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BATF Transgenic Mice Reveal a Role for Activator Protein-1 in NKT Cell Development

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The importance of regulated AP-1 activity during T cell development was assessed using transgenic mice overexpressing BATF, a basic leucine zipper transcription factor and an AP-1 inhibitor. BATF transgenic animals possess normal thymic cellularity and all major T cell subsets, but show impaired thymocyte proliferation in vitro and no induction of IL-2, IL-4, IL-5, IL-10, and IL-13 expression. Since NKT cells are largely responsible for cytokine production in the thymus, this population was examined by detection of the Vα14-Jα281 TCR, flow cytometry of NK1.1+ TCRβ+ cells, and analysis of cytokine production by heat-stable Aglow thymocytes and peripheral NKT cells stimulated in vivo. Results show a severe under-representation of NKT cells in BATF mutants where, for the most part, functional compensation by other family members has masked the emergence of well-defined phenotypes (8, 9). Most recently, the effects of inhibiting AP-1 activity specifically in the thymus have been studied using transgenic mice expressing TAM-67, a truncated version of c-Jun lacking the trans-activation domain (10). TAM-67 mice display a decrease in total thymic cellularity due to the inability of DN thymocytes to progress in normal numbers to the DP stage. This result is similar to what was observed when c-jun null cells were used to reconstitute the immune system in recombinase-activating gene 2-deficient mice (8) and reflects the need for adequate c-Jun-dependent AP-1 activity primarily during the DN to DP transition.

BATF is a nuclear basic leucine zipper protein and a negative regulator of AP-1 activity (11, 12). BATF forms heterodimers with all Jun proteins (c-Jun, JunD, and JunB) and effectively blocks AP-1 trans-activation in vitro and in vivo (11–13). BATF mRNA is detected in very few tissues, with the highest levels observed in hemopoietic organs such as the spleen, thymus, and lymph nodes (12). BATF gene expression, like that of other AP-1 family members, is decreased dramatically in DP thymocytes and re-emerges as development progresses to the SP stage (7, 13). BATF mRNA continues to be expressed in naive and CD4+ Th1 and Th2 cells in the periphery and is up-regulated after T cell activation (13). These data strongly suggest that T cells use BATF to modulate the trans-activation of AP-1 target genes whose products play a role in T cell lineage establishment and expansion.

In this study the phenotype of transgenic mice expressing BATF under transcriptional control of the p56lck proximal promoter (14) was examined. Our goal was to use this negative AP-1 regulator to address the need for precise control of AP-1 activity during T cell development in the thymus. We show that BATF transgenic animals possess normal numbers of DN, DP, and SP thymocytes, but display a significant decrease in thymocyte proliferation and no induction of IL-2, IL-4, IL-5, IL-10, or IL-13 gene expression in vitro. This pattern of impaired cytokine production by thymocytes suggests a defect in NKT cell development and/or function (15–17), which was supported by the lack of IL-4 production from transgenic heat-stable Ag (HSA)low thymocytes (of which ~10% are potent cytokine-producing NKT cells) and by the lack of IL-4 and IL-13 gene expression following the stimulation of peripheral NKT cells in vivo. Additional molecular evidence for an NKT

T cells are key regulators of the immune responses associated with autoimmunity, allergies, transplant rejection, and infectious disease. The development of T cells in the thymus proceeds through defined steps, characterized by changes in the expression and/or activity of lineage-specific receptors, intracellular signaling molecules, and transcription factors. Numerous investigations have shown that the activities of specific transcription factors, including Ikaros, GATA-3, c-Myb, Ets1, TCF1, and members of the AP-1 superfamily of basic leucine zipper proteins, play important roles in the development and function of T cells (1, 2).

AP-1 transcription complexes function as dimers composed of the Fos, Jun, and ATF families of basic leucine zipper proteins and demonstrate preferential affinity for AP-1 DNA sites (3). The DNA-binding and transcriptional activities of AP-1 complexes is a feature of double-negative (DN) thymocytes (CD4−CD8−TCR−) and of single-positive (SP) T cells (CD4+TCR+ or CD8+TCR+) and is induced further in SP T cells following stimulation (4–7). AP-1 activity is not detected in double-positive (DP) thymocytes (CD4+CD8+TCR−) (6), suggesting that a dramatic alteration of AP-1 target gene expression is critical to this transition in T cell development.

Approaches for examining the importance of AP-1 activity during T cell development have focused primarily on gene disruption

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3 Abbreviations used in this paper: DN, double negative; DP, double positive; HA, hemagglutinin; HSA, heat-stable Ag; RPA, RNase protection assay; SP, single positive; Thp, peripheral Th cells.

