The Journal of Immunology

GATA-3 Regulates the Development and Function of Invariant NKT Cells

Peter J. Kim, Sung-Yun Pai, Manfred Brigl, Gurdyal S. Besra, Jenny Gumperz and I-Cheng Ho

J Immunol 2006; 177:6650-6659; ;
doi: 10.4049/jimmunol.177.10.6650
http://www.jimmunol.org/content/177/10/6650

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

GATA-3 Regulates the Development and Function of Invariant NKT Cells[1]

Peter J. Kim,*† Sung-Yun Pai,†‡ Manfred Brigl,*† Gurdyal S. Besra,§ Jenny Gumperz,¶ and I-Cheng Ho[2]*†

Although invariant NKT (iNKT) cells participate in many aspects of immune responses, the molecular mechanisms regulating their development, maturation, and activation are still poorly understood. GATA-3 is a T cell-specific transcription factor that is also expressed in iNKT cells. The critical role of GATA-3 in conventional $\alpha\beta$ T cells has been well documented, but whether GATA-3 also regulates the development and function of iNKT cells is unknown. In the present study, we report that deficiency of GATA-3 results in cell-intrinsic defects in the thymic development and peripheral maturation of murine iNKT cells. In addition, GATA-3 is also required for survival, activation, and effector functions of this unique population of T cells. Our data also reveal a previously unidentified peripheral maturation step that is GATA-3 dependent. *The Journal of Immunology,* **2006, 177: 6650–6659.**

Invariant NKT (iNKT)[3] cells constitute a small subset of T cells expressing Vα14-Jα18 (mouse) or Vα24-Jα18 (human) TCR, which recognize lipid Ags presented by CD1d (1). iNKT cells are descendents of CD4 and CD8 double-positive (DP) thymocytes (2, 3), but unlike conventional $\alpha\beta$ T cells, which are selected by MHC molecules expressed on the surface of thymic stromal cells, iNKT cells are selected by cortical DP thymocytes expressing CD1d (4, 5). The most immature iNKT cells, as defined by CD1d tetramer staining, are $CD44^{-}NK1.1^{-}$ cells, which mature through a transitional $CD44^{+}NK1.1^{-}$ stage to become $CD44^{+}NK1.1^{+}$ cells (6, 7). While the interactions between invariant TCR and CD1d are essential for iNKT development, other signaling pathways are also required. For example, the transcription factor, RelB, is required in thymic epithelial cells but not iNKT precursors for iNKT cell development (8, 9). The required interaction between thymic epithelium and iNKT precursors does not require CD1d because CD1d-deficient (CD1d knockout (KO)) $RelB^{+/+}$ thymic epithelial cells are capable of supporting the differentiation of $RelB^{-/-}$ iNKT cells.

iNKT cells can be found in various peripheral organs, including spleen, lymph node, and liver. In particular, >20% of mononuclear cells found in the liver are iNKT cells. Peripheral iNKT cells display a phenotype of activated memory T cells with high CD44 and CD69 expression (10) and are known to rapidly produce large amounts of cytokines, including IFN-γ, IL-4, and IL-13, upon activation (11). For example, significant levels of serum IL-4 and IFN-γ can be detected hours after i.v. injection of α-galactosylceramide (αGalCer), a phenomenon called "cytokine storm" (12, 13). The production of cytokines by iNKT cells has been demonstrated to play critical roles in the pathogenesis of various autoimmune, allergic, and infectious diseases (14–16). In addition to mounting a cytokine storm, iNKT cells can also influence the maturation of DC through CD40-CD40L interactions and mediate tumor rejection (17, 18).

Mature iNKT cells can be further divided into $CD4^{+}$ and double-negative (DN) subsets. Recent studies indicate that these two subsets of iNKT cells are functionally distinct. In human, both subsets of iNKT cells are capable of producing type 1 cytokines, whereas type 2 cytokines are mainly expressed by $CD4^{+}$ subset (11). In an animal model of tumor rejection, DN iNKT cells, particularly those obtained from the liver, are most potent in rejecting implanted tumors (19). Despite the functional distinction, it remains to be determined whether the differentiation of these two subsets of iNKT cells requires different genetic programs.

Deficiency of several transcription factors are known to cause a profound defect in iNKT cell development (7). For example, T-bet deficiency causes a block in iNKT cell development possibly through a CD122-dependent mechanism (20), and RelB decreases IL-15 production by thymic epithelial cells, which is required for iNKT cell to develop (9). For other transcription factors, however, the mechanism for impaired iNKT cell development is unclear. So far, deficiency of transcription factors involved in iNKT cell development have caused a near total block in their development and have thereby precluded analysis of their role in the survival, activation, and function of iNKT cells.

GATA-3 is a C2C2 type zinc finger transcription factor whose hemopoietic expression is limited to T cells and NK cells in adult mice (21, 22). We and others (22–24) have found that GATA-3 plays crucial roles in multiple stages of T cell development. In addition, GATA-3 is also essential for the differentiation of Th2 cells and can serve as a direct transcription factor for several Th2 cytokine genes (25–29). GATA-3 is expressed in iNKT cells (20,

*Division of Rheumatology, Immunology, and Allergy, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115; †Harvard Medical School, Boston, MA 02115; ‡Division of Hematology/Oncology, Children's Hospital Boston and Department of Pediatric Oncology, Dana-Farber Cancer Institute; §School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom; and ¶Department of Medical Microbiology and Immunology, University of Wisconsin-Madison Medical School, Madison, WI 53706

Received for publication April 18, 2006. Accepted for publication August 26, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was made possible by grants from the National Institutes of Health (Rheumatology Fellowship Training Grant (to P.J.K.), K08-AI050601 (to S.-Y.P.), and R01-AI054451 (to I.-C.H.)), American College of Rheumatology-Physician Scientist Development Award (to P.J.K.), Lister Institute-Jenner Research Fellowship (to G.S.B.), Medical Research Council (to G.S.B.), and The Wellcome Trust (to G.S.B.).

[2] Address correspondence and reprint requests to Dr. I-Cheng Ho, One Jimmy Fund Way, Smith Building, Room 524, Boston, MA 02115. E-mail address: iho@partners.org

[3] Abbreviations used in this paper: iNKT, invariant NKT; 7-AAD, 7-aminoactinomycin D; DN, double negative; DP, double positive; αGalCer, α-galactosylceramide; KO, knockout; MFI, mean fluorescence intensity; PKC, protein kinase C; qPCR, quantitative PCR; SP, single positive; WT, wild type.

0022-1767/06/$02.00

30), but no study has directly addressed the role of GATA-3 in iNKT cell development and function. In the present study, we report that deficiency of GATA-3 alters, but does not block, the thymic development of iNKT cells and that GATA-3-deficient mice have a selective deficiency of the $CD4^+$ subset. GATA-3 is also required for the peripheral maturation and survival of iNKT cells. The remaining GATA-3-deficient peripheral iNKT cells are not functionally normal and display profound defects in ligand-induced activation and cytokine production. Taken together, our data indicate that GATA-3 plays a critical and broad role in iNKT cells.

Materials and Methods

Mice

Generation of the GATA-3 conditional KO mouse has been described previously (23). The mice were backcrossed onto the C57BL/6 background for five generations and housed in specific-pathogen free conditions. Experiments were conducted in accordance with institutional guidelines for animal care at the Dana-Farber Cancer Institute under approved protocols. Mice were bred using a homozygous floxed *Gata3* (FF) by FF/CD4-cre (FF/cre) strategy with the intent to generate a 1:1 mix of littermate matched FF (equivalent to wild-type (WT)) and FF/CD4-cre (GATA-3KO) mice. All WT mice, unless indicated otherwise, are littermates of GATA-3KO mice. Experiments were conducted at 4–8 wk unless indicated. CD1d-null (CD1d KO) mice were donated by Dr. M. Grusby (Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA). C57BL/6 Thy1.1 congenic and RAG-1KO mice were obtained from The Jackson Laboratory.

FACS analysis

Single-cell suspensions from the thymus and spleen were washed in PBS with 1% FCS before fluorescent Ab staining. Liver suspensions were purified using a Percoll (Amersham Biosciences) gradient. Hepatic mononuclear cells were collected from the 80–53% interface, red cell lysed, and then stained for FACS analysis. All fluorescently labeled Abs were from BD Pharmingen, except biotinylated anti-IL-13 from R&D Systems. Unloaded and lipid (PBS-57)-loaded murine CD1d tetramer was provided by the National Institutes of Health Tetramer Facility. To identify thymic iNKT cells, thymocytes were stained with fluorochrome-conjugated anti-TCR-β and loaded and unloaded CD1d tetramer. To identify splenic and hepatic iNKT cells, fluorochrome-conjugated anti-CD19 (1D3) was also added to gate out B cells. Anti-CD1d (1B1), anti-CD4 (L3T4), anti-CD25 (PC61), anti-CD40L (MR1), anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-CD122 (TM-β1), anti-NK1.1 (PK136), and anti-Thy1.2 (30-H12) were also used (BD Pharmingen).

