALB/c mice with a dominant-negative form of GATA-3 are resistant to such induction[40], it has been suggested that the T_H2 skewing of conventional $CD4^+$ T cells is responsible for the strain specificity of disease induction. However, CD1d-deficient BALB/c mice are resistant to asthma induction[41], and deficiency in T-bet or GATA-3 facilitates the development of NKT2 cells or NKT1 cells, respectively[31] (as reported here). Moreover, T-bet-deficient BALB/c mice still require *i*NKT cells for the induction of airway hyper-responsiveness[42]. Therefore, NKT2 cells may have a role in the strain differences of mouse models of asthma.

*i*NKT cells influence the outcome of pathogenic infection in various ways. They can recognize pathogens both directly and indirectly, and the cytokines they produce can subsequently skew adaptive immune responses[43]. Some evidence suggests that *i*NKT cells can secrete IL-4 to enhance $CD8^+$ T cell responses. In a model of infection with respiratory syncytial virus, *i*NKT cells have been shown to activate virus-specific $CD8^+$ T cells and CD1d-deficient BALB/c mice have been shown to develop a less-severe response at the expense of delayed viral clearance[44]. In a model of malarial infection, BALB/c mice have been shown to be more resistant than B6 mice, with the protective response dependent on receptors for IL-4 on $CD8^+$ T cells[45]. Furthermore, IL-4-deficient or CD1d-deficient BALB/c mice are more susceptible to parasite infection and develop severe pathology[46]. Such findings suggest that *i*NKT cells provide IL-4 required for proper activation of $CD8^+$ T cells after infection with malarial parasites. However, it is not clear whether IL-4 and *i*NKT cells act during the infection or developmentally (i.e., by inducing $Eomes^+$ memory-like $CD8^+$ T cells). More sophisticated experimental systems are needed to properly address these issues.

In this report, we have provided a new transcription factor–based classification of *i*NKT cells that may be useful in various strains of mice and that correlates closely with function. We suggest that our lineage-diversification model, while possibly oversimplified, is more appropriate for understanding *i*NKT cell biology than is a linear-stages model. Unexpectedly, B6 mice showed one extreme of *i*NKT cell development, with NKT1 cells dominating. We found that NKT2 cells dominated in many other inbred strains of mice, and the consequence of this was greater basal production of IL-4, with broad effects on immune-response potential.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.J.L. designed and did experiments, analyzed data and wrote the manuscript; K.L.H. did experiments and provided input for interpretation; J.Z. provided reagents and research interpretation; S.C.J. provided input for research design and interpretation; and K.A.H. conceptualized the research, directed the study, analyzed data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. B6 (C57BL/6NCr) and B6.SJL (B6-LY5.2/Cr) mice were from the National Cancer Institute. Other mouse strains were from Jax: *Tbx21*$^{-/-}$ B6 (B6.129S6-*Tbx21tm1Glm*/J), *Cd1d*$^{-/-}$ B6 (B6.129S6-Cd1d1/Cd1d2tm1Spb/J), 129 (129X1/SvJ), DBA2 (DBA/2J), CBA (CBA/J), NOD (NOD/ShiLtJ), BALB/c (BALB/cByJ or BALB/cJ), *Il4ra*$^{-/-}$ BALB/c (BALB/c-*Il4ratm1Sz*/J) and *Cd1d*$^{-/-}$ BALB/cJ (C.129S2-*Cd1tm1Gru*/J). *Tbx21*$^{+/-}$*Cd1d*$^{-/-}$ CB6 mice were generated by the crossing of *Tbx21*$^{+/-}$*Cd1d*$^{+/-}$ B6 mice and *Cd1d*$^{-/-}$ BALB/cJ mice. KN2 mice on the B6 and BALB/c background were obtained from M. Mohrs (Trudeau Institute). Nur77^{GFP} C57BL/6 mice were generated before[25] and were backcrossed onto BALB/cByJ mice. Mice with a ZsGreen (GFP) reporter for T-bet expression (T-bet–GFP) mice have been described[21] and were backcrossed onto a BALB/cByJ background. All the mice experiments were done according to protocols approved by the Institutional Animal Care and Use Committee of the University of Minnesota and the NIAID Animal Care and Use Committee.

