Sensitivity of NK1.1-Negative NKT Cells to Transgenic BATF Defines a Role for Activator Protein-1 in the Expansion and Maturation of Immature NKT Cells in the Thymus[1]

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NKT cells are glycolipid-reactive lymphocytes that express markers and perform functions common to both T lymphocytes and NK cells. Although the genetic events controlling conventional T cell development are well defined, the transcription factors and genetic programs regulating NKT cell development are only beginning to be elucidated. Previously, we described the NKT cell-deficient phenotype of transgenic (Tg) mice constitutively expressing B cell-activating transcription factor (BATF), a basic leucine zipper protein and inhibitor of AP-1. In this study, we show that Tg BATF targets the majority of Vα14Jα281 (Vα14i^7) NKT cells, regardless of CD4 expression and *Vβ* gene usage. The residual NKT cells in the thymus of *BATF-Tg* mice are CD44$^+$, yet are slow to display the NK1.1 marker characteristic of mature cells. As a population, BATF-expressing NKT cells are TCRβ/CD3ε^{low}, but express normal levels of CD69, suggesting a failure to expand appropriately following selection. Consistent with the sensitivity of NKT cells to BATF-induced changes in AP-1 activity, we detect a full complement of AP-1 basic leucine zipper proteins in wild-type NKT cells isolated from the thymus, spleen, and liver, and show that AP-1 DNA-binding activity and cytokine gene transcription are induced in NKT cells within a few hours of glycolipid Ag exposure. This study is the first to characterize AP-1 activity in NKT cells and implicates the integrity of this transcription factor complex in developmental events essential to the establishment of this unique T cell subset in the thymus. *The Journal of Immunology,* **2007, 178: 58–66.**

The Vα14i NKT cells are an atypical lymphocyte subset that expresses markers and performs functions common to both conventional T lymphocytes (TCRβ^+) and innate NK cells (NK1.1$^+$). In contrast to conventional T cells, which are MHC restricted and respond to peptide ligands, Vα14i NKT cells interact with glycolipid ligands such as synthetic α-galactosylceramide (α-GalCer),[4] endogenous iGB3, and bacterially derived glycolipids presented by CD1d (1). Although NKT cells impact a myriad of immune system functions in response to glycolipid ligands (2), Vα14i NKT cells are best known for their rapid and robust production of the Th1 and Th2 cytokines, IFN-γ and IL-4, respectively (2). Consistent with this unique pattern of dual cytokine production, there is evidence supporting a role for NKT cells in the defense against pathogens, the prevention of autoimmune diseases, and the hypersensitivity to airway allergens (3, 4).

The discovery of α-GalCer (5) and the development of fluorescent α-GalCer multimers (6–8) as a tool to detect, stimulate, and purify NKT cells have permitted many laboratories to identify over two dozen genes whose products are essential for the development and function of Vα14i NKT cells. Included in this group are a number of genes encoding transcription factors, including members of the NF-κB family (9–12), T-bet (13, 14), Ets-1 (15), Mef (16), Irf-1 (17), Runx (18), and RORγt (18, 19). Previously, our laboratory described the NKT cell-deficient phenotype of transgenic (Tg) mice expressing a T cell-specific, *p56^{lck}* transgene encoding a hemagglutinin (HA) Ag-tagged B cell-activating transcription factor (BATF) (20), an inhibitory component of the AP-1 family of basic leucine zipper (bZIP) transcription factors (21). As predicted, the constitutive expression of BATF in thymocytes results in altered AP-1 DNA-binding activity, reduced expression of a stably integrated AP-1 reporter gene, and the absence of a mitogenic response following stimulation in vitro (20, 22, 23). Interestingly, whereas conventional T cell development remains intact in these animals, thymic and peripheral TCRβ^+NK1.1$^+$ NKT lymphocytes are dramatically underrepresented in *BATF-Tg* mice (20). The selective sensitivity of the NKT cell lineage to the perturbation of AP-1 activity by BATF was an intriguing result that clearly warranted further investigation.

In this study, we extend our analysis of *BATF-Tg* mice to show that expression of the *p56^{lck}-HA-BATF* transgene influences the majority of α-GalCer-reactive Vα14i NKT cells, including the predominant Vβ8 and Vβ7 subsets and both CD4$^+$ and double-negative NKT cells. Maturation analysis using CD1d-α-GalCer tetramers with anti-CD44 and anti-NK1.1 Abs revealed that as early as 3 wk of age, *BATF-Tg NKT* cells are reluctant to adopt a mature, NK1.1-positive phenotype. In addition, the NKT cells populating in the thymus of *BATF-Tg* mice show a bias toward being TCR^{low}, express reduced levels of CD3ε, but express normal levels of CD69. Undoubtedly, the lack of NKT cells in the thymus of *BATF-Tg* mice most likely reflects the ability of BATF, as an

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[4] Abbreviations used in this paper: α-GalCer, α-galacotsylceramide; BATF, B cell-activating transcription factor; bZIP, basic leucine zipper; HA, hemagglutinin; Tg, transgenic.

AP-1 inhibitor, to restrict cell growth and precursor expansion. Interestingly, the Tg NKT cells that do complete maturation in the thymus persist throughout the life of the animals as stable, BATF-expressing populations in the spleen and the liver. These observations prompted us to characterize the AP-1 activity that functions downstream of Vα14i TCR signaling in wild-type NKT cells and NKT cell hybridomas. We show that NKT cells possess transcripts for the major Jun and Fos family proteins, and that, within hours of stimulation, they display the characteristic induction of sequence-specific DNA binding associated with AP-1 transcriptional activity. Furthermore, this induction of AP-1 DNA binding is correlated temporally with both the rate-limiting presentation of glycolipid Ag and the enhanced transcription of *IL-4*, a well-established AP-1 target gene (24, 25). Taken together, these experiments are the first to demonstrate that the AP-1 transcription complex is an endogenous regulator of NKT cell gene expression, and that the proper in vivo regulation of this activity is critical during NKT cell development within the thymus.

Materials and Methods

Mice

The generation of *p56^{lck}-HA-BATF* (*BATF-Tg*) mice has been described previously (20). *BATF-Tg* mice were backcrossed eight generations and were maintained on the C57BL/6 background. *CD1d$^{-/-}$* and *Jα18$^{-/-}$* mice (26–29) were obtained from R. Brutkiewicz (Indiana University School of Medicine, Indianapolis, IN). *Vα14-Tg* mice, on a C57BL/6 genetic background (30), were obtained from L. Van Kaer (Vanderbilt University, Nashville, TN). All mice were housed in the pathogen-free conditions of the Purdue University Transgenic Mouse Core Facility, according to institutional guidelines. All procedures involving these animals have been reviewed and approved by the Purdue University Animal Care and Use Committee. Unless otherwise noted in the figure legends, all experiments were performed using age- and sex-matched animals between 6 and 12 wk of age.

Cell culture

The isolation and culturing of primary T lymphocytes have been described previously (22). The DN32.D3 (Vα14$^+$) and 431.A11 (Vα14$^-$) NKT cell hybridomas have been described (31, 32) and were maintained at a density no greater than 1×10^6 cells/ml in IMDM, 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Antibodies

The following Abs used in this study were purchased from BD Biosciences: anti-mouse IgG PE (A85-1), Fc block (2.4G2), anti-Vβ8.1/8.2 FITC (MR5-2), anti-Vβ7 FITC (TR310), anti-HSA FITC (M1/69), anti-CD4 FITC, anti-CD4 PE (RM45), anti-CD8 FITC, anti-CD8 PerCP (53.6.7), anti-TCRβ FITC, anti-PE-Cy5 (H57-597), anti-NK1.1 PE, anti-NK1.1 PE-Cy7 (PK136), anti-CD3ε PE-Cy5 (145-2C11), anti-CD44 PE-Cy5 (IM7), and anti-CD69 PE Cy7 (H1.2F3).