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cell-deficient phenotype in BATF transgenic animals was provided by the reduced number of TCRβ+ cells coexpressing the NK marker NK1.1 in thymus, spleen, and liver and by the inability to detect significant levels of DNA or RNA representing the NKT Vα14-Jα281 TCR. We conclude that the overexpression of BATF, a known negative regulator of AP-1 activity, disrupts the development of thymus-derived NKT cells. These studies are the first to link the precise control of AP-1-mediated transcription with the emergence of this unique T cell subset in the mouse.

Materials and Methods

Mice

A BamHI DNA fragment containing the cDNA for hemagglutinin Ag (HA)-tagged human BATF was cloned into expression vector pT17 (18)’ to the mouse p50ω proximal promoter. A Nol fragment containing the BATF transgene was used to generate transgenic mice of the FVB/N strain. For genotyping, tail DNA was restricted with SpeI and analyzed by Southern blot hybridization as previously described (13). Two independent founders were bred to establish FVB-lk line 2 and FVB-lk line 4. FVB-lk line 2 mice were crossed with C57BL6 mice to generate F2 hybrids carrying the BATF transgene in a NK1.1 background. All experiments were performed on age- and sex-matched animals between 8 and 12 wk of age. Where possible, the comparison of transgenic and wild-type (non-transgenic) animals was performed using littermates. The CD1d null mice have been described previously (19).

Immunoblotting

Mouse tissues were lysed in RIPA buffer plus protease inhibitors (Sigma-Aldrich, St. Louis, MO), and protein content was determined by a protein assay (Bio-Rad, Hercules, CA). Forty micrograms of each extract was resolved by 15% SDS-PAGE and transferred to a polyvinylidene di fluoride membrane. HA-BATF was detected with an anti-HA mAb (Roche, Indianapolis, IN) as previously described (20).

Flow cytometry

Single-cell suspensions of 1 x 10^6 cells in 100 μl of medium were incubated on ice with 1 μg of anti-CD3e (2.4G2) and then 1 μg of the following mAbs as indicated: PE-anti-CD4 (RM4-5), FITC-anti-CD3e/β2 (53-58), PE-anti-CD44 (IM7), FITC-anti-CD3e (145-2C11), PE-anti-CD45R/B220 (RA3-6B2), FITC-anti-HSA (M1/69), FITC- or CyChrome-antiCD28 (CT2816), FITC-anti-CD1d (1B1), or the κ isotype control rat IgG2b (A95-1). All Abs were purchased from BD PharMingen (San Diego, CA). Results were analyzed with an XL flow cytometer ( Coulter, Hialeah, FL).

Cell proliferation assay

Thymocytes and splenocytes, plated at 2 x 10^5 cells/well in 96-well plates, were activated with 1 μg/ml plate-bound anti-CD3e mAb (145-2C11) plus 1 μg/ml soluble anti-CD28 mAb (37.51; BD PharMingen). After 48 h, 1 μCi of [3H]thymidine (6.7 Ci/mmol; Amersham Pharmacia Biotech, Arlington, Heights, IL) was added to the wells and incubated at room temperature for 2 h. Immune cell enrichment was achieved by selecting HSAlow cells as previously described (21). Two independent founders were bred to establish FVB-lk line 2 and FVB-lk line 4. FVB-lk line 2 mice were crossed with C57BL6 mice to generate F2 hybrids carrying the BATF transgene in a NK1.1 background. All experiments were performed on age- and sex-matched animals between 8 and 12 wk of age. Where possible, the comparison of transgenic and wild-type (non-transgenic) animals was performed using littermates. The CD1d null mice have been described previously (19).

In vivo cytokine production assay

Intravenous tail vein injection was performed as previously described (21) using 100 μl of PBS or PBS plus 1.33 μg of anti-CD3e mAb. After 90 min, total RNA was prepared from spleens using TRIzol reagent (Life Technologies) and homogenized by sonication. Northern blot hybridization was performed as previously described (13). Two independent founders were bred to establish FVB-lk line 2 and FVB-lk line 4. FVB-lk line 2 mice were crossed with C57BL6 mice to generate F2 hybrids carrying the BATF transgene in a NK1.1 background. All experiments were performed on age- and sex-matched animals between 8 and 12 wk of age. Where possible, the comparison of transgenic and wild-type (non-transgenic) animals was performed using littermates. The CD1d null mice have been described previously (19).

