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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

Alfred J. Zullo,* Kamel Benlagha,** Albert Bendelac,† and Elizabeth J. Taparowsky3*

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 (Vα14iT) NKT cells, regardless of CD4 expression and Vβ gene usage. The residual NKT cells in the thymus of BATF-Tg mice are CD44low, yet are slow to display the NK1.1 marker characteristic of mature cells. As a population, BATF-expressing NKT cells are TCRβ/CD3ε-expressing, 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 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 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), Mef2 (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, p56lck-HA-BATF transgene encoding a hemagglutinin (HA) Ag-tagged B cell-activating transcription factor (BATF) (20), an inhibitory component of the AP-1 DNA-binding activity, reduced expression of a stably integrated AP-1 reporter gene, and the absence of a mi- togenic response following stimulation in vitro (20, 22, 23). Interestingly, whereas conventional T cell development remains intact in these animals, thymic and peripheral TCRβNK1.1+ 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 p56lck-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 TCRβlow, 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...
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 Va14i 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 p56lck-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 JaaB−/− mice (26–29) were obtained from R. Brutkiewicz (Indiana University School of Medicine, Indianapolis, IN). Va14i-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 isolation and culturing of primary cells in 3 ml of 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 (A58-1), 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-CD3e PE-Cy5 (145-2C11), anti-CD44 PE-Cy5 (IM7), and anti-CD69 PE Cy7 (H1.2F3).

Staining with CD1d-lg-α-GalCer dimers and tetrastims

Dimeric CD1-d-Ig-α-GalCer (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 7 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 × 106 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 the time 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 tetrastems 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 Va14i alleles 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, Gaqβδ, and the NKT cell-enriched T-bet and NK1.1 transcripts. For the analysis of α-GalCer-induced IL-4 expression, 1 × 106 thymocytes and splenocytes from non-Tg, CD1d−/−, and BATF-Tg mice were cultured for 18 h at 37°C, 5% CO2 in the presence of 1 μg of α-GalCer. RNA was prepared, converted to cDNA, and analyzed for HA-BATF and Cd4 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 Va14i-induced AP-1 DNA-binding activity

CD1d-lg-α-GalCer dimers or anti-CD3e 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 × 106 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 32P-labeled AP-1 oligonucleotide probe has been described previously (23). To assay APC-induced, AP-1 activity, 5 × 105 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 × 106 DN32.D3 (Va14i+) or 431A11 (Va14i−) 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 Va14i NKT cells

p56lck-HA-BATF-Tg mice express an HA-tagged, human BATF protein from the constitutive, T cell-specific p56lck 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 Va14i 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. 1A, thymocytes from BATF-Tg mice exhibited a marked reduction in α-GalCer-reactive NKT cells compared with non-Tg control animals. Thymocytes from JaaB−/− mice, which lack the Ja18 cassette needed to form the Va14i 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

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cells, the NKT cell deficiency is not as severe as in \(\text{J}a \text{I}8^8-/-\) or \(CD1d^{-/-}\) mice, in which the NKT cell lineage fails to develop (Fig. 1A and data not shown). Consistent with the observation that residual NKT cells are present in the thymus of \(\text{BATF-Tg}\) mice, semiquantitative RT-PCR on total RNA purified from thymocytes stimulated in vitro with \(\alpha\text{-GalCer}\) revealed a level of \(IL-4\) transcription that is dramatically less than \(\text{non-Tg}\), but above the level in \(CD1d^{-/-}\) mice (Fig. 1B). We conclude that Tg expression of BATF, an in vivo inhibitor of AP-1 transcriptional activity (21, 22), profoundly impacts the population of \(\alpha\text{-GalCer}\)-reactive NKT cells in the thymus.

Although \(\alpha\text{-GalCer}\)-reactive NKT cells show invariant expression of the \(\text{V}\alpha14\text{J}a281\) (\(\text{V}\alpha14i\)) TCR\(\alpha\) chain, NKT cells pair this \(\alpha\)-chain predominantly with either \(V\beta8\) or \(V\beta7\). Additionally, some \(\text{V}\alpha14i\) cells are \(CD4^+\), whereas others are \(CD4^-CD8^+\) (18, 19, 40 – 42). We extended our examination of \(\text{V}\alpha14i\) NKT cells in \(\text{BATF-Tg}\) and \(\text{non-Tg}\) mice using anti-CD8 and \(\alpha\text{-GalCer}\) tetramers (Fig. 2A) and anti-\(V\beta8\) or anti-\(V\beta7\) Abs with anti-NK1.1 (Fig. 2B) 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 \(\text{V}\alpha14i\) NKT cells in the thymus and in the periphery.

**NKT cell maturation is delayed in \(\text{BATF-Tg}\) mice**

In response to positive selection by CD1d and glycolipid ligands (18, 19, 40 – 42), \(\text{V}\alpha14i\) 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 establish whether constitutive BATF expression targets discrete NKT cell subsets within the thymus, T cells from \(\text{non-Tg}\) and \(\text{BATF-Tg}\) mice were stained with dimeric CD1d-Ig-\(\alpha\text{-GalCer}\) and Abs against \(V\beta8.1/2\), \(V\beta7\), CD4, and CD8. Results show that the \(V\beta8^+, V\beta7^+, CD4^+,\) and \(CD4^-CD8^-\) NKT subtypes are all decreased in \(\text{BATF-Tg}\) mice (Fig. 1, C and D).