Staining with CD1d-Ig-α-GalCer dimers and tetramers

Dimeric CD1d-Ig (BD Biosciences) was loaded at neutral pH, according to manufacturers' instructions, using a 40-fold molar excess of α-GalCer (Axxora). The loaded dimers were labeled by incubating for 1 h at room temperature with PE anti-mouse IgG, followed by unlabeled mouse IgG. The amount used for staining was determined experimentally for each batch of dimers prepared. For staining, 1×10^6 cells were resuspended in 50 μl of PBS, 2% FBS, and were incubated for 15 min at 4°C with 1 μg of Fc block, followed by 1.5 h at 4°C with prepared dimers. For costaining, Abs were added for another 30-min incubation, after which time the cells were washed twice in staining buffer, once in PBS, and resuspended in PBS for analysis by flow cytometry using a Coulter FC500 (Beckman Coulter). PE-CD1d-α-GalCer tetramers were prepared and used to stain thymocytes and splenocytes, according to the procedures described by Benlagha et al. (6, 33).

Analysis of gene expression in NKT cells

Thymic (CD1d-Ig-α-GalCer$^+$TCRβ$^+$), splenic (TCRβ$^+$NK1.1$^+$), and liver (CD4$^+$NK1.1$^+$) NKT cells from C57BL/6 mice were isolated using a FACS. Cells were recovered in T cell medium, and RNA prepared using TRIzol with 3 μg of yeast tRNA (BD Biosciences) was added as a carrier. As a nonsorted control, RNA was prepared from an equal number of total thymocytes, splenocytes, or liver lymphocytes. One microgram of each RNA sample was converted to cDNA, and the levels of *Cα* and *Vα14Jα281* transcripts were determined by PCR, as described (27). Fifteen microliters of the same cDNA prep was used with a custom-designed SuperArray PCR kit (SuperArray) to detect the transcripts for the indicated AP-1 components, *Gapdh*, and the NKT cell-enriched *T-bet* and *NK1.1* transcripts. For the analysis of α-GalCer-induced *IL-4* expression, 1×10^7 thymocytes and splenocytes from *non-Tg*, *CD1d$^{-/-}$*, and *BATF-Tg* mice were cultured for 18 h at 37°C, 5% CO_2 in the presence of 1 μg of α-GalCer. RNA was prepared, converted to cDNA, and analyzed for *HA-BATF* and *Cα* transcripts by PCR. The following primers were used to detect *IL-4*: forward, 5′-GATGGATGTGCCAAACGTCC-3′; reverse, 5′-CAT GATGCTCTTTAGGCTTTCC-3′. For the time course experiments, parallel cultures of 1×10^7 thymocytes or splenocytes were rested overnight and then left unstimulated, or treated with 1 μg of α-GalCer or vehicle as a control. RNA harvested at the indicated time points was analyzed for *IL-4* and *Cα* transcripts by PCR. The number of cycles used was adjusted to allow inducible *IL-4* gene expression to be distinguished from the basal transcript levels that are characteristic of resting NKT cells (34–36). All PCR-generated DNA products were resolved by 3% agarose gel electrophoresis and visualized with ethidium bromide.

Intracellular staining of HA-BATF protein

Splenocytes from non-*Tg* and *BATF-Tg* mice were isolated and stained, as described in Thornton et al. (23). Briefly, splenocytes were blocked with 2.4G2, surface stained with anti-TCRβ PE-Cy5 and anti-NK1.1 PE-Cy7, and fixed, as recommended by eBioscience. The cells were permeabilized using eBioscience permeabilization buffer and were incubated with anti-HA FITC clone 3F10 (Roche) to detect intracellular HA-BATF. After extensive washing in PBS, the cells were analyzed by flow cytometry. NKT cells (TCR$^+$NK1.1$^+$), NK cells (TCR$^-$NK1.1$^+$), and conventional T cells (TCR$^+$NK1.1$^-$) were identified, and levels of intracellular HA-BATF (anti-HA FITC) fluorescence were measured for each population.

Analysis of Vα14i-induced AP-1 DNA-binding activity

CD1d-Ig-α-GalCer dimers or anti-CD3ε at a concentration of 2 μg/ml in PBS were added to 60-mm tissue culture dishes and incubated at 37°C for 2 h (dimers) or overnight (CD3) to allow plate binding. Plates were washed three times with PBS before adding 1×10^7 hybridoma or primary cells in 5 ml of their respective medium. Cultures were incubated for 6 h, after which nuclear extracts were prepared using the NE-PER Kit (Pierce) and protein was quantified using the bicinchoninic acid protein assay (Bio-Rad). The procedure for EMSA using a ^{32}P-labeled AP-1 oligonucleotide probe has been described previously (23). To assay APC-induced, AP-1 activity, 5×10^6 thymocytes prepared from C57BL/6 mice were incubated overnight at 37°C with 1 μg of α-GalCer or vehicle control (0.5% Tween 20, PBS). On the following day, 5.0×10^6 DN32.D3 (Vα14i$^+$) or 431.A11 (Vα14i$^-$) cells were added, and after 6 h, nuclear extracts were prepared and EMSA was performed, as described above.

Results

HA-BATF targets all classes of Vα14i NKT cells

p56^{lck}-HA-BATF-Tg mice express an HA-tagged, human BATF protein from the constitutive, T cell-specific *p56^{lck}* proximal promoter (20, 22). Previous studies have shown that all T cell subsets within the thymus of *BATF-Tg* mice express the transgene (23), yet the only thymic T cell population that is underrepresented in these animals is the TCR$^+$NK1.1$^+$ (NKT) cells (20). To firmly establish that the thymus of *BATF-Tg* mice contains reduced numbers of glycolipid-reactive Vα14i NKT cells, primary thymocytes were stained with either anti-CD8, anti-TCRβ, or anti-CD44, and dimeric CD1d loaded with the synthetic NKT cell Ag, α-GalCer. As shown in Fig. 1*A*, thymocytes from *BATF-Tg* mice exhibited a marked reduction in α-GalCer-reactive NKT cells compared with non-*Tg* control animals. Thymocytes from *Jα18$^{-/-}$* mice, which lack the Jα18 cassette needed to form the Vα14i NKT cell TCR, and *CD1d$^{-/-}$* mice, which lack the ability to positively select NKT cell development in the thymus, were included as controls. Although *BATF-Tg* mice possess reduced numbers of thymic NKT