Flow cytometry and antibodies. Single-cell suspensions were prepared from thymi and spleens, and hepatic mononuclear cells were separated by Percoll gradient centrifugation. CD1d monomers loaded with biotinylated PBS57 or unloaded monomers were obtained from the tetramer facility of the US National Institutes of Health. For staining of human CD2 and PLZF, surfaces of cells were stained with antibody to human CD2 (S5.2, mouse IgG2a; BD Biosciences) and fixed with a Foxp3 staining buffer set (eBioscience), and were incubated with antibody to PLZF (anti-PLZF; D-9, mouse IgG1; Santa Cruz), followed by staining with antibody to mouse IgG1 (A85-1 or X-56; BD Biosciences). T-bet (4B10, mouse IgG1; eBioscience) was stained after blockade of the free arm of antibody to mouse IgG1 with mouse serum or anti-PLZF. For some staining, Alexa Fluor 488–labeled anti-PLZF (Mags.21F7; a gift from D.B. Sant'Angelo) or phycoerythrin-conjugated anti-PLZF (Mags.21F7; eBioscience) was used. Antibody to mouse IL-17RB was from R&D Systems (752101, rat IgG1) or MBL (B5F6, rat IgG2a). The complete list of other antibodies used is as follows: anti-CD4 (GK1.5 or RM4-4), anti-CD24 (M1/69), anti-NK1.1 (PK136) and anti-Thy-1.2 (30-H12; all from Biolegend); CD8α (53-6.7), anti-IFN-γ (XMG1.2), anti-IL-17 (eBio17B7), anti-RORγt (AFKJS-9 or B2D), anti-Eomes (Dan11mag), anti-IRF4 (3E4), anti-GATA-3 (TWAJ), anti-CD11c (N418), antibody to major histocompatibility complex class II (M5/114.15.2), anti-CD3 (145-2C11), anti-B220 (RA3-6B2), anti-CD122 (TM-β1) and anti-CD27 (LG.7F9; all from eBioscience); anti-SIRPα (p84), anti-TCRβ (H57-597), anti–TCR $V_β$2 (B20.6), anti–TCR $V_β$7 (TR310), antibody to TCR $V_β$8.1 and $V_β$8.2 (MR5-2), anti–TCR $V_β$8.3 (1B3.3) anti-CD44 (IM7) and anti-CD49b (DX5; all from BD Biosciences). Cells were analyzed on an LSR II (Becton Dickinson) and data were processed with FlowJo software (TreeStar).

***In vitro* and *in vivo* cytokine production.** For *in vitro* stimulation with PMA and ionomycin, total thymocytes or thymocyte samples depleted of CD8$^+$ and CD24$^+$ cells by immunomagnetic selection were plated at a density of 1×10^6 cells per ml in RPMI medium plus 10% (vol/vol) FCS. Cells were incubated for 4 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (1.5 μM) with monensin (eBioscience) added for the final 2 h, and intracellular cytokines were analyzed by flow cytometry. For *in vivo* stimulation of *i*NKT cells, mice were injected intravenously with 5 μg α-galactosylceramide (KRN7000, Avanti Polar Lipids) and spleens and livers were analyzed 3 h later.

Mixed–bone marrow chimeras. Total bone marrow cells were prepared from the femurs and tibias of wild-type B6 (CD45.1$^+$CD45.1$^+$) or *Tbx21*$^{-/-}$ B6 (CD45.1$^+$CD45.2$^+$) donor mice, and samples were depleted of mature T cells with anti-Thy1.2 (30-H12; Biolegend) and complement. Recipient mice (CD45.2$^+$CD45.2$^+$) were lethally irradiated (1,000 rads) and received 1×10^7 adult bone marrow cells (samples depleted of T cells). Chimeras were analyzed 6 weeks after transplantation.