In vitro Th cell skewing

$CD4^+$ T cells were isolated and skewed as described previously (31). Cells were unstimulated or stimulated with phorbol myristate acetate (PMA) and ionomycin for 4 h before RNA extraction with TRIzol (Invitrogen Life Technologies).

Sorting and quantitative PCR (qPCR)

Thymocytes were extracellularly stained with lipid-loaded CD1d tetramer, CD4, and TCRβ and sorted on a MoFlo (DakoCytomation). Total RNA was extracted using TRIzol (Invitrogen Life Technologies) and then reverse transcribed using iScript (Bio-Rad). qPCR analysis was done on an Mx3005P cycler (Stratagene) using Brilliant SYBR Green PCR reagent (Stratagene). Cycling conditions were 95°C at 10 min, 40 cycles of 95°C at 15 s, and 60°C at 60 s. Primers for GATA-3 were 5′-AGAACCGGC CCCTTATCAA-3′ (sense) and 5′-AGTTCGCGCAGGATGTCC-3′ (antisense) (32); and for β-actin, 5′-GCTCTGGCTCCTAGCACCAT-3′ (sense) and 5′-GCCACCGATCCACACCGCGT-3′ (antisense) (23).

NKT hybridoma stimulation

Thymocytes from WT and GATA-3KO mice were purified using CD8 magnetic beads (Miltenyi Biotec) to enrich DP cells. CD1d KO thymocytes were used without enrichment. The purified thymocytes (5×10^4 cells) were then incubated in a 1:1 ratio with variant (14S.6 and 14S.15) or invariant (24.7 and 24.8) NKT hybridomas (33). Cells were incubated in a 96-well plate overnight, and supernatants were assayed for IL-2 using a sandwich ELISA (BD Pharmingen). Secondary detection was performed using streptavidin-conjugated alkaline phosphatase and *p*-nitrophenyl phosphate (Sigma-Aldrich).

Bone marrow transfer

Bone marrow cells were harvested from the femur, tibia, and humerus of GATA-3KO or C57BL/6 Thy1.1 congenic mice. Cells were depleted of RBC and washed twice with RPMI 1640. Recipient mice were gamma irradiated (single dose of 400 rad) and were infused with 4×10^6 cells by tail vein injection. Mice were analyzed 6 wk later.

Apoptosis assay

Thymocytes or splenocytes were first stained for iNKT cells as described above. The stained cells were washed twice with PBS and then resuspended in annexin V binding buffer. Cells were stained with annexin V-PE and 7-aminoactinomycin D per BD Pharmingen protocol and analyzed by FACS.

In vivo αGalCer-induced activation assays

Two micrograms of αGalCer or vehicle (DMSO) in 100 μl of PBS were injected into the tail vein. For serum cytokine determination, mice were euthanized and bled at 2 and 5 h. The blood was allowed to clot, and the serum was separated from the clot by centrifugation. IL-4 and IFN-γ were detected by sandwich ELISA using capture and biotinylated Abs from BD Pharmingen. For secondary stimulation of B and CD8 cells, splenocytes were collected 5 h after injection and stained with CD69, anti-CD8, and anti-B220. For intracellular cytokine staining, splenocytes were collected at 40 min after injection and cultured in T cell medium (RPMI 1640 with 10% FCS, HEPES, penicillin and streptomycin, pyruvate, nonessential amino acids, L-glutamine, and 2-ME). Monensin was added to a final concentration of 3 μM, and the cells were incubated for an additional hour. Cells were extracellularly stained with anti-TCRβ, anti-CD4, and CD1d-tetramer. After washing and fixing with 2% paraformaldehyde, cells were permeabilized with 0.1% saponin and stained with anti-IFN-γ, anti-IL4, or anti-IL13 Abs and analyzed by FACS. For determination of activation markers (CD25, CD40L, and CD69) and TCR down-regulation, splenocytes were collected 2 h after injection and stained with anti-TCRβ, anti-CD4, CD1d-tetramer, as well as anti-CD25, anti-CD40L, or anti-CD69 Abs.

In vitro PMA- and ionomycin-induced activation assays

For in vitro stimulation, splenocytes were stimulated and cultured in T cell medium. For intracellular cytokine staining, monensin was added after 1 h to a final concentration of 3 μM and allowed to incubate for an additional hour. The remainder of the assay is identical to the aforementioned protocol for in vivo protocol. For determination of activation markers, splenocytes were cultured for 2 h and stained identically to the in vivo protocol.

Results

Expression of GATA-3 in subsets of iNKT cells

GATA-3 has been reported to be expressed in iNKT cells (20, 30), but their subset specific expression and their levels relative to Th1 and Th2 cells have not been reported previously. To address these questions, we sorted WT thymic iNKT cells into $CD4^+$ and DN subsets and measured GATA-3 expression with qPCR. As shown in Fig. 1*a*, both subsets expressed comparable levels of GATA-3 and the levels were comparable to that of stimulated Th2 cells, which are known to express high levels of GATA-3.

GATA-3 is required for the development of $CD4^+$ iNKT cells and the survival of peripheral iNKT cells

To further investigate the role of GATA-3 in the development of iNKT cells, we compared the number and phenotype of iNKT cells between GATA-3KO mice and control littermates (WT). We found that GATA-3KO mice did have iNKT cells that stained specifically for lipid-loaded CD1d tetramer (Fig. 1*b*). The absolute number of iNKT cells in the thymus of GATA-3KO mice was very comparable to that of WT mice (Fig. 1*c*), although there was a 2-fold reduction in their surface TCR expression as measured by