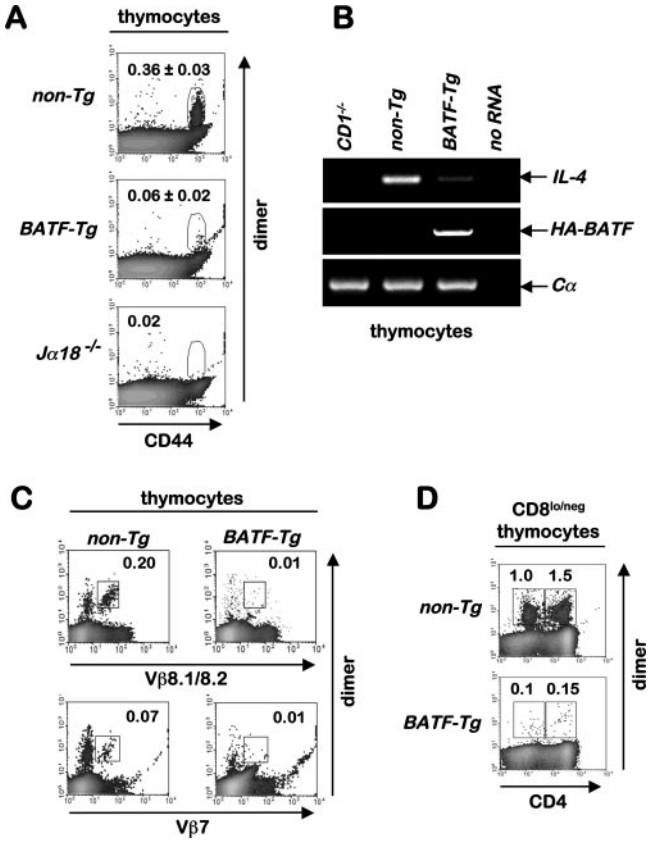

FIGURE 1. HA-BATF impacts all classes of thymic Vα14i NKT cells. *A*, Thymocytes were isolated from two *non-Tg*, two *BATF-Tg*, and two *Jα18*$^{-/-}$ mice; stained with CD1d-Ig-α-GalCer dimers, anti-CD8, anti-TCR, and anti-CD44; and analyzed by flow cytometry to quantify the percentage of NKT cells. Representative plots identifying dimer^{+}CD44^{+} NKT cells (inscribed circles) in each sample are shown. The percentages quantifying NKT cells (±SD, where possible) represent the averages from three independent experiments performed in duplicate, with the exception of the *Jα18*$^{-/-}$ control, which was performed once. *B*, Thymocytes from *non-Tg*, *CD1d*$^{-/-}$, and *BATF-Tg* mice were cultured for 18 h in the presence of α-GalCer. RT-PCR was used to detect *IL-4*, *HA-BATF*, and *Cα* transcripts. Parallel reactions without RNA (no RNA) are included as controls. The experiment was repeated a second time, and similar results were obtained. *C*, Thymocytes from one *non-Tg* and one *BATF-Tg* mice were stained with dimers and anti-Vβ8.1/8.2 or anti-Vβ7 Abs. Dimer^{+} Vβ8.1/2^{+} (*upper*) and dimer^{+} Vβ7^{+} (*lower*) cells are identified by the inscribed squares on the representative plots. Similar results were obtained using two additional mice and anti-Vβ7, anti-Vβ8, and anti-NK1.1 Abs. *D*, Thymocytes from one *non-Tg* and one *BATF-Tg* mouse were stained with dimers, anti-CD4, and anti-CD8. After gating on CD8$^{low/negative}$ cells, the indicated percentages of double-negative (*left inscribed square*) and CD4 single-positive (*right inscribed square*) NKT cells were determined.

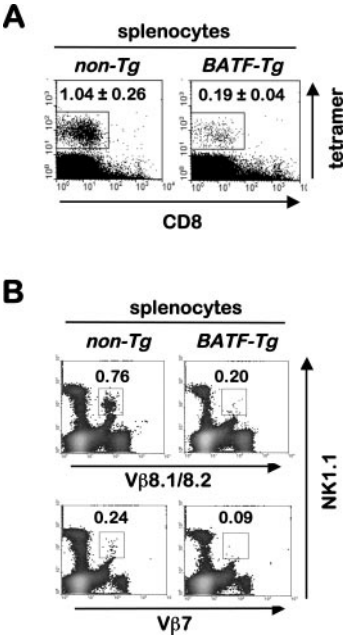

FIGURE 2. Splenic Vα14i NKT cells are decreased in *BATF-Tg* mice. *A*, Splenocytes from two *non-Tg* and two *BATF-Tg* mice were stained with α-GalCer tetramers and anti-CD8 and analyzed by flow cytometry. NKT cells are identified by the inscribed squares. The average percentage (±SD) was calculated from three independent experiments. *B*, Splenocytes from two *non-Tg* and two *BATF-Tg* mice were stained with anti-Vβ7, anti-Vβ8.1/2, and anti-NK1.1 Abs, and analyzed by flow cytometry. NKT cells were identified (inscribed squares) with the percentages representing the average obtained from two, independent experiments.

cells, the NKT cell deficiency is not as severe as in *Jα18*$^{-/-}$ or *CD1d*$^{-/-}$ mice, in which the NKT cell lineage fails to develop (Fig. 1*A* and data not shown). Consistent with the observation that residual NKT cells are present in the thymus of *BATF-Tg* mice, semiquantitative RT-PCR on total RNA purified from thymocytes stimulated in vitro with α-GalCer revealed a level of *IL-4* transcription that is dramatically less than non-*Tg*, but above the level in *CD1d*$^{-/-}$ mice (Fig. 1*B*). We conclude that Tg expression of BATF, an in vivo inhibitor of AP-1 transcriptional activity (21, 22), profoundly impacts the population of α-GalCer-reactive NKT cells in the thymus.

Although α-GalCer-reactive NKT cells show invariant expression of the Vα14Jα281 (Vα14i) TCRα chain, NKT cells pair this α-chain predominantly with either Vβ8 or Vβ7. Additionally, some Vα14i cells are CD4^{+}, whereas others are CD4^{-}CD8^{-}. Recently, it has been shown that these Vα14i subtypes possess different patterns of cytokine expression (37, 38) and, upon migration to the periphery, orchestrate different immune system functions (39). To establish whether constitutive BATF expression targets discrete NKT cell subsets within the thymus, T cells from non-*Tg* and *BATF-Tg* mice were stained with dimeric CD1d-Ig-α-GalCer and Abs against Vβ8.1/8.2, Vβ7, CD4, and CD8. Results show that the Vβ8^{+}, Vβ7^{+}, CD4^{+}, and CD4^{-}CD8^{-} NKT subtypes are all decreased in *BATF-Tg* mice (Fig. 1, *C* and *D*).

We extended our examination of Vα14i NKT cells in *BATF-Tg* and non-*Tg* mice using anti-CD8 and α-GalCer tetramers (Fig. 2*A*) and anti-Vβ8 or anti-Vβ7 Abs with anti-NK1.1 (Fig. 2*B*) to detect NKT cells in the spleen. The results parallel what was observed in the thymus, leading us to conclude that BATF acts globally to impact the development of all classes of Vα14i NKT cells in the thymus and in the periphery.

NKT cell maturation is delayed in BATF-Tg mice

In response to positive selection by CD1d and glycolipid ligands (18, 19, 40–42), Vα14i NKT cells undergo a series of expansion and maturation events that correlate first with the expression of the activation/memory marker, CD44, and later with the coexpression of NK markers such as NK1.1 (33, 43, 44). To determine how HA-BATF might impact NKT cell maturation, flow cytometry with CD1d-α-GalCer tetramers was used to track the emergence of CD44^{-}NK1.1^{-}, CD44^{+}NK1.1^{-}, and CD44^{+}NK1.1^{+} NKT cells over time. Consistent with published reports (33), 70% of the tetramer-positive cells in control, *non-Tg* mice coexpress CD44 and NK1.1 as early as 3 wk of age, and this percentage increases to 85% by 12 wk of age (Fig. 3). In contrast, thymic

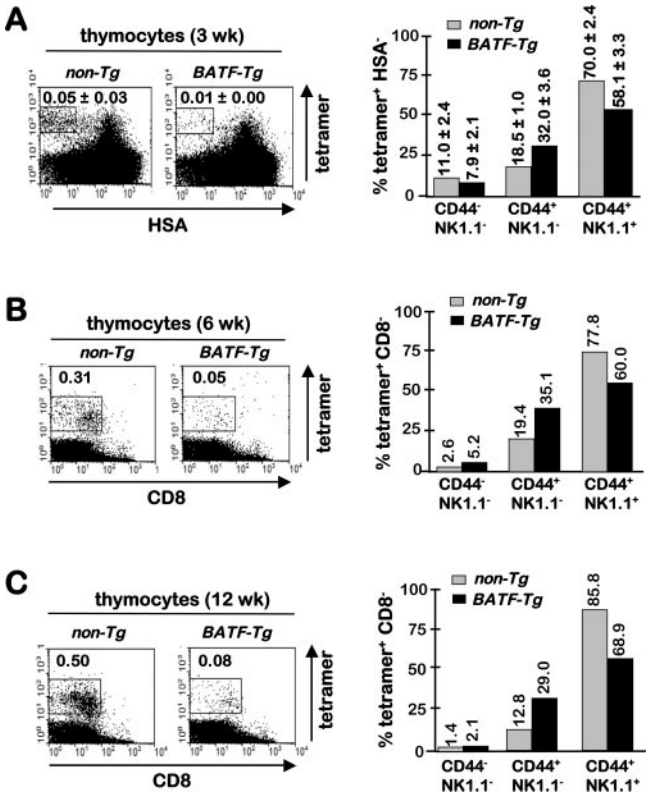

FIGURE 3. NKT cell expansion and maturation are impaired in *BATF-Tg* mice. Thymocytes from *non-Tg* and *BATF-Tg* mice at 3 wk (*A*), 6 wk (*B*), and 12 wk (*C*) of age were stained with CD1d-α-GalCer tetramers, anti-CD8, anti-CD44, anti-HSA, and anti-NK1.1. NKT cells were identified as tetramer$^+$CD8$^{low/negative}$ or tetramer$^+$HSA$^{low/negative}$ cells (inscribed squares, *left panels*) and then were reanalyzed for expression of CD44 and NK1.1 (*right panels*). This experiment was performed three times for *A*, two times for *B*, and a single time for *C*. For each age and genotype, the average percentages (±SD, where possible) are noted for total NKT cells (*left*), and for the CD44$^-$NK1.1$^-$, CD44$^+$NK1.1$^-$, and CD44$^+$ NK1.1$^+$ NKT cell subsets (*right*).