Cytotoxicity assay

A MLR assay was used to differentiate splenocytes into CTL. Briefly, 8 x 10^5 splenocytes (responders) were cocultured with 4 x 10^5 irradiated C57BL/6 splenocytes (stimulators) for 5 days in 30 ml of IMDM supplemented with 10% FBS, 1-glutamine, and antibiotics. Live cells were collected and cocultured at the ratios indicated in Fig. 5C using round-bottom microtiter plates with 1 x 10^5 MC57G target fibroblasts/well, prelabeled for 1 h at 37°C with 100 μCi of Na251CrO4 (NEN, Boston, MA) x 10^5 cells. After 6 h cells were pelleted in the plates by centrifugation, and 10 μl of supernatant from each well was counted in a gamma counter. For spontaneous and maximum release, designated wells containing only the MC57G target cells received 100 μl of complete IMDM or 1% Nonidet P-40, respectively. The percent lysis (percent specific release) was calculated as follows: [(experimental release – spontaneous release) / (maximum release – spontaneous release)] x 100.

Analysis of Vα14-Jα281 expression

Genomic DNA (1 μg) from the thymus, spleen, and liver of wild-type and BATF transgenic mice was subjected to 35 cycles of amplification by PCR (95°C for 1 min, 62°C for 1 min, and 72°C for 1 min) using primers for Vα14 and Ja281 (26). DNA from the NKT cell line DN32D3 hybridoma (23) and from a CD1d null mouse spleen were used as controls. PCR controls included amplification by 35 cycles (95°C for 1 min, 65°C for 4 min, and 72°C for 1 min) of the d3K gene (5'-AGGACTGGTGGCTTCAACTCCAGAACAC and 3'-AGCTTCTATGTGGCCGCAGTTGCAGG) and the HA-BATF transgene (5'-ATAAGAAATTGCGGCGCATGCTTCTGATCTAAT and 3'-ATATGTTGAGCCCGTCAGGGTG). RT-PCR was used to detect Vα14-Jα281 transcripts. Five micrograms of total RNA was reverse transcribed using random hexamer primers and was subjected to 38 cycles of amplification by PCR (94°C for
Results

**BATF transgenic mice display a normal profile of T and B cell development**

To examine how BATF-mediated negative control of AP-1 activity influences specific transitions in thymic T cell development, the p56\(^{lck}\) proximal promoter was used to drive expression of an HA Ag-tagged BATF protein in mice. Two founder animals positive for the transgene were bred to generate the FVB-lck line 2 and FVB-lck line 4 mice used in these studies (Fig. 1A). Both lines were characterized for BATF transgene expression by immunoblotting with anti-HA mAb. In agreement with prior studies on the expression of the p56\(^{lck}\) proximal promoter in vivo (14, 28), BATF transgene expression was high in the thymus, low in the spleen, and not detected in any other tissue examined (Fig. 1B and data not shown).

To determine whether the overexpression of BATF changes the profile of AP-1 DNA binding activity, thymocytes from transgenic and wild-type (nontransgenic) FVB-lck line 2 littermates were stimulated for 6 h with anti-CD3e and anti-CD28 mAbs. Nuclear extracts were prepared and used for EMSA with an AP-1 DNA probe. The results show a characteristic stimulation-induced DNA shift (5) in both wild-type and transgenic extracts, with BATF overexpression causing a more prominent shift of increased mobility due to the difference in mass of BATF:Jun vs Fos:Jun heterodimers (11, 12) (Fig. 1C). The AP-1 DNA binding activity detected in transgenic thymocytes was specific, since it was reduced by adding unlabeled AP-1 competitor DNA. The presence of BATF in the complex was demonstrated by a supershift with anti-BATF Ab (Fig. 1C). Combined with our previous work characterizing BATF as a Jun dimerization partner and an AP-1 inhibitor (12, 13), these data indicate that the majority of the AP-1 binding activity detected in FVB-lck line 2 contains BATF and should exhibit decreased trans-activation potential.

Comparison of thymic index (thymus weight/body weight) and total thymic cellularity between age- and sex-matched wild-type and transgenic FVB-lck line 2 and line 4 mice revealed no significant differences associated with the expression of the BATF transgene (data not shown). To investigate whether BATF overexpression affects the emergence of the major T cell populations, total thymocytes and splenocytes from transgenic and wild-type mice were analyzed by flow cytometry. A portion of these cells were sorted, and protein extracts were analyzed by anti-HA immunoblot for the expression of the BATF transgene. The results for FVB-lck line 2