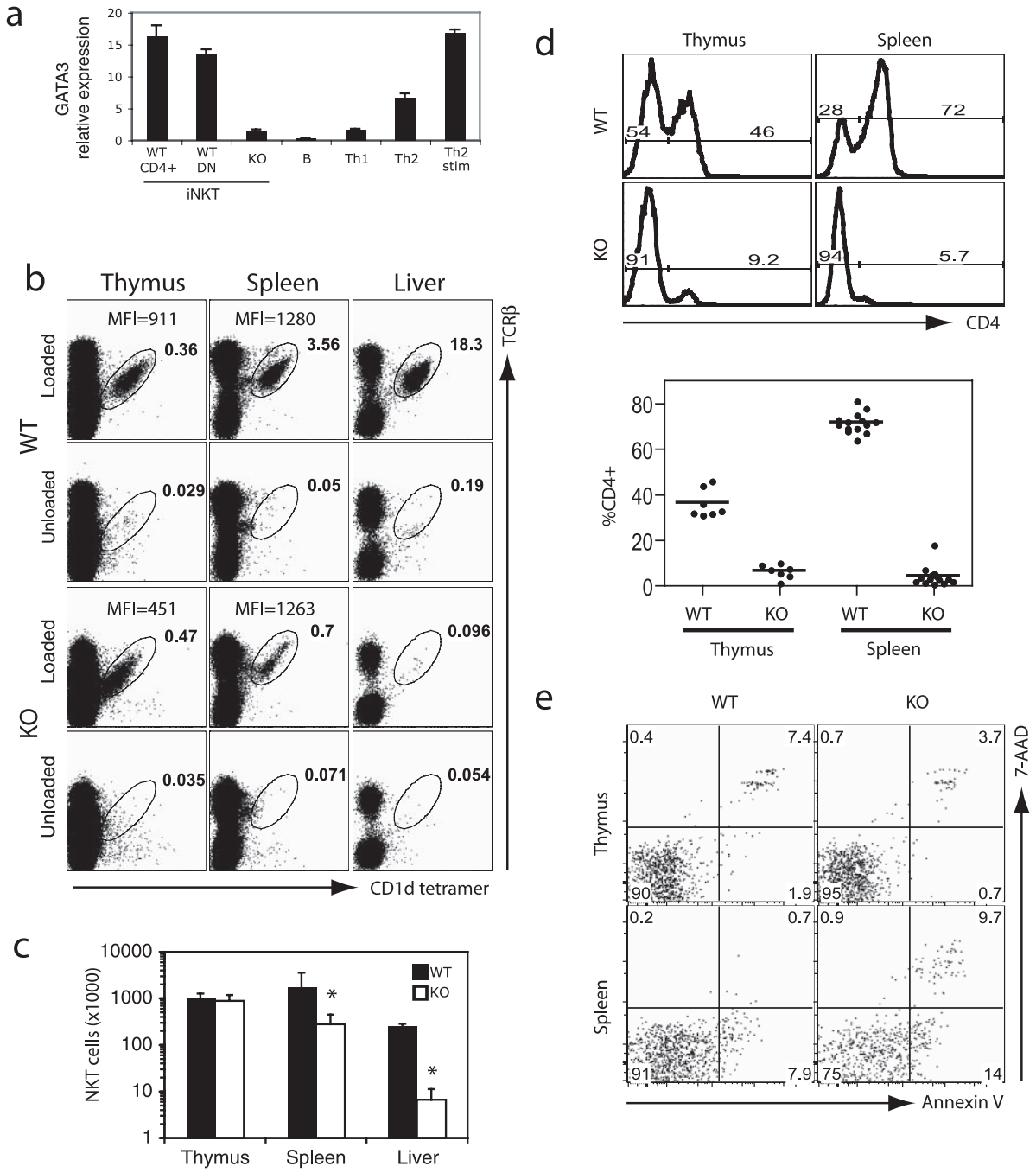

FIGURE 1. Characterization of iNKT cells in GATA-3KO mice. *a*, qPCR quantitation of GATA-3 transcript in indicated cell populations. WT iNKT cells were separated into $CD4^+$ and DN subsets. GATA-3KO iNKT cells (KO) were not further separated. Results are plotted relative to β-actin expression. WT B cells (B) were purified from whole splenocytes using B220 magnetic beads (Miltenyi Biotec). WT Th1 and Th2 cells were generated as described in *Materials and Methods*. A fraction of Th2 cells (Th2 stim) were stimulated with PMA and ionomycin for 4 h before analysis. *b*, Cells from the indicated organs from WT and GATA-3KO mice were stained for TCRβ-allophycocyanin (H57-597) and CD1d tetramer-PE (either loaded with lipid or unloaded). Spleen and liver plots were gated on CD19-allophycocyanin-Cy7-negative cells. Numbers beside the oval gates indicate percentage of iNKT cells. MFI of TCRβ is shown for the iNKT population where indicated. Results are representative of six (thymus and spleen) or three (liver) experiments. *c*, Total iNKT cells were enumerated from six (thymus and spleen) or three (liver) experiments and are shown plotted on a logarithmic scale. The differences marked with an asterisk were statistically different with $p < 0.01$ using a paired, two-tailed Student's *t* test. *d*, Representative CD4-PerCP distributions of iNKT cells from the thymus and spleen are shown from WT and GATA-3KO mice. The percentage of $CD4^+$ iNKT cells from thymus and spleen from multiple experiments is shown below. Both comparisons between WT and GATA-3KO were statistically different with $p < 0.00002$ using a paired, two-tailed Student's *t* test. *e*, Annexin V and 7-AAD staining on WT and GATA-3KO iNKT cells from thymus and spleen. Results are representative of two independent experiments. In the thymus, cells were gated for TCR-FITC-positive, CD1d tetramer-allophycocyanin-positive cells. In the spleen, cells were additionally gated for CD19-allophycocyanin-Cy7-negative cells.

mean fluorescence intensity (MFI) when compared with WT iNKT cells (Fig. 1*b*). This is in contrast to the periphery where the number of iNKT cells was decreased (Fig. 1*c*), but their TCR expression was normal (Fig. 1*b*). iNKT cells were drastically reduced in the spleens and livers of GATA-3KO mice (Fig. 1*c*)—a 6-fold decrease in the spleen and almost complete absence (>40-fold

reduction) in the liver. The reduced number of peripheral GATA-3KO iNKT cells was also confirmed by qPCR measuring the level of Vα14-Jα18 transcripts (data not shown). Another striking phenotype of the GATA-3KO iNKT cells was the marked reduction in the $CD4^+$ subset (Fig. 1*d*). In the thymus, only 6.44 ± 2.99% of GATA-3 KO iNKT cells were $CD4^+$ vs 36 ± 6.2% in the WT ($n = 7$). This reduction was also seen in the splenic iNKT cells: 4.5 ± 4.3% vs 72.2 ± 4.4% for GATA-3KO and WT, respectively ($n = 14$). As Cre-mediated gene deletion is often incomplete, the residual GATA-3KO iNKT cells may represent cells that have escaped Cre-mediated deletion of the *Gata3* gene. This scenario is very unlikely because the level of GATA-3 transcript in the residual GATA-3KO iNKT cells was as low as that of B cells, which do not express GATA-3 (Fig. 1*a*). Thus, the GATA-3KO iNKT cells have likely undergone Cre-mediated deletion of *Gata3*.

The expansion of iNKT cells takes place mainly at the thymic $NK1.1^-$ stage (6) and mature thymic and peripheral iNKT cells proliferate rarely in naive mice (3, 6, 34, 35). Thus, the sharp drop in the number of iNKT cells in the peripheral organs of GATA-3KO mice implies a net loss, instead of lack of proliferation, of mature iNKT cells. In agreement with this notion, we found more GATA-3KO splenic iNKT cells than WT cells were undergoing apoptosis, as evidenced by positive staining with annexin V and 7-AAD (Fig. 1*e*). In contrast, the percentages of apoptotic cells were very comparable between WT and GATA-3KO thymic iNKT populations, suggesting that a large number of GATA-3KO iNKT cells die after exiting the thymus. These data indicate that increased death in the periphery contributes to the decreased number of iNKT cells seen in the GATA-3KO mice but does not exclude the possibility of defects in thymic egress or peripheral proliferation.

Deficiency of GATA-3 alters the thymic development of iNKT cells

To further characterize the development of iNKT cells in the absence of GATA-3, we examined the expression of several stage-dependent surface markers by iNKT cells obtained from mice at different ages (Fig. 2*a*). At 1 wk of age, there was a small population of cells that stained specifically with lipid-loaded CD1d-tetramer in the WT mouse. No such population could be seen in the

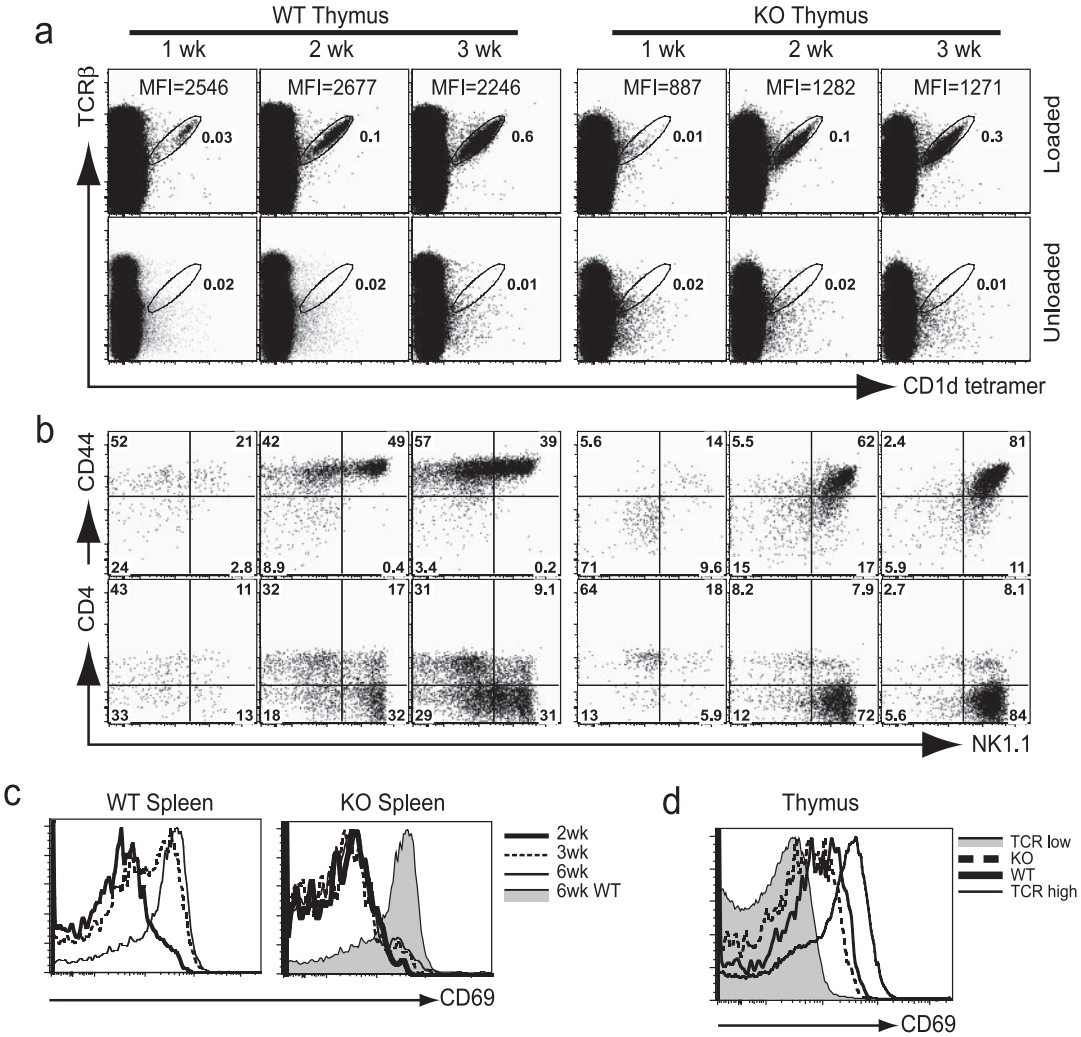

FIGURE 2. iNKT development in the GATA-3KO mouse is altered. *a*, Thymocytes from mice aged 1, 2, and 3 wk were stained with lipid loaded or unloaded CD1d tetramer. Numbers next to the gates represent percent iNKT cells. TCRβ MFI are shown above the iNKT gate. Cells were gated as in Fig. 1*b*. *b*, iNKT cells from *a* were analyzed for the maturation markers CD4-PerCP, CD44-allophycocyanin, and NK1.1-PE-Cy7. For this combination, TCR-FITC and CD1d tetramer-PE were used. *c*, iNKT cells from the spleens of 2-, 3-, and 6-wk-old mice were analyzed for CD69-PE-Cy7 expression. The shaded histogram in the right plot is the CD69 profile of splenic iNKT cells from 6-wk-old WT mice for comparison. *d*, CD69 expression on WT immature (TCR low) and mature (TCR high) thymocytes, WT, and GATA-3KO thymic iNKT cells from 3-wk-old mice. The results in Fig. 2 are representative of two independent experiments.