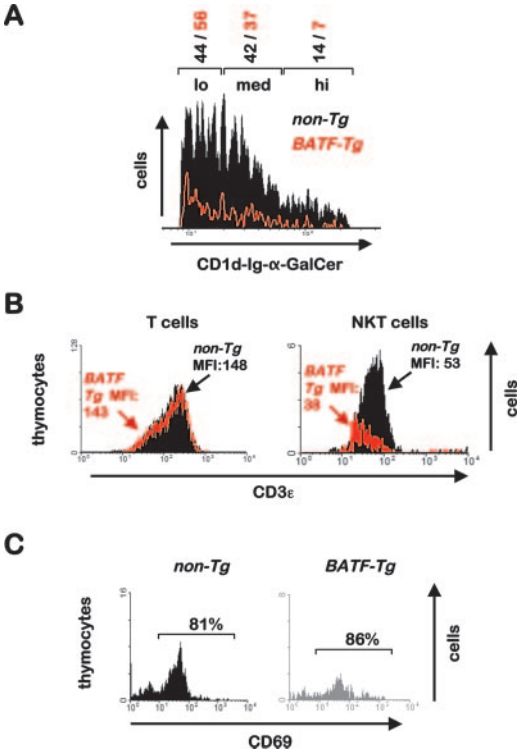

FIGURE 4. Residual NKT cells in the thymus of *BATF-Tg* mice are TCR/CD3^{low} and CD69 positive. *A*, Thymocytes from two *non-Tg* (black) and two *BATF-Tg* (red) mice were stained with CD1d-Ig-α-GalCer dimers and anti-CD8. Dimer$^+$CD8$^{low/negative}$ were replotted to compare the distribution of NKT cells exhibiting low (lo), medium (med), and high (hi) staining intensity. Brackets denote the range of cells counted. The percentages indicated for *non-Tg* and *BATF-Tg* NKT cells falling within each range represent the average of results obtained from two, independent experiments. *B*, Thymocytes from two *non-Tg* (black) and two *BATF-Tg* (red) mice were stained with dimers, anti-TCRβ, and anti-CD3ε. Dimer$^-$TCRβ$^+$ T cells (*right panel*) and dimer$^+$TCRβ$^+$ NKT cells (*left panel*) were reevaluated for CD3ε expression. Mean fluorescence intensity (MFI) was determined, and the results are presented as the average MFI. *C*, Thymocytes from one *non-Tg* and two *BATF-Tg* mice were stained with dimers, anti-CD8, and anti-CD69. The percentage of CD69$^+$ Tg NKT cells is indicated on each plot.

NKT cells from *BATF-Tg* mice express CD44$^+$, but do not transition efficiently to a CD44$^+$NK1.1$^+$ phenotype, even by 12 wk of age (Fig. 3). As a result, the maturation profile of *BATF-Tg* NKT cells undergoes minimal change between 3 and 12 wk of age. The reduced numbers of all classes of NKT cells in *BATF-Tg* mice, coupled with the relative inability of the BATF-expressing cells to adopt a CD44$^+$NK1.1$^+$ profile, demonstrate that both NKT cell expansion and maturation are impaired in these animals.

The residual Vα14i NKT population in BATF-Tg mice is TCR/CD3^{low} and CD69$^+$

It was noted during our analysis of Vα14i NKT cells from *BATF-Tg* mice using CD1d-Ig-α-GalCer dimers and tetramers that the level of staining displayed by these cells was consistently less intense than that of control cells. This observation suggested that *BATF-Tg* NKT cells express reduced quantities of the α-GalCer-reactive TCR and its associated proteins. To investigate this further, NKT cells (dimer$^+$ CD8$^{low/negative}$) from *non-Tg* and *BATF-Tg* mice were identified and then quantified based on staining intensity as dimer^{low}, dimer^{medium}, or dimer^{high}. Results show that 56% of the residual NKT cells present in the thymus of *BATF-Tg* mice are dimer^{low} and only 7% of the cells are dimer^{high} (Fig. 4*A*). This contrasts with the profile of normal NKT cells, which show that 44% are dimer^{low} and 14% are dimer^{high}. To validate these results, thymocytes from *non-Tg* and *BATF-Tg* mice were stained with CD1d-Ig-α-GalCer, anti-TCRβ, and anti-CD3ε, and the Vα14i NKT cells (dimer$^+$TCRβ$^+$) were reanalyzed for CD3ε staining intensity (Fig. 4*B*). Consistent with reduced levels of the α-GalCer-reactive TCR, *BATF-Tg* NKT cells show a 28% reduction in CD3ε mean fluorescence intensity compared with control cells. The reduced level of surface TCR expression on these cells does not correlate with the inability of cells to receive, or to interpret, positive selection signals. As reported previously, *BATF-Tg* thymocytes express normal levels of CD1d (20), and the cells were responsive initially to Ag presentation, as CD69 expression is equivalent to that of *non-Tg* controls (Fig. 4*C*). These findings support the previous observation that *BATF-Tg* NKT cells acquire expression of CD44 and thus are committed to the NKT cell lineage (Fig. 2).

NKT cells in the spleen of BATF-Tg mice are stable over time

To examine whether peripheral NKT cells in *BATF-Tg* mice are persistently low, decline further, or perhaps recover over time, flow cytometry with NK1.1 and TCRβ Abs was used to establish

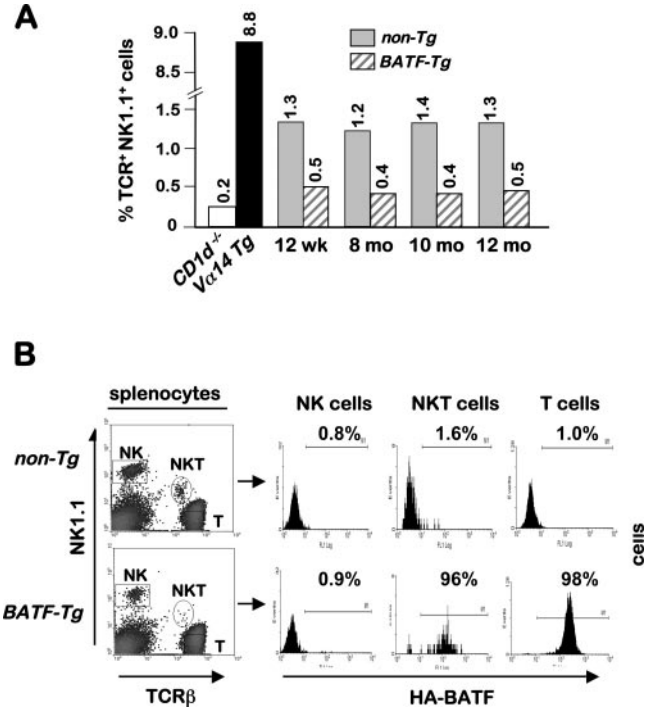

FIGURE 5. Peripheral NKT cells in *BATF-Tg* mice express HA-BATF and are stable over time. *A*, Splenocytes from *non-Tg* and *BATF-Tg* animals of the indicated ages, as well as splenocytes from control *CD1d*$^{-/-}$ and *Vα14-Tg* animals at 12 wk of age, were stained with anti-TCRβ and anti-NK1.1. TCRβ^+ NK1.1$^+$ cells were identified by flow cytometry, and the average percentages for each genotype at each age are presented as a bar graph. *B*, Splenocytes from *non-Tg* and *BATF-Tg* mice (*left panels*) were stained with anti-TCRβ and anti-NK1.1. After washing, fixation, and permeabilization, the cells were stained with anti-HA FITC. After identifying NK cells (NK1.1$^+$ TCRβ^-), NKT cells (TCRβ^+ NK1.1$^+$), and T cells (TCRβ^+ NK1.1$^-$), the level of anti-HA fluorescence (*right panels*) was determined. The percentages represent the number of cells expressing HA-BATF obtained from the analysis of five *BATF-Tg* and five *non-Tg* mice.