We extended our examination of \(\text{V}\alpha14i\) NKT cells in \(\text{BATF-Tg}\) and \(\text{non-Tg}\) mice using anti-CD8 and \(\alpha\text{-GalCer}\) tetramers (Fig. 2A) and anti-\(V\beta8\) or anti-\(V\beta7\) Abs with anti-NK1.1 (Fig. 2B) 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 \(\text{V}\alpha14i\) NKT cells in the thymus and in the periphery.

**FIGURE 1.** HA-BATF impacts all classes of thymic \(\text{V}\alpha14i\) NKT cells. A. Thymocytes were isolated from two \(\text{non-Tg}\), two \(\text{BATF-Tg}\), and two \(\text{Ja}18^-/-\) mice; stained with CD1d-Ig-\(\alpha\text{-GalCer}\) 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 (\(\pm SD\), where possible) represent the averages from three independent experiments performed in duplicate, with the exception of the \(\text{Ja}18^-/-\) control, which was performed once. B. Thymocytes from \(\text{non-Tg}, CD1d^{-/-}\), and \(\text{BATF-Tg}\) mice were cultured for 18 h in the presence of \(\alpha\text{-GalCer}\). RT-PCR was used to detect \(IL-4, HA-BATF,\) and \(Ca\) 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 \(\text{non-Tg}\) and one \(\text{BATF-Tg}\) mice were stained with dimers and anti-\(V\beta8.1/2\) or anti-\(V\beta7\) Abs. Dimer+ \(V\beta8.1/2\) (upper) and dimer- \(V\beta7^+\) (lower) cells are identified by the inscribed squares on the representative plots. Similar results were obtained using two additional mice and anti-\(V\beta7\), anti-\(V\beta8\), and anti-NK1.1 Abs. D. Thymocytes from one \(\text{non-Tg}\) and one \(\text{BATF-Tg}\) mouse were stained with dimers, anti-CD4, and anti-CD8. After gating on CD8\text{high}Cells, the indicated percentages of double-negative (left inscribed square) and CD4 single-positive (right inscribed square) NKT cells were determined.

**FIGURE 2.** Splenic \(\text{V}\alpha14i\) NKT cells are decreased in \(\text{BATF-Tg}\) mice. A. Splenocytes from two \(\text{non-Tg}\) and two \(\text{BATF-Tg}\) mice were stained with \(\alpha\text{-GalCer}\) tetramers and anti-CD8 and analyzed by flow cytometry. NKT cells are identified by the inscribed squares. The average percentage (\(\pm SD\)) was calculated from three independent experiments. B. Splenocytes from two \(\text{non-Tg}\) and two \(\text{BATF-Tg}\) mice were stained with anti-\(V\beta7\), anti-\(V\beta8.1/2\), 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.
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/CD3low 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+CD8low/negative) from non-Tg and BATF-Tg mice were identified and then quantified based on staining intensity as dimerlow, dimeronmed, or dimerhigh. Results show that 56% of the residual NKT cells present in the thymus of BATF-Tg mice are dimerlow and only 7% of the cells are dimerhigh (Fig. 4A). This contrasts with the profile of normal NKT cells, which show that 44% are dimerlow and 14% are dimerhigh. To validate these results, thymocytes from non-Tg and BATF-Tg mice were stained with CD1d-Ig-α-GalCer dimers and CD69+ Abs was used to establish 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+T cells (right panel) and dimer+TCRβ+ NKT cells (left panel) were reevaluated for CD3e expression. Mean fluorescence intensity (MFI) was determined, and the results are presented as the average MFI.

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.
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<sup>−/−</sup> and Vα14i-Tg animals were used. In Fig. 5A, 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 identify peripheral NK (NK1.1<sup>+</sup> TCRβ<sup>+</sup>), NKT (NK1.1<sup>+</sup> TCRβ<sup>+</sup>), and T (NK1.1<sup>−</sup> TCRβ<sup>+</sup>) cells and an anti-HA Ab to detect HA-BATF protein, we show that the p56<sup>lck</sup>-HA-BATF transgene continues to be expressed in >95% of peripheral T and NKT cells (Fig. 5B), suggesting that BATF continues to exert 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<sup>T</sup> TCR<sup>+</sup>; TCR<sup>+</sup>NK1.1<sup>+</sup>; CD4<sup>+</sup>NK1.1<sup>+</sup>) 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 Vα14i TCRα chain, the T-bet transcription factor, and the NK1.1 surface marker was used as NKT cell expression controls. GAPDH, Csa, 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
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 (empty), or dimeric CD1d-Ig-α-GalCer (α-GalCer). After 6 h, nuclear extracts were prepared and used in EMSA with [32P]-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 Ca transcripts are included as the controls. The gels shown are representative of results obtained from two, independent experiments.

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. 7C, 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. 7D, 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. 5B), are less abundant in BATF-Tg mice than in control mice (20) (Figs. 2B and 5B). 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 p56lck-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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