GATA-3KO mouse at the same age. At 2 and 3 wk of age, there were increasing numbers of iNKT cells that could be clearly distinguished from the background in both WT and GATA-3KO mice. As in the adult mice, the GATA-3KO thymic iNKT cells had lower surface TCR expression when compared with WT cells.

Immature iNKT cells undergo several well-defined developmental stages in the thymus. The most immature iNKT cells are $CD44^{-}NK1.1^{-}$ (stage 1), which differentiate through the $CD44^{+}NK1.1^{-}$ stage (stage 2) to become $CD44^{+}NK1.1^{+}$ (stage 3) cells. At 1 wk after birth, the earliest time point that we could convincingly identify thymic iNKT in WT mice, ~20, 50, and 20% of CD1d tetramer^{+} cells are stage 1, 2, and 3, respectively (Fig. 2*b*). The percentage of immature stage 1 cells gradually decreased as mice aged, and at 2 and 3 wk of age, 49 and 40% of iNKT cells had reached stage 3. At 1 wk of age, 54% of iNKT cells are $CD4^{+}$ and at 2 wk, this falls slightly to 49%. By 3 wk of age, 40% are $CD4^{+}$, approximating the average value for adult mice (36% from Fig. 1*d*).

The development of iNKT cells was altered greatly in the absence of GATA-3. The CD44/NK1.1 and CD4/NK1.1 plots are shown for the 1-wk-old GATA-3KO mice for completeness, but these cells likely represent background rather than true iNKT cells. At 2 wk of age, a significant portion (~17%) of GATA-3KO iNKT cells expressed NK1.1 before the up-regulation of CD44 (Fig. 2*b*), apparently bypassing stage 2. Despite the altered maturation program, 81% of GATA-3KO thymic iNKT cells eventually entered stage 3 by 3 wk of age. However, the percentage of thymic iNKT cells expressing the NK1.1 marker is still significantly higher (90 vs 40–50%) in GATA-3KO mice than in WT mice. Thus, deficiency of GATA-3 altered the maturation program rather than causing a block in the development of iNKT cells. Furthermore, the reduction in the $CD4^{+}$ subset was seen from the earliest times we could convincingly distinguish iNKT cells (2 wk).

GATA-3 is required for the peripheral maturation of iNKT cells

Splenic iNKT cells of WT mice at 1 and 2 wk after birth only expressed a medium level of CD69 (Fig. 2*c* only shows the 2-wk profile for clarity but the 1-wk profile was identical). At 3 wk, a bimodal distribution was seen with both intermediate and high populations, and by 6 wk of age, nearly all splenic iNKT cells expressed high levels CD69. In contrast, GATA-3KO splenic iNKT cells had an intermediate CD69 expression, which did not change with age. CD69 expression on WT thymic iNKT cells was intermediate when compared with the expression seen on immature (TCR low) and mature (TCR high) thymocytes (Fig. 2*d*). Furthermore, the levels were comparable between the WT and GATA-3KO thymic iNKT cells and did not change with age (data not shown). Our results indicate that iNKT cells, after exiting the thymus, undergo further maturation by up-regulating CD69 and that this peripheral maturation step is dependent on GATA-3.

Abnormal iNKT development in GATA-3KO animals is cell intrinsic

iNKT precursor cells originate from DP thymocytes and are selected by DP thymocytes in a CD1d-dependent manner (2–4). In addition, thymic epithelial cells also play a crucial role in the positive selection of iNKT cells (8, 9). As iNKT precursors and selectors both express GATA-3, the abnormal development of GATA-3KO iNKT could be due to a defect in either iNKT precursors or selectors. We first found that the level of CD1d in GATA-3KO thymocytes was comparable to that of control thymocytes (Fig. 3*a*). We then went on to determine whether GATA-3KO thymocytes could efficiently express and present autoantigens to iNKT cells. As deficiency of GATA-3 results in a profound

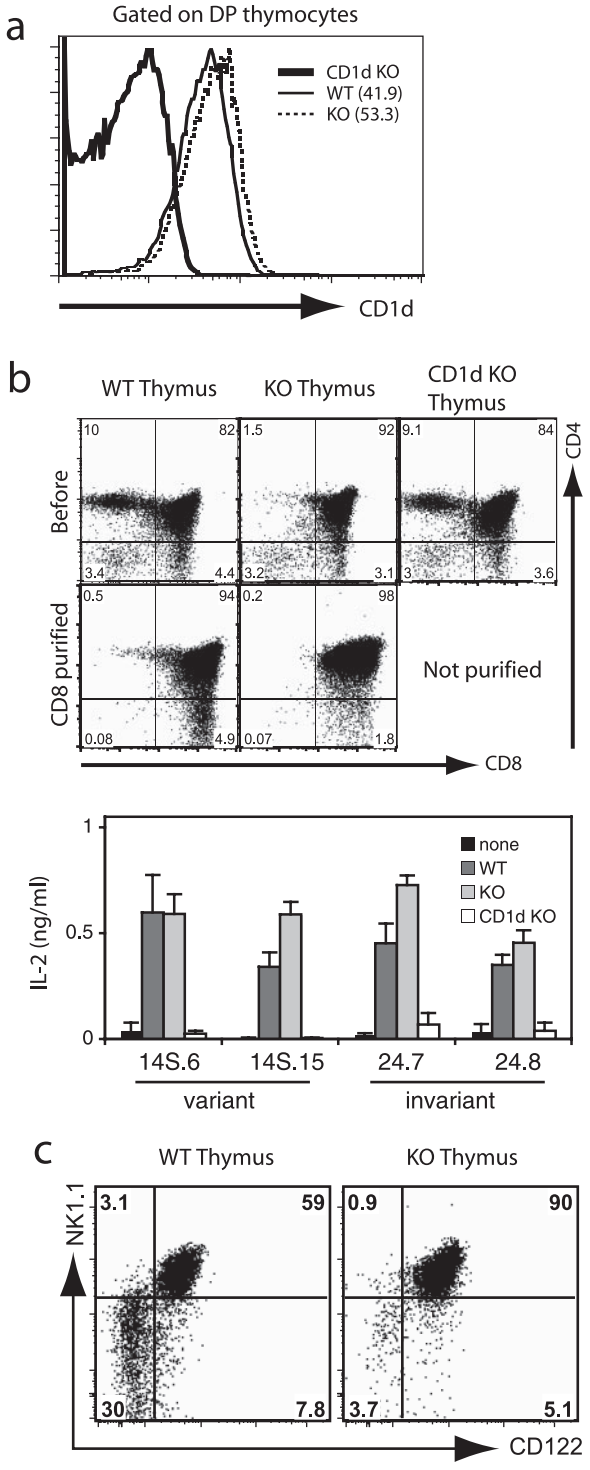

FIGURE 3. *a*, CD1d expression on DP thymocytes from WT, GATA-3KO, and CD1d KO mice are comparable. CD1d MFI is shown in parenthesis in the figure legend. Data are representative of three independent experiments. *b*, CD4/CD8 profiles of WT and GATA-3KO thymocytes before and after CD8 MACS purification are shown. CD1d KO thymocytes were not purified. These thymocytes were incubated with autoreactive variant NKT (14S.6 and 14S.15) or iNKT (24.7 and 24.8) hybridomas overnight. IL-2 produced by the stimulated NKT hybridomas was measured by ELISA. The NKT hybridomas were also incubated in the absence of thymocytes (none) as an additional negative control. Data shown are representative of two independent experiments. *c*, CD122 and NK1.1 expression is shown for WT and GATA-3KO thymic iNKT cells. Cells were stained with TCRβ-FITC, CD122-PE, CD1d tetramer-allophycocyanin, and NK1.1-PE-Cy7 and gated on TCRβ^{+}, tetramer^{+} events. Results are representative of two independent experiments.

defect in the generation of CD4 single-positive (SP) thymocytes (23), we used CD8 magnetic beads to enrich DP and CD8SP thymocytes to make the WT and GATA-3KO thymocyte subsets more comparable (Fig. 3*b*). The DP and CD8SP thymocytes were then incubated with variant and invariant NKT hybridomas, and the production of IL-2 was measured as an indicator of NKT hybridoma activation. As shown in Fig. 3*b*, the enriched GATA-3KO DP thymocytes were at least as competent as WT thymocytes in stimulating autoreactive NKT hybridomas. The activation of NKT hybridomas required CD1d on thymocytes because CD1d KO thymocytes failed to elicit any IL-2 response. These data indicate that the Ag-presenting function of GATA-3KO thymocytes is intact and suggest that the aberrant development of GATA-3KO iNKT cells is caused by a cell-intrinsic defect.