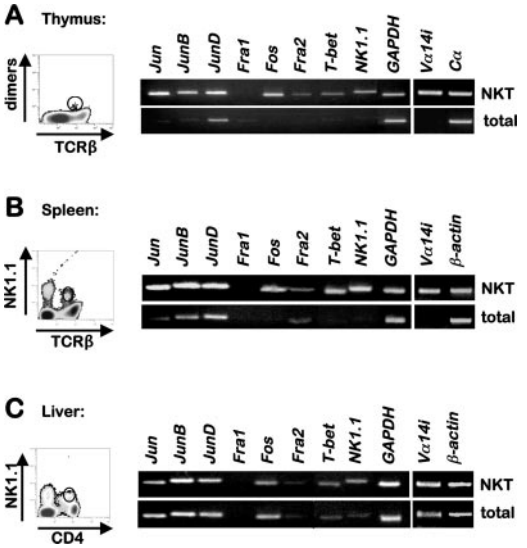

FIGURE 6. AP-1 factors are present in wild-type NKT cells. *A*, 1×10^4 dimer$^+$TCRβ^+ cells from thymus; *B*, 7×10^4 TCRβ^+NK1.1$^+$cells from spleen; and *C*, 2×10^4 CD4$^+$NK1.1$^+$ cells from liver were identified (*left flow cytometry plots*, inscribed circles) and purified using a FACS. RNA isolated from these purified NKT cells and from an equivalent number of nonsorted cells from the same tissues (total) was used with a custom SuperArray PCR kit to detect transcription of the indicated AP-1 family member genes and the *T-bet*, *NK1.1*, and *GAPDH* genes as controls. Standard, semiquantitative RT-PCR was used with the same RNA samples to detect transcripts from the *Vα14i TCR* gene, as a unique identifier of NKT cells, and from either the *Cα* or *β-actin* genes as a control for sample integrity. The gels shown in each panel are independent experiments, with NKT cells (in each experiment) purified from pools of 5–10 C57BL/6 mice.

the percentages of NKT cells in the spleens of *BATF-Tg* and *non-Tg* mice at 12 wk, 8 mo, 10 mo, and 1 year of age. As controls, the profiles of splenic NKT cells from *CD1d*$^{-/-}$ (26–28) mice and Tg mice biased for overproduction of NK1.1$^+$ Vα14i NKT cells (*Vα14-Tg*) (30) were used. As shown in Fig. 5*A*, there is no significant change in the NKT cell-deficient phenotype of *BATF-Tg* mice over time. This result appears to conflict with a previous study in which it was shown that NKT cells will divide to fill a deficient niche (45). However, using the same Abs to purify peripheral NK (NK1.1$^+$TCRβ^-), NKT (NK1.1$^+$TCRβ^+), and T (NK1.1$^-$TCRβ^+) cells and an anti-HA Ab to detect HA-BATF protein, we show that the *p56*^{lck}*-HA-BATF* transgene continues to be expressed in >95% of peripheral T and NKT cells (Fig. 5*B*), suggesting that BATF continues to exert its negative influence on the dynamics of NKT cell expansion in the periphery. Additional factors possibly contributing to the maintenance of a stable NKT cell deficiency in *BATF-Tg* animals will be considered in *Discussion*.

AP-1 components and activity are present in NKT cells

BATF inhibits AP-1 activity by forming heterodimers with the Jun proteins and displacing the canonical Jun partners (i.e., Jun or Fos proteins) associated with the transcriptional activation of AP-1 target genes (21, 46, 47). Because constitutive expression of BATF disrupts the development of NKT cells, the mechanism underlying this defect most likely involves the Jun and Fos family members that are expressed in NKT cells. To characterize the AP-1 factors that are expressed in NKT cells, a FACS was used to purify NKT cells from the thymus, spleen, and liver of C57BL/6 mice using marker combinations (dimer$^+$TCR$^+$; TCR$^+$NK1.1$^+$; CD4$^+$NK1.1$^+$) validated previously for the isolation of NKT cells from these tissues (6, 7, 48). RNA was prepared from the sorted cells and from equivalent numbers of unsorted cells prepared from the same tissues. To manipulate the low RNA yield from small numbers of purified NKT cell samples without significant losses, 3 μg of carrier yeast tRNA was added to each sample and subsequently used in all assays as a control (data not shown). RT-PCR with primers specific for the transcripts of six major *Jun* and *Fos* family genes was performed initially with RNA prepared from PMA- and ionomycin-treated mouse EL-4 cells, in which all six genes are expressed (49) (data not shown), and then on each of the NKT cell samples. Detection of transcripts representing the Vα14Jα281 TCRα chain, the T-bet transcription factor, and the NK1.1 surface marker was used as NKT cell expression controls. *GAPDH*, *Cα*, or *β-actin* primers were used as a control for RNA integrity. As shown in Fig. 6, all three *Jun* and two of the three *Fos* genes are expressed in NKT cells.

The presence of AP-1 component transcripts in purified NKT cells should correlate with the production of Jun and Fos proteins and with the presence of AP-1 DNA-binding activity. Because the phenotype of *BATF-Tg* mice suggests that the proper activation of AP-1 target genes is required for NKT cell responses, we predict that NKT cells stimulated through the Vα14i TCR should have

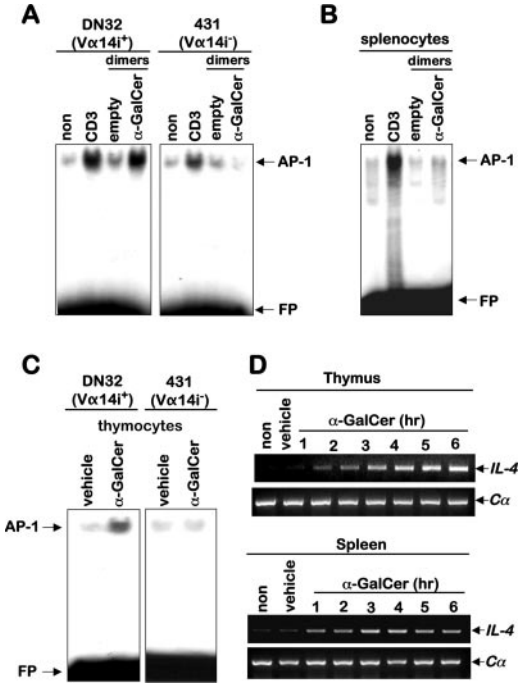

FIGURE 7. AP-1 DNA-binding activity and *IL-4* gene expression in NKT cells. *A*, DN32.D3 and A431.A11 cells were cultured without stimulation (non) or with plate-bound anti-CD3ε (CD3), dimeric CD1d-Ig (empty), or dimeric CD1d-Ig-α-GalCer (α-GalCer). After 6 h, nuclear extracts were prepared and used in EMSA with ^{32}P-labeled AP-1 DNA (see *Materials and Methods* for details). *B*, Nuclear extracts prepared from total splenocytes exposed for 6 h to the indicated stimuli were used for EMSA, as described in *A*. *C*, DN32.D3 and 431.A11 cells were cultured for 6 h together with wild-type C57BL/6 thymocytes that had been stimulated overnight with 1 μg of α-GalCer or solvent (vehicle) as a control. Nuclear extracts were prepared and analyzed for AP-1 DNA-binding activity, as described in *A*. *D*, Thymocytes and splenocytes from wild-type C57BL/6 mice were cultured in the absence (non) or presence of solvent (vehicle) or with α-GalCer for the indicated times (hr). RNA was prepared from the cultures and used in standard semiquantitative RT-PCR to evaluate levels of *IL-4* gene expression (see *Materials and Methods* for details). RT-PCR to detect *Cα* transcripts are included as the controls. The gels shown are representative of results obtained from two, independent experiments.