Intrathymic injection. Thymocyte samples from B6-KN2 or BALB/c-KN2 T-bet–GFP mice were depleted of CD8$^+$ and CD24$^+$ cells by immunomagnetic selection for enrichment of thymic *i*NKT cells. CD4$^+$T-bet–GFP$^-$ NKT2 cells positive and negative for human CD2 were sorted with a FACSAria. Host (B6 or BALB/c) mice were anesthetized by intraperitoneal injection of ketamine (90 mg per kg body weight) and xylazine (9 mg per kg body weight). Sorted cells were mixed with splenic CD8$^+$ T cells from B6 or BALB/c CD1d-deficient mice, labeled with CellTrace Violet (Invitrogen) and injected into thymi of B6 or BALB/c recipients. In some experiments, enriched *i*NKT cells were labeled with CellTrace Violet and injected into wild-type or CD1d-deficient BALB/c mice together with splenic CD8$^+$ T cells labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester; Sigma).

Cell sorting and real-time RT-PCR. Thymocyte samples were enriched for CD11c$^+$ cells by immunomagnetic selection, and CD8α$^+$ or SIRPα$^+$ DCs were purified with a FACSAria (Becton Dickinson) after gating CD11c$^+$MHCII$^+$ Thy-1.2$^-$CD3$^-$B220$^-$ cells. Cells with more than 95% purity of the target population were used for experiments. An RNeasy mini or micro kit (Qiagen) and SuperScript III First Strand Synthesis SuperMix for qRT-PCR (Invitrogen) were used for the isolation of RNA and production of cDNA. FastStart Universal SYBR Green Master (Roche) and an ABI PRISM 7000 sequence detection system (Applied Bioscience) were used for amplification and detection. Primers were as follows: GAPDH forward, 5′-TGGCCTACATGGCCTCCA-3′, and reverse, 5′-TCCCTAGGCCCCTCCTGTTAT-3′; IL-4 forward, 5′-GAGACTCTTTCGGGCTTTTC-3′, and reverse, 5′-TGATGCTCTTTAGGCTTTCCA-3′; and Nur77 forward, 5′-ATGCTTCGTGTCAGCACTAT-3′, and reverse, 5′-GTACTTGGCGCTTTTCTGTA-3′. Primers for CCL17 and CCL22 have been described[28].

Lipid-pulsed bone marrow DCs. Bone marrow cells from the femurs of mice were cultured for 7 d (at a density of 5×10^6 cells per well) in six-well cell culture dishes with complete RPMI medium in the presence of recombinant mouse granulocyte-macrophage colony-stimulating factor (50 ng/ml; PeproTech) and IL-4 (10 ng/ml; PeproTech). On day 6, cells were pulsed for 12–15 h with 100 ng/ml of α-galactosylceramide (KRN7000; Avanti Polar Lipids), 1 μg/ml of β-glucosylceramide (C24:1 glucosyl-(β)-ceramide (d18:1/24:1(15Z)); Avanti Polar Lipids) or 1 μl/ml of solvent (2:1, methanol/chloroform). Bone marrow DCs were injected intravenously into Nur77^{GFP} mice (0.5×10^6 to 1.0×10^6 cells per mouse), and endogenous splenic *i*NKT cells were analyzed 16 h later.

Enzyme-linked immunosorbent assay. A mouse IgE ELISA MAX kit (Biolegend) was used for quantification of IgE in serum. All samples were serially diluted three times for an optimal range of dilution.

Statistical analysis. Prism software (GraphPad) was used for statistical analysis. Unpaired or paired two-tailed *t*-tests, linear regression and one-way ANOVA were used for data analysis and the generation of *P* or r^2 values. Blinding, randomization and data exclusion were not used in this study. Data sets (in Prism format) are available on request.

Corrigendum: Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of *i*NKT cells

You Jeong Lee, Keli L Holzapfel, Jinfang Zhu, Stephen C Jameson & Kristin A Hogquist

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In the version of this article initially published, the upper graph of Figure 1b was incorrectly duplicated as the lower graph. The error has been corrected in the HTML and PDF versions of the article.