IL-2Rβ (CD122) is necessary for iNKT cell development and is up-regulated between stages 2 and 3 of thymic iNKT development (7, 36). Failure to up-regulate CD122 may be responsible for the iNKT cell phenotype seen in the T-bet KO mice (20). Thymic iNKT cells were analyzed to determine whether GATA-3 deficiency caused a similar defect. In WT mice, NK1.1$^-$ iNKT cells (stage 1 and 2) were predominantly CD122 negative whereas NK1.1$^+$ iNKT cells (stage 3) were CD122 positive (Fig. 3*c*). In the GATA-3 deficient thymus, 90% of iNKT cells were positive for both CD122 and NK1.1 indicating that failure to up-regulate CD122 is not responsible for the developmental phenotype. There was no appreciable difference in CD122 expression between WT and GATA-3KO splenic iNKT cells (data not shown).

Although these findings are suggestive of a cell-intrinsic defect in iNKT development, they do not exclude the possible role of subtle yet important defects in the thymic microenvironment of GATA-3KO mice. To confirm the cell-intrinsic defect, we generated bone marrow chimeric mice. RAG-1-deficient (RAG-1KO) mice were sublethally irradiated and then received bone marrow cells derived from GATA-3KO and/or WT Thy1.1 congenic mice. In the RAG-1KO hosts that received both GATA-3KO and congenic bone marrow, GATA-3KO and congenic iNKT precursors develop in the same microenvironment composed of RAG-1KO thymic epithelium, GATA-3KO, and congenic DP thymocytes. Differences seen between the WT and GATA-3KO iNKT cells in these mice must therefore be due to cell-intrinsic effects. Six weeks after transfer, host mice were sacrificed, and the development of iNKT cells was analyzed. Because RAG-1KO mice do not have endogenous iNKT cells, all iNKT cells in the host animals were derived from donor bone marrow cells. Both GATA-3KO and congenic bone marrow were able to reconstitute iNKT cells in the RAG-1KO mice (Fig. 4*a*). Similar to the results seen in the Fig. 1*b*, the GATA-3KO iNKT cells had lower surface expression of TCR when compared with the congenic iNKT cells, and this difference was not rescued in the host receiving bone marrow from both donors.

We found that host animals that received only WT bone marrow cells contained both CD4$^+$ and DN iNKT cells (Fig. 4*b*). In contrast, GATA-3KO bone marrow gave rise mainly to the DN subset. In RAG-1KO mice that received both congenic and GATA-3KO bone marrow cells, the GATA-3KO iNKT cells once again were predominantly DN, indicating a cell-intrinsic defect.

The requirement of GATA-3 for optimal peripheral iNKT survival is also cell intrinsic. In mice receiving both GATA-3KO and congenic bone marrow, both lineages contributed comparably to thymic iNKT cells. Thymic iNKT cells (48 ± 12%) were from the GATA-3KO bone marrow (Thy1.2$^+$) vs 52 ± 12% from the congenic (Thy1.2$^-$) (Fig. 4*c*). This is in sharp distinction to the spleen of these mice where only 8 ± 5.5% of iNKT cells were from the GATA-3KO bone marrow ($p < 0.05$ for comparison to thymus by

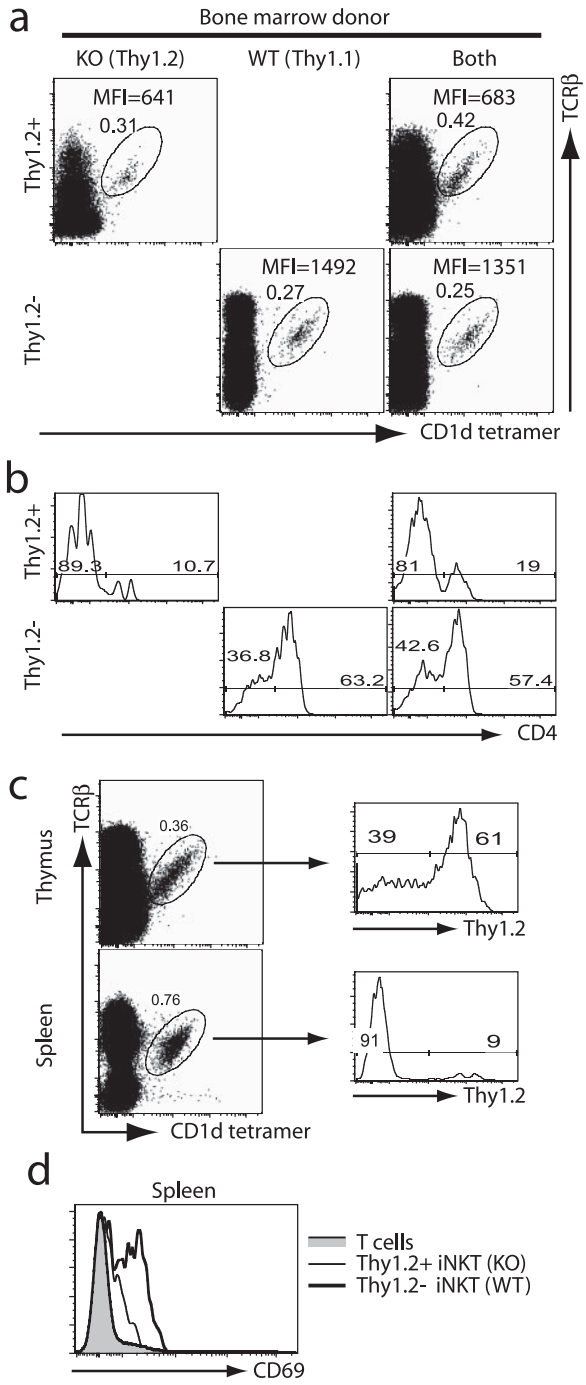

FIGURE 4. The defects seen in GATA-3KO iNKT cells are cell intrinsic. RAG-1KO mice were sublethally irradiated and received bone marrow cells harvested from GATA-3KO (KO Thy1.2) mice and/or WT congenic (WT Thy1.1) mice. Six weeks after bone marrow transplantation, iNKT cells of recipient mice were analyzed. The thymic iNKT cells, as identified by CD1d tetramer and TCRβ staining, and the MFI of TCRβ staining are shown in *a*. The distribution of CD4$^+$ and DN subsets of thymic iNKT cells, as distinguished by CD4 staining, is shown in *b*. *c* and *d*, Thymic and splenic iNKT cells in the host animals receiving both bone marrow populations were gated and the contribution of each bone marrow population to the gated cells was analyzed by Thy1.2-FITC staining and shown in *c*. The expression of CD69 on splenic iNKT cells from Thy1.2$^+$ and Thy1.2$^-$ subsets is shown in *d*. Resting T cells obtained from the same animals were also stained for CD69 as negative controls. Results are representative of three mice for mixed bone marrow chimeras and two mice each for single bone marrow chimeras.