enhanced AP-1 activity, which is most reliably assessed by detecting an increase in AP-1 DNA binding. To test this prediction, EMSA were performed with nuclear extracts and a radiolabeled, double-stranded oligonucleotide containing an AP-1 consensus site. A pair of NKT cell hybridomas, DN32.D3 (Vα14i$^+$) and 431.A11 (Vα14i$^-$), was used as the source of nuclear extracts following a 6-h exposure to plate-bound CD1d-Ig-α-GalCer. As controls, the hybridomas were cultured on plates coated with anti-CD3ε Ab (positive) or empty CD1d-Ig (negative). Resolution of the DNA:protein complexes demonstrates a clear increase in AP-1 DNA-binding activity in DN32.D3 cells (Fig. 7*A*), but not in 431.A11 cells, indicating that AP-1 induction by α-GalCer depends on the Vα14i TCR. The experiment also was performed with nuclear extracts prepared from total splenocytes stimulated in the same manner (Fig. 7*B*). As expected, due to the reduced target cell number, α-GalCer-stimulated AP-1 activity is not as prominent as in the DN32 cell line, but is increased compared with the empty CD1d-Ig control. These results clearly indicate that AP-1 DNA-binding activity is present in NKT cells and is induced by signaling downstream of the glycolipid-reactive Vα14i TCR.

The ability of TCR signaling to efficiently activate a transcription factor within a given time frame is dependent upon the kinetics of Ag presentation by APCs. To confirm that thymocytes, an APC population relevant to the development of Vα14i NKT cells in vivo, can present glycolipid that induces AP-1 activity in NKT cells within 6 h, thymocytes from wild-type mice were incubated with α-GalCer overnight. The following day, either DN32.D3 (Vα14i$^+$) or 431.A11 (Vα14i$^-$) cells were added to the cultures for 6 h and nuclear extracts were prepared for use in EMSA. As shown in Fig. 7*C*, α-GalCer-pulsed thymocytes caused a significant increase in the AP-1 DNA-binding activity in DN32.D3 cells. In contrast, the AP-1 activity in 431.A11 cells was unchanged following exposure to α-GalCer-pulsed thymocytes. As predicted, the presentation of α-GalCer by thymocytes isolated from *CD1d*$^{-/-}$ mice had no effect on the AP-1-binding activity in Vα14i$^+$ DN32.D3 cells (data not shown).

As a demonstration that these EMSA results correlate with the kinetics of Vα14i NKT cell activation, thymocytes and splenocytes were treated with α-GalCer. At various time points following treatment, the cells were harvested and RNA was prepared. RT-PCR was used to track changes in the expression of *IL-4*, a well-characterized, AP-1 target gene (24, 25) that encodes a cytokine that is produced by NKT cells within 90 min of stimulation (50). For these experiments, PCR cycle number was reduced to distinguish induced *IL-4* mRNA expression from the basal levels of *IL-4* mRNA expressed by resting NKT cells (34–36). As shown in Fig. 7*D*, after only 1 h of exposure to α-GalCer, both splenocytes and thymocytes show a marked increase in *IL-4* transcription. This induced expression peaked between 2 and 4 h and remained high throughout the 6-h duration of the experiment. These results are consistent with what is known about cytokine production by NKT cells and correlate well with the induction of AP-1 DNA binding downstream of the Vα14i TCR (Fig. 7, *A–C*). Therefore, we conclude that TCR-induced AP-1 activity is linked temporally to NKT cell gene expression. Furthermore, perturbation of AP-1 activity (through the constitutive expression of BATF) identifies AP-1 as a critical regulator of the earliest stages of NKT cell development within the thymus.

Discussion

Previously, our laboratory reported the NKT cell-deficient phenotype of Tg mice expressing a T cell-restricted transgene encoding BATF, a negative regulator of the AP-1 family of bZIP transcription factors. BATF dimerizes with the Jun proteins to generate sequence-specific DNA-binding complexes with little to no transactivation potential and promotes a slow growth phenotype that has been linked to the inhibition of mitogenic signaling (21, 23) and to the initiation of cellular differentiation (51). Our observation that constitutive expression of BATF in vivo results in a NKT-specific phenotype, rather than the anticipated, broad impact on proliferation and signaling in all T lymphocytes, warranted further investigation.

In the current work, we extend our analysis of *BATF-Tg* mice to show that BATF expression results in reduced numbers of all α-GalCer-reactive NKT cells, regardless of Vβ gene usage or CD4 coexpression. This indicates that the effects of BATF are not dependent on particular ligand affinities or on the specific functionalities described recently for distinct NKT cell subsets (37–39). Fortunately, the Vα14i NKT cell-deficient phenotype of *BATF-Tg* mice is not completely penetrant, thus providing us with an opportunity to examine the properties of the residual NKT cells found in the thymus and periphery.

Using the CD44 and NK1.1 markers to follow the developmental profile of thymic NKT cells in *BATF-Tg* mice, we found that

maturation of these cells was delayed. In addition, the residual NKT cell population in the thymus of *BATF-Tg* mice had a clear bias toward reduced surface expression of both the Vα14i TCR and the associated signaling subunit CD3ε. Following normal activation, NKT cells naturally lack surface TCR expression for a period of time (52–55). However, a reduction in surface TCR expression during T cell development also can be diagnostic of aberrant signal transduction during positive selection (56). In this regard, our observation that *BATF-Tg* NKT cells express normal levels of CD69 and then, ultimately, CD44, indicates that the earliest presentation and signaling events are taking place. Therefore, it appears that expression of BATF skews the NKT cells that eventually emerge toward a population with a reduced avidity for ligand. Although these cells do develop over time, they progress with delayed kinetics, as might be predicted for cells expressing an AP-1 inhibitor. The mechanism underlying this skewing is unknown, although recent data from our lab show that α-Gal-Cer-reactive thymic NKT cells from *BATF-Tg* mice display increased staining with annexin V, an indication that apoptosis might play a role (A. Zullo and E. Taparowsky, unpublished data).

There is a clear, negative effect of HA-BATF expression on the development of the NKT cell lineage in the thymus. In addition, we have shown that the *BATF* transgene continues to be expressed in the periphery, where the NKT cells that complete maturation are maintained as a small, but stable, population of cells. The observation that the NKT cells in the spleens of *BATF-Tg* mice fail to expand over time is consistent with the documented negative influence of BATF on cell growth (21, 23). However, we have recent evidence that *BATF-Tg* splenocytes contain twice the number of $CD8^+$ lymphocytes coexpressing the activation/memory marker, CD44 (A. Zullo and E. Taparowsky, unpublished data). These peripheral memory-type $CD8^+$ cells have been shown by Matsuda et al. (45) to engage in direct and rigorous competition with both NK and NKT cells for the cytokine IL-15, which is required for both NK and NKT cells to expand following stimulation. In support of this, we have noted in all of our studies that NK cells ($TCR^-NK1.1^+$), which are negative for expression of the *HA-BATF* transgene (Fig. 5*B*), are less abundant in *BATF-Tg* mice than in control mice (20) (Figs. 2*B* and 5*B*). Clearly, understanding why the inhibition of AP-1 activity by BATF leads to an increase in memory-type, $CD8^+CD44^+$ cells is an interesting future avenue of research. Additionally, future studies using *BATF-Tg* animals to examine the responsiveness of peripheral Vα14i NKT cells to glycolipid Ag must take into account potential effects generated from the increased presence of these potent IL-15 consumers.