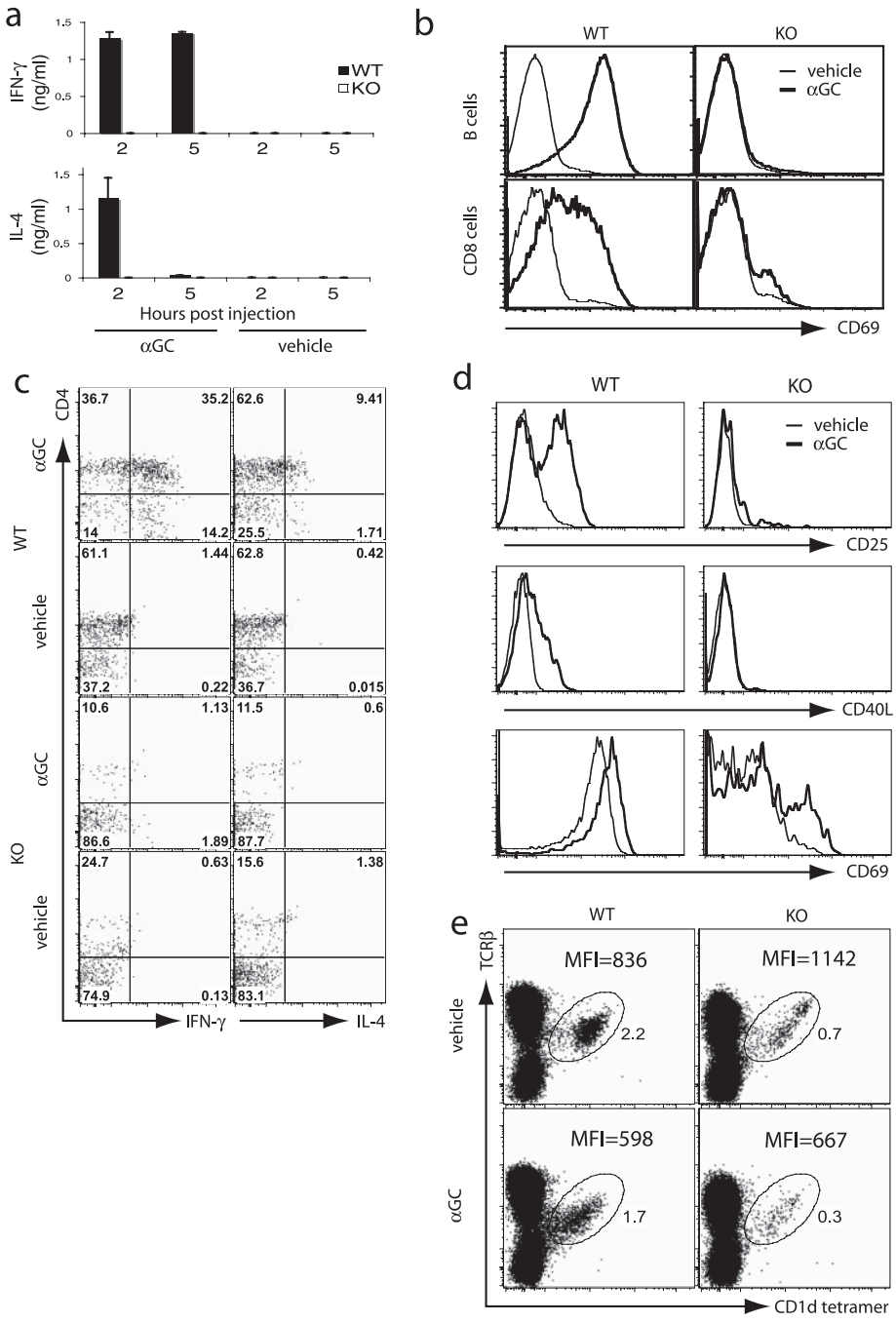

FIGURE 5. GATA-3KO iNKT cells have impaired response to i.v. GalCer injection. GATA-3KO and WT mice were injected with 2 μg of GalCer or vehicle and subjected to the following analyses. *a*, Serum was collected at 2 and 5 h for detection of IFN-γ and IL-4 by ELISA. *b*, The expression of CD69 by splenic B and CD8$^+$ T cells was analyzed 5 h after injection. *c*, IFN-γ and IL-4 production by CD4$^+$ and DN subsets of splenic iNKT cells was analyzed by intracellular cytokine staining. Events shown are gated on CD19-allophycocyanin-Cy7 negative, TCR-FITC-positive, CD1d tetramer-allophycocyanin-positive events. Anti-cytokine Abs conjugated to PE and CD4-PerCP were also used. *d*, The expression of CD25-PE, CD40L-PE, and CD69-PE-Cy7 by splenic iNKT cells was analyzed 2 h after injection (gated on CD19-allophycocyanin-Cy7-negative, TCR-FITC-positive, CD1d tetramer-allophycocyanin-positive events). *e*, The level of TCRβ of splenic iNKT cells (the gated populations), as assessed by MFI, were analyzed 2 h after injection. Percentages of iNKT cells among CD19-allophycocyanin-Cy7 negative splenocytes are also shown. The results in Fig. 5 are representative of two to five independent experiments.

two tailed Student *t* test). This dramatic drop in peripheral GATA-3KO iNKT cells despite normal numbers in the thymus recapitulates what was seen in the GATA-3KO mouse (Fig. 1*c*). Furthermore, the level of CD69 expression on splenic GATA-3KO iNKT cells was lower than that of congenic iNKT cells (Fig. 4*d*). These findings show that all of the defects in the GATA-3-deficient iNKT cells—loss of CD4$^+$ subset, decreased number in the periphery, and defective up-regulation of CD69 in the periphery—are due to cell-intrinsic defects rather than due to defects in the microenvironment.

Impaired responses of GATA-3KO iNKT cells to i.v. αGalCer

One unique feature of iNKT cells is the prompt production of large amounts of cytokine in response to TCR signaling. To determine whether deficiency of GATA-3 also affects the function of iNKT cells, we injected both GATA-3KO and WT mice with αGalCer i.v. It has been demonstrated that i.v. injection of αGalCer results in a sharp rise in the serum levels of cytokines, including IL-4 and IFN-γ, produced by iNKT cells (12, 13). Significant levels of IL-4 and IFN-γ were readily detected in the serum of WT mice 2 h after injection (Fig. 5*a*). The level of IFN-γ remained high 5 h after injection. In addition, we detected up-regulation of CD69 in B and T cells (Fig. 5*b*), a consequence of the iNKT-induced cytokine storm (37). In contrast, no IL-4 or IFN-γ was detected in the serum of GATA-3KO mice even 5 h after injection, nor did we detect up-regulation of CD69 in B or T cells in those mice. GATA-3KO mice are unable to mount a cytokine storm in response to i.v. αGalCer.

The lack of cytokine response to i.v. αGalCer in GATA-3KO mice could be explained by the markedly reduced numbers of iNKT cells. In addition, GATA-3KO iNKT cells may have an intrinsic defect precluding them from producing cytokines. To test the latter scenario, we again injected mice with i.v. αGalCer and

then examined the production of cytokines by iNKT with ex vivo intracellular cytokine staining. As shown in Fig. 5*c*, ~50% of WT iNKT cells stained positive for IFN-γ and ~10% of iNKT cells also expressed IL-4. Unlike WT iNKT cells, iNKT cells from GATA-3KO mice produced neither IL-4 nor IFN-γ in response to i.v. αGalCer injection even when examined on a single-cell basis.

GATA-3 plays an important role in the production of Th2 cytokines in T cells (27) and IFN-γ in NK cells (21), and this may explain the lack of cytokine storm in response to αGalCer stimulation in GATA-3KO mice. An alternative, but not mutually exclusive explanation, is that GATA-3 deficiency causes a defect in TCR signaling, thereby affecting the activation of iNKT cells. We therefore investigated whether GATA-3KO iNKT cells were activated by i.v. αGalCer. As shown in Fig. 5*d*, i.v. αGalCer induced the up-regulation of CD25, CD40L, and CD69, downstream markers of TCR signals, in WT iNKT cells. In contrast, i.v. αGalCer only induced a modest but reproducible increase in the level of CD69 in GATA-3KO iNKT cells and no induction of CD25 or CD40L. These observations argue strongly that deficiency of GATA-3 leads to a defect in TCR signaling. Nevertheless, GATA-3KO iNKT cells obtained from αGalCer-injected mice still displayed ligand-induced down-modulation of TCR, as evidenced by the dimmer staining with CD1d tetramer and TCRβ Ab (Fig. 5*e*). Thus, GATA-3KO iNKT cells did encounter αGalCer and were not sequestered away from APC in vivo.

Bypassing proximal TCR signaling induces IFN-γ but not Th2 cytokine production in GATA-3KO iNKT cells

The data shown in Fig. 5 suggest that deficiency of GATA-3 leads to a block in TCR-mediated signal transduction. To localize the block in TCR-mediated signal transduction, we stimulated GATA-3KO iNKT cells in vitro with PMA and ionomycin, which bypass proximal TCR-mediated signaling events. Two hours after stimulation with PMA and ionomycin, 22, 9, and 10% of splenic iNKT cells from WT mice stained positive for IFN-γ, IL-4, and IL-13, respectively (Fig. 6*a*). In the GATA-3KO iNKT cells, 29% stained positive for IFN-γ after stimulation, demonstrating that the machinery for producing IFN-γ is still functional in GATA-3KO iNKT cells. In contrast to IFN-γ production, PMA and ionomycin were still unable to induce IL-4 or IL-13 production in GATA-3KO iNKT cells.

In addition to inducing IFN-γ production, PMA and ionomycin up-regulated CD69 in GATA-3KO iNKT cells to a level nearly equivalent to that of stimulated WT iNKT cells (Fig. 6*b*). This indicates that the cellular machinery necessary to up-regulate CD69 is intact in GATA-3KO iNKT cells. Somewhat surprisingly, despite being able to induce IFN-γ production and up-regulate of CD69, PMA and ionomycin could not up-regulate CD25 or CD40L even in WT iNKT cells. Taken together, GATA-3 is not only absolutely required for the production of type 2 cytokines but is also critical for the activation of iNKT cells through the TCR, most likely at a stage proximal to protein kinase C (PKC) and calcium flux, the pathways activated by PMA and ionomycin. Our data also indicate that in vitro stimulation with PMA and ionomycin does not fully recapitulate the activation of iNKT cells by in vivo αGalCer injection.

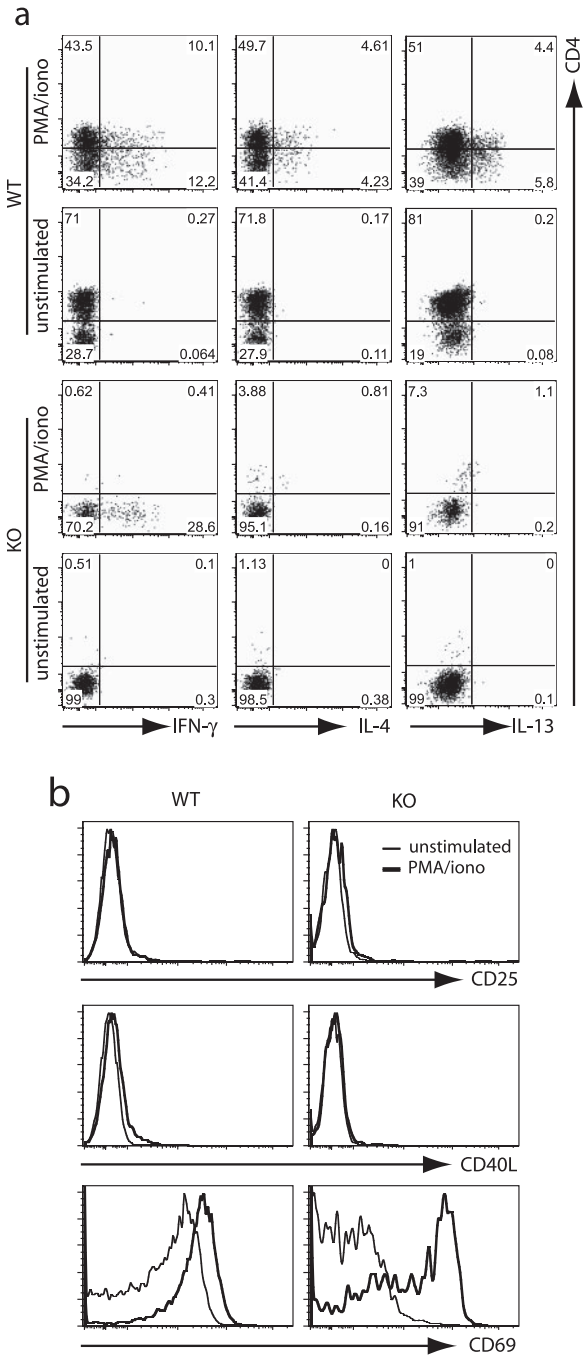

FIGURE 6. PMA and ionomycin can induce IFN-γ production and CD69 up-regulation in GATA-3KO iNKT cells. *a*, Whole splenocytes of WT or GATA-3KO mice were left unstimulated or stimulated for 2 h in vitro with PMA and ionomycin. The production of IFN-γ, IL-4, and IL-13 by splenic iNKT cells were then analyzed with intracellular cytokine staining. Results are representative of five independent experiments. Cells were stained and gated as in Fig. 5*c*. *b*, The unstimulated or PMA/ionomycin-stimulated splenic iNKT cells, as described in *a*, were also analyzed for the expression of indicated activation markers. Cells were stained and gated as in Fig. 5*d*. Results are representative of three independent experiments.

Discussion

iNKT cells, a distinct subpopulation of T cells, play a critical role in a variety of normal and abnormal immune responses. The transcriptional control of iNKT cell development, and even more so iNKT cell function, remains very poorly understood in comparison to that of conventional αβ T cells. Using a T cell-specific, gene-deficient animal model, we show here that GATA-3, a transcription factor required for the development and function of conventional CD4 Th cells, also critically regulates the development, survival, activation, and effector function of iNKT cells in a cell-intrinsic manner.

Previously described defects in iNKT cell maturation have led to either complete loss or severe reduction in their number. In

contrast, we show that GATA-3 deficiency selectively affects the generation of the $CD4^+$ subset of iNKT cells in both the thymus and the periphery. In the GATA-3-deficient mouse, iNKT cells have a higher rate of apoptosis in the periphery, but the failure of $CD4^+$ iNKT cells to develop in the thymus remains unexplained. The selective nature of this deficiency is reminiscent of the lack of conventional CD4SP thymocytes in GATA-3KO mice, raising the possibility that the molecular mechanisms regulating the generation of both $CD4^+$ T cell subsets could be similar. In addition to GATA-3, the development of conventional $CD4^+$ $\alpha\beta$ T cells requires lineage commitment signals, controlled by factors such as c-Krox/Th-POK (38, 39), and postcommitment survival signals, such as those generated by CD83-CD83L interactions (40). Whether these factors operate in iNKT cells as in conventional CD4 thymocytes has yet to be explored.

The ability of iNKT cells to generate a cytokine storm hours after stimulation is likely related to their state of activation as measured by their high expression of CD69. The factors that initiate and maintain CD69 expression are unclear but the maintenance of high CD69 on iNKT cells in the absence of peripherally expressed CD1d argues that the maintenance, but not necessarily the initiation, is CD1d independent (5, 34). In peripheral iNKT cells, CD69 expression is intermediate before 3 wk of age but then transitions to the high state seen in adult mice. CD69 is regulated during thymic development in conventional and iNKT cells (41–43), but to our knowledge, it has never been described as an age-dependent maturation marker in peripheral iNKT cells. This peripheral maturation step is completely blocked in the absence of GATA-3. Interestingly, this is in sharp contrast to GATA-3KO Th cells, which readily express high levels of CD69 even in unimmunized mice (29). Because GATA-3KO peripheral iNKT cells fail to up-regulate CD69, we are unable to determine whether GATA-3 is also required for the maintenance of CD69 after it has been up-regulated.

Given the role of GATA-3 as a chromatin remodeling factor (44) and direct transactivator of type 2 cytokine genes (25–27), the failure of GATA-3-deficient iNKT cells to produce IL-4 and IL-13 after stimulation is not surprising. In contrast to GATA-3KO Th cells, which produce more IFN-γ under type 2 culture conditions (28), GATA-3KO iNKT cells are defective in IFN-γ production when stimulated with αGalCer. Although NK cells also require GATA-3 for efficient IFN-γ production (21), the mechanisms for this similarity are different between NK and iNKT cells. In NK cells, GATA-3 deficiency causes decreased expression of T-bet, a factor known to be critical for IFN-γ production, and IFN-γ production was impaired even in response to PMA and ionomycin stimulation. In iNKT cells, PMA and ionomycin can restore IFN-γ production, demonstrating that the transcriptional and translational machinery for IFN-γ production is intact. Consistent with this observation, the expression of T-bet in thymic iNKT cells was comparable between WT and GATA-3KO mice (data not shown).

Deficiency of GATA-3 caused a profound defect in transducing TCR-mediated signals as measured by activation markers and cytokine production in iNKT cells stimulated in vivo with αGalCer. The paucity of peripheral GATA-3KO iNKT cells has thus far hampered more detailed biochemical analyses of the TCR-signaling pathways in these cells. However, the data above allow us to make some preliminary attempts to characterize the pathways leading to cytokine production and induction of activation. GATA-3-deficient iNKT cells display ligand-induced down-modulation of TCR, suggesting that proximal TCR signaling events are at least partially intact and that activation and recruitment of molecules such as Lck, Fyn, and Zap-70, important in conventional T cells, may also be preserved (45). These early events in TCR signaling culminate in the activation of PKC and calcium flux, which can be pharmacologically induced with PMA and ionomycin (46). The fact that in vitro stimulation with PMA and ionomycin can induce cytokine production and up-regulation of CD69 in GATA-3KO iNKT cells indicates that the signaling defect caused by GATA-3 deficiency lies downstream of Lck, Fyn, and Zap-70 but before the activation of PKC and calcium flux. Intriguingly, while some downstream effects of in vivo stimulation with αGalCer, such as IFN-γ production and CD69 up-regulation, were recapitulated by in vitro PMA and ionomycin stimulation, others, such as CD25 and CD40L up-regulation, were not, even in WT iNKT cells. This unexpected observation suggests that signals responsible for up-regulating CD25 and CD40L in iNKT cells, while initiated by TCR/CD1d interactions, may not involve activation of PKC or calcium flux. Alternatively, the signals responsible for CD25 and CD40L up-regulation may be initiated from a CD1d independent source but act synergistically with TCR-mediated signals. In such a case, it will be of interest to determine whether this CD1d-independent pathway is the same one that is needed to maintain the $CD69^{high}$ phenotype in peripheral iNKT cells. Our work underscores the similarities and differences between iNKT and conventional $\alpha\beta$ T cells, and further dissection of the transcriptional and signaling networks involved will further our understanding of iNKT cell biology.