Based upon the phenotype of *BATF-Tg* mice, we predicted that AP-1 family bZIP proteins would be expressed in NKT cells and that increased DNA binding by AP-1 complexes would be observed in response to Vα14i TCR signaling. Indeed, purified NKT cells possess transcripts for five of the six AP-1 factors examined, including the *c-Fos* and *c-Jun* transcripts that were detected in a recent profiling of NKT cell transcripts using microarrays (14). Most importantly, we detect expression of all three *Jun* genes, indicating that NKT cells possess sufficient quantities of the bZIP proteins that are the preferred dimerization partners for BATF (46, 57, 58). In this regard, endogenous *BATF* mRNA is expressed in purified NKT cells (data not shown), although at much reduced levels compared with the level expressed from the *p56^{lck}-HA-BATF* transgene (23). Within 6 h of exposure of NKT cells to α-GalCer presented either by dimers or by primary APCs, there is a substantial increase in the binding of AP-1 complexes to DNA. The timing of this induction correlates well with increased expression of *IL-4*, a well-established AP-1 target gene (24), providing solid evidence to link signaling through the Vα14i TCR with AP-1 activity and a relevant, NKT cell response.

The role of AP-1 in conventional T lymphocyte function has been the focus of intense research for many years. In conventional T cells, signals transduced through both the TCR and CD28 coreceptor collaborate to generate maximum AP-1 transcriptional activity in response to Ag (59). In that way, complete AP-1 activity is a tangible demonstration of the two-signal hypothesis. Although our data demonstrate biochemically that signaling through the Vα14i TCR induces AP-1 activity, recent studies from other groups have shown that NKT cells, like other T cells, rely on signals from additional receptors and pathways (60–63). The extent to which these other pathways activate AP-1 is not known. Although we plan to continue to use experimental approaches with AP-1 inhibitors to further explore this question, establishing the dependence of any one set of signaling events on AP-1 will be a challenge given the modular nature of the multiple bZIP proteins that comprise this dimeric transcription factor (64) and the fact that vectors designed to target gene knockouts exclusively to the NKT cell lineage are not yet readily available.

In recent years, a number of studies have been focused on NKT cell transcription factor networks to further understand the unique features associated with signaling through the Vα14i TCR. These studies have shown that Runx and RORγt function are required for the earliest stages of NKT selection (18, 19), that members of the NF-κB family are required extrinsically (and in a cell autonomous fashion), throughout NKT cell maturation (9–12), and that T-bet is essential for production of mature, NK1.1-expressing NKT cells in thymus (13, 14). Although not as precisely defined, the NKT compartment does not develop properly in mice lacking the Ets-1 (15), Mef (16), or Irf-1 (17) transcription factors. In addition, the unique and plastic properties of NKT cell transcription have been exemplified by recent work showing differential use of GATA-3, Stat6, and NF-AT2 by $TCR^+NK1.1^+$ NKT cells (48). This further supports the idea that broadly used transcription factor networks, such as AP-1, can be reconfigured in NKT cells to generate new models of gene regulation. As a result of our studies, we now know that the positive action of AP-1 complexes is critical to the establishment and maturation of thymic NKT cells. Ultimately, with continued effort, a complete map of the signal transduction cascades active in all NKT cell subsets will be generated and provide new opportunities to predict the transcriptional output of this important class of T lymphocyte.

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Disclosures

The authors have no financial conflict of interest.

References

1. Brutkiewicz, R. R. 2006. CD1d ligands: the good, the bad, and the ugly. *J. Immunol.* 177: 769–775.
2. Van Kaer, L. 2005. α-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat. Rev. Immunol.* 5: 31–42.
3. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23: 877–900.
4. 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.