Acknowledgments

We thank Dr. Michael Brenner for critical review of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* 278: 1626–1629.
2. Benlagha, K., D. G. Wei, J. Veiga, L. Teyton, and A. Bendelac. 2005. Characterization of the early stages of thymic NKT cell development. *J. Exp. Med.* 202: 485–492.
3. Gapin, L., J. L. Matsuda, C. D. Surh, and M. Kronenberg. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat. Immunol.* 2: 971–978.
4. Bendelac, A. 1995. Positive selection of mouse $NK1^+$ T cells by CD1-expressing cortical thymocytes. *J. Exp. Med.* 182: 2091–2096.
5. Wei, D. G., H. Lee, S. H. Park, L. Beaudoin, L. Teyton, A. Lehuen, and A. Bendelac. 2005. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J. Exp. Med.* 202: 239–248.
6. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NK T cell lineage. *Science* 296: 553–555.
7. Matsuda, J. L., and L. Gapin. 2005. Developmental program of mouse Vα14i NKT cells. *Curr. Opin. Immunol.* 17: 122–130.
8. Elewaut, D., R. B. Shaikh, K. J. Hammond, H. De Winter, A. J. Leishman, S. Sidobre, O. Turovskaya, T. I. Prigozy, L. Ma, T. A. Banks, et al. 2003. NIK-dependent RelB activation defines a unique signaling pathway for the development of Vα14i NKT cells. *J. Exp. Med.* 197: 1623–1633.
9. Sivakumar, V., K. J. Hammond, N. Howells, K. Pfeffer, and F. Weih. 2003. Differential requirement for Rel/nuclear factor κ B family members in natural killer T cell development. *J. Exp. Med.* 197: 1613–1621.
10. Park, S. H., K. Benlagha, D. Lee, E. Balish, and A. Bendelac. 2000. Unaltered phenotype, tissue distribution and function of $V\alpha14^+$ NKT cells in germ-free mice. *Eur. J. Immunol.* 30: 620–625.
11. Gumperz, J. E., S. Miyake, T. Yamamura, and M. B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J. Exp. Med.* 195: 625–636.
12. Fujii, S., K. Shimizu, M. Kronenberg, and R. M. Steinman. 2002. Prolonged IFN-γ-producing NKT response induced with α-galactosylceramide-loaded DCs. *Nat. Immunol.* 3: 867–874.
13. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23: 877–900.
14. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9: 582–588.
15. Heller, F., I. J. Fuss, E. E. Nieuwenhuis, R. S. Blumberg, and W. Strober. 2002. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* 17: 629–638.

16. Kinjo, Y., and M. Kronenberg. 2005. Vα14i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes. *J. Clin. Immunol.* 25: 522–533.
17. Vincent, M. S., D. S. Leslie, J. E. Gumperz, X. Xiong, E. P. Grant, and M. B. Brenner. 2002. CD1-dependent dendritic cell instruction. *Nat. Immunol.* 3: 1163–1168.
18. Nishimura, T., H. Kitamura, K. Iwakabe, T. Yahata, A. Ohta, M. Sato, K. Takeda, K. Okumura, L. Van Kaer, T. Kawano, et al. 2000. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int. Immunol.* 12: 987–994.
19. Crowe, N. Y., J. M. Coquet, S. P. Berzins, K. Kyparissoudis, R. Keating, D. G. Pellicci, Y. Hayakawa, D. I. Godfrey, and M. J. Smyth. 2005. Differential antitumor immunity mediated by NKT cell subsets in vivo. *J. Exp. Med.* 202: 1279–1288.
20. Townsend, M. J., A. S. Weinmann, J. L. Matsuda, R. Salomon, P. J. Farnham, C. A. Biron, L. Gapin, and L. H. Glimcher. 2004. T-bet regulates the terminal maturation and homeostasis of NK and Vα14i NKT cells. *Immunity* 20: 477–494.
21. Samson, S. I., O. Richard, M. Tavian, T. Ranson, C. A. Vosshenrich, F. Colucci, J. Buer, F. Grosveld, I. Godin, and J. P. Di Santo. 2003. GATA-3 promotes maturation, IFN-γ production, and liver-specific homing of NK cells. *Immunity* 19: 701–711.
22. Ting, C. N., M. C. Olson, K. P. Barton, and J. M. Leiden. 1996. Transcription factor GATA-3 is required for development of the T cell lineage. *Nature* 384: 474–478.
23. Pai, S. Y., M. L. Truitt, C. N. Ting, J. M. Leiden, L. H. Glimcher, and I. C. Ho. 2003. Critical roles for transcription factor GATA-3 in thymocyte development. *Immunity.* 19: 863–875.
24. Hernandez-Hoyos, G., M. K. Anderson, C. Wang, E. V. Rothenberg, and J. Alberola-Ila. 2003. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* 19: 83–94.
25. Kishikawa, H., J. Sun, A. Choi, S. C. Miaw, and I. C. Ho. 2001. The cell type-specific expression of the murine IL-13 gene is regulated by GATA-3. *J. Immunol.* 167: 4414–4420.
26. Zhang, D. H., L. Cohn, P. Ray, K. Bottomly, and A. Ray. 1997. Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J. Biol. Chem.* 272: 21597–21603.
27. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587–596.
28. Zhu, J., B. Min, J. Hu-Li, C. J. Watson, A. Grinberg, Q. Wang, N. Killeen, J. F. Urban, Jr., L. Guo, and W. E. Paul. 2004. Conditional deletion of Gata3 shows its essential function in Th1-Th2 responses. *Nat. Immunol.* 5: 1157–1165.
29. Pai, S. Y., M. L. Truitt, and I. C. Ho. 2004. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc. Natl. Acad. Sci. USA* 101: 1993–1998.
30. Wilson, S. B., S. C. Kent, H. F. Horton, A. A. Hill, P. L. Bollyky, D. A. Hafler, J. L. Strominger, and M. C. Byrne. 2000. Multiple differences in gene expression in regulatory Vα24JαQ T cells from identical twins discordant for type I diabetes. *Proc. Natl. Acad. Sci. USA* 97: 7411–7416.
31. Grenningloh, R., B. Y. Kang, and I. C. Ho. 2005. Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. *J. Exp. Med.* 201: 615–626.
32. Grogan, J. L., M. Mohrs, B. Harmon, D. A. Lacy, J. W. Sedat, and R. M. Locksley. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* 14: 205–215.
33. Gumperz, J. E., C. Roy, A. Makowska, D. Lum, M. Sugita, T. Podrebarac, Y. Koezuka, S. A. Porcelli, S. Cardell, M. B. Brenner, and S. M. Behar. 2000. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* 12: 211–221.
34. McNab, F. W., S. P. Berzins, D. G. Pellicci, K. Kyparissoudis, K. Field, M. J. Smyth, and D. I. Godfrey. 2005. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J. Immunol.* 175: 3762–3768.
35. Pellicci, D. G., K. J. Hammond, A. P. Uldrich, A. G. Baxter, M. J. Smyth, and D. I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent $NK1.1^{-}CD4^{+}$ CD1d-dependent precursor stage. *J. Exp. Med.* 195: 835–844.
36. Ohteki, T., S. Ho, H. Suzuki, T. W. Mak, and P. S. Ohashi. 1997. Role for IL-15/IL-15 receptor β-chain in natural killer 1.1^{+} T cell receptor-$\alpha\beta^{+}$ cell development. *J. Immunol.* 159: 5931–5935.
37. Kitamura, H., A. Ohta, M. Sekimoto, M. Sato, K. Iwakabe, M. Nakui, T. Yahata, H. Meng, T. Koda, S. Nishimura, et al. 2000. α-Galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cell. Immunol.* 199: 37–42.
38. He, X., X. He, V. P. Dave, Y. Zhang, X. Hua, E. Nicolas, W. Xu, B. A. Roe, and D. J. Kappes. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* 433: 826–833.
39. Sun, G., X. Liu, P. Mercado, S. R. Jenkinson, M. Kypriotou, L. Feigenbaum, P. Galera, and R. Bosselut. 2005. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nat. Immunol.* 6: 373–381.
40. Fujimoto, Y., L. Tu, A. S. Miller, C. Bock, M. Fujimoto, C. Doyle, D. A. Steeber, and T. F. Tedder. 2002. CD83 expression influences $CD4^{+}$ T cell development in the thymus. *Cell* 108: 755–767.
41. Dao, T., D. Guo, A. Ploss, A. Stolzer, C. Saylor, T. E. Boursalian, J. S. Im, and D. B. Sant'Angelo. 2004. Development of CD1d-restricted NKT cells in the mouse thymus. *Eur. J. Immunol.* 34: 3542–3552.
42. Gadue, P., and P. L. Stein. 2002. NK T cell precursors exhibit differential cytokine regulation and require Itk for efficient maturation. *J. Immunol.* 169: 2397–2406.
43. Yamashita, I., T. Nagata, T. Tada, and T. Nakayama. 1993. CD69 cell surface expression identifies developing thymocytes which audition for T cell antigen receptor-mediated positive selection. *Int. Immunol.* 5: 1139–1150.
44. Lee, H. J., N. Takemoto, H. Kurata, Y. Kamogawa, S. Miyatake, A. O'Garra, and N. Arai. 2000. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. *J. Exp. Med.* 192: 105–115.
45. Davanture, S., J. Leignadier, P. Milani, P. Soubeyran, B. Malissen, M. Malissen, A. M. Schmitt-Verhulst, and C. Boyer. 2005. Selective defect in antigen-induced TCR internalization at the immune synapse of CD8 T cells bearing the ZAP-70(Y292F) mutation. *J. Immunol.* 175: 3140–3149.
46. Truneh, A., F. Albert, P. Golstein, and A. M. Schmitt-Verhulst. 1985. Calcium ionophore plus phorbol ester can substitute for antigen in the induction of cytolytic T lymphocytes from specifically primed precursors. *J. Immunol.* 135: 2262–2267.