5. Natori, T., K. Akimoto, K. Motoki, Y. Koezuka, and T. Higa. 1997. Development of KRN7000, derived from agelasphin produced by Okinawan sponge. *Nippon Yakurigaku Zasshi* 110(Suppl. 1): 63P–68P.
6. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.* 191: 1895–1903.
7. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192: 741–754.
8. Schumann, J., R. B. Voyle, B. Y. Wei, and H. R. MacDonald. 2003. Cutting edge: influence of the TCR Vβ domain on the avidity of CD1d:α-galactosylceramide binding by invariant Vα14 NKT cells. *J. Immunol.* 170: 5815–5819.
9. Nakagawa, K., K. Iwabuchi, K. Ogasawara, M. Ato, M. Kajiwara, H. Nishihori, C. Iwabuchi, H. Ishikura, R. A. Good, and K. Onoe. 1997. Generation of NK1.1$^+$ T cell antigen receptor $\alpha\beta^+$ thymocytes associated with intact thymic structure. *Proc. Natl. Acad. Sci. USA* 94: 2472–2477.
10. Sivakumar, V., K. J. Hammond, N. Howells, K. Pfeffer, and F. Weih. 2003. Differential requirement for Rel/nuclear factor κB family members in natural killer T cell development. *J. Exp. Med.* 197: 1613–1621.
11. Stanic, A. K., J. S. Bezbradica, J. J. Park, L. Van Kaer, M. R. Boothby, and S. Joyce. 2004. Cutting edge: the ontogeny and function of Vα14Jα18 natural T lymphocytes require signal processing by protein kinase Cθ and NF-κB. *J. Immunol.* 172: 4667–4671.
12. 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.
13. 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.
14. Matsuda, J. L., Q. Zhang, R. Ndonye, S. K. Richardson, A. R. Howell, and L. Gapin. 2005. T-bet concomitantly controls migration, survival and effector functions during the development of Vα14i NKT cells. *Blood* 107: 2797–2805.
15. Walunas, T. L., B. Wang, C. R. Wang, and J. M. Leiden. 2000. Cutting edge: the Ets1 transcription factor is required for the development of NK T cells in mice. *J. Immunol.* 164: 2857–2860.
16. Lacorazza, H. D., Y. Miyazaki, A. Di Cristofano, A. Deblasio, C. Hedvat, J. Zhang, C. Cordon-Cardo, S. Mao, P. P. Pandolfi, and S. D. Nimer. 2002. The ETS protein MEF plays a critical role in perforin gene expression and the development of natural killer and NK-T cells. *Immunity* 17: 437–449.
17. Ohteki, T., H. Yoshida, T. Matsuyama, G. S. Duncan, T. W. Mak, and P. S. Ohashi. 1998. The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of natural killer 1.1$^+$ T cell receptor-$\alpha\beta^+$ (NK1$^+$ T) cells, natural killer cells, and intestinal intraepithelial T cells. *J. Exp. Med.* 187: 967–972.
18. Egawa, T., G. Eberl, I. Taniuchi, K. Benlagha, F. Geissmann, L. Hennighausen, A. Bendelac, and D. R. Littman. 2005. Genetic evidence supporting selection of the Vα14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* 22: 705–716.
19. Bezbradica, J. S., T. Hill, A. K. Stanic, L. Van Kaer, and S. Joyce. 2005. Commitment toward the natural T (iNKT) cell lineage occurs at the CD4$^+$8$^+$ stage of thymic ontogeny. *Proc. Natl. Acad. Sci. USA* 102: 5114–5119.
20. Williams, K. L., A. J. Zullo, M. H. Kaplan, R. R. Brutkiewicz, C. D. Deppmann, C. Vinson, and E. J. Taparowsky. 2003. BATF transgenic mice reveal a role for activator protein-1 in NKT cell development. *J. Immunol.* 170: 2417–2426.
21. Echlin, D. R., H. J. Tae, N. Mitin, and E. J. Taparowsky. 2000. B-ATF functions as a negative regulator of AP-1 mediated transcription and blocks cellular transformation by Ras and Fos. *Oncogene* 19: 1752–1763.
22. Williams, K. L., I. Nanda, G. E. Lyons, C. T. Kuo, M. Schmid, J. M. Leiden, M. H. Kaplan, and E. J. Taparowsky. 2001. Characterization of murine BATF: a negative regulator of activator protein-1 activity in the thymus. *Eur. J. Immunol.* 31: 1620–1627.
23. Thornton, T. M., A. J. Zullo, K. L. Williams, and E. J. Taparowsky. 2006. Direct manipulation of activator protein-1 controls thymocyte proliferation in vitro. *Eur. J. Immunol.* 36: 160–169.
24. Rooney, J. W., T. Hoey, and L. H. Glimcher. 1995. Coordinate and cooperative roles for NF-AT and AP-1 in the regulation of the murine IL-4 gene. *Immunity* 2: 473–483.
25. Macian, F., C. Garcia-Rodriguez, and A. Rao. 2000. Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. *EMBO J.* 19: 4783–4795.
26. Smiley, S. T., M. H. Kaplan, and M. J. Grusby. 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* 275: 977–979.
27. Chen, Y. H., N. M. Chiu, M. Mandal, N. Wang, and C. R. Wang. 1997. Impaired NK1$^+$ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* 6: 459–467.
28. Mendiratta, S. K., W. D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6: 469–477.
29. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623–1626.
30. Bendelac, A., R. D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J. Exp. Med.* 184: 1285–1293.
31. Lantz, O., and A. Bendelac. 1994. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4$^+$ and CD4$^-$8$^-$ T cells in mice and humans. *J. Exp. Med.* 180: 1097–1106.
32. Bendelac, A., O. Lantz, M. E. Quimby, J. W. Yewdell, J. R. Bennink, and R. R. Brutkiewicz. 1995. CD1 recognition by mouse NK1$^+$ T lymphocytes. *Science* 268: 863–865.
33. 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.
34. Tanaka, S., J. Tsukada, W. Suzuki, K. Hayashi, K. Tanigaki, M. Tsuji, H. Inoue, T. Honjo, and M. Kubo. 2006. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* 24: 689–701.
35. Matsuda, J. L., L. Gapin, J. L. Baron, S. Sidobre, D. B. Stetson, M. Mohrs, R. M. Locksley, and M. Kronenberg. 2003. Mouse Vα14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc. Natl. Acad. Sci. USA* 100: 8395–8400.
36. Stetson, D. B., M. Mohrs, R. L. Reinhardt, J. L. Baron, Z. E. Wang, L. Gapin, M. Kronenberg, and R. M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J. Exp. Med.* 198: 1069–1076.
37. 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.
38. Lee, P. T., K. Benlagha, L. Teyton, and A. Bendelac. 2002. Distinct functional lineages of human Vα24 natural killer T cells. *J. Exp. Med.* 195: 637–641.
39. 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.
40. 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.
41. Zhou, D., J. Mattner, C. Cantu III, N. Schrantz, N. Yin, Y. Gao, Y. Sagiv, K. Hudspeth, Y. P. Wu, T. Yamashita, et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306: 1786–1789.
42. 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.
43. 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.
44. 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.
45. Matsuda, J. L., L. Gapin, S. Sidobre, W. C. Kieper, J. T. Tan, R. Ceredig, C. D. Surh, and M. Kronenberg. 2002. Homeostasis of Vα14i NKT cells. *Nat. Immunol.* 3: 966–974.
46. Dorsey, M. J., H. J. Tae, K. G. Sollenberger, N. T. Mascarenhas, L. M. Johansen, and E. J. Taparowsky. 1995. B-ATF: a novel human bZIP protein that associates with members of the AP-1 transcription factor family. *Oncogene* 11: 2255–2265.
47. Foletta, V. C., D. H. Segal, and D. R. Cohen. 1998. Transcriptional regulation in the immune system: all roads lead to AP-1. *J. Leukocyte Biol.* 63: 139–152.
48. Wang, Z. Y., S. Kusam, V. Munugalavadla, R. Kapur, R. R. Brutkiewicz, and A. L. Dent. 2006. Regulation of Th2 cytokine expression in NKT cells: unconventional use of Stat6, GATA-3, and NFAT2. *J. Immunol.* 176: 880–888.
49. Kim, H. P., and W. J. Leonard. 2002. The basis for TCR-mediated regulation of the IL-2 receptor α chain gene: role of widely separated regulatory elements. *EMBO J.* 21: 3051–3059.
50. Yoshimoto, T., A. Bendelac, J. Hu-Li, and W. E. Paul. 1995. Defective IgE production by SJL mice is linked to the absence of CD4$^+$, NK1.1$^+$ T cells that promptly produce interleukin 4. *Proc. Natl. Acad. Sci. USA* 92: 11931–11934.
51. Senga, T., T. Iwamoto, S. E. Humphrey, T. Yokota, E. J. Taparowsky, and M. Hamaguchi. 2002. Stat3-dependent induction of BATF in M1 mouse myeloid leukemia cells. *Oncogene* 21: 8186–8191.
52. Crowe, N. Y., A. P. Uldrich, K. Kyparissoudis, K. J. Hammond, Y. Hayakawa, S. Sidobre, R. Keating, M. Kronenberg, M. J. Smyth, and D. I. Godfrey. 2003. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J. Immunol.* 171: 4020–4027.
53. Wilson, M. T., C. Johansson, D. Olivares-Villagomez, A. K. Singh, A. K. Stanic, C. R. Wang, S. Joyce, M. J. Wick, and L. Van Kaer. 2003. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc. Natl. Acad. Sci. USA* 100: 10913–10918.
54. Harada, M., K. Seino, H. Wakao, S. Sakata, Y. Ishizuka, T. Ito, S. Kojo, T. Nakayama, and M. Taniguchi. 2004. Down-regulation of the invariant Vα14 antigen receptor in NKT cells upon activation. *Int. Immunol.* 16: 241–247.
55. Uldrich, A. P., N. Y. Crowe, K. Kyparissoudis, D. G. Pellicci, Y. Zhan, A. M. Lew, P. Bouillet, A. Strasser, M. J. Smyth, and D. I. Godfrey. 2005. NKT

cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J. Immunol.* 175: 3092–3101.

56. Alberola-Ila, J., and G. Hernandez-Hoyos. 2003. The Ras/MAPK cascade and the control of positive selection. *Immunol. Rev.* 191: 79–96.
57. Newman, J. R., and A. E. Keating. 2003. Comprehensive identification of human bZIP interactions with coiled-coil arrays. *Science* 300: 2097–2101.
58. Vinson, C., M. Myakishev, A. Acharya, A. A. Mir, J. R. Moll, and M. Bonovich. 2002. Classification of human B-ZIP proteins based on dimerization properties. *Mol. Cell. Biol.* 22: 6321–6335.
59. Rincon, M., and R. A. Flavell. 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J.* 13: 4370–4381.
60. Nichols, K. E., J. Hom, S. Y. Gong, A. Ganguly, C. S. Ma, J. L. Cannons, S. G. Tangye, P. L. Schwartzberg, G. A. Koretzky, and P. L. Stein. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat. Med.* 11: 340–345.
61. Pasquier, B., L. Yin, M. C. Fondaneche, F. Relouzat, C. Bloch-Queyrat, N. Lambert, A. Fischer, G. de Saint-Basile, and S. Latour. 2005. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J. Exp. Med.* 201: 695–701.
62. Chung, B., A. Aoukaty, J. Dutz, C. Terhorst, and R. Tan. 2005. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J. Immunol.* 174: 3153–3157.
63. Larkin, J., G. J. Renukaradhya, V. Sriram, W. Du, J. Gervay-Hague, and R. R. Brutkiewicz. 2006. CD44 differentially activates mouse NK T cells and conventional T cells. *J. Immunol.* 177: 268–279.
64. Deppmann, C. D., R. S. Alvania, and E. J. Taparowsky. 2006. Cross-species annotation of basic leucine zipper factor interactions: insight into the evolution of closed interaction networks. *Mol. Biol. Evol.* 23: 1480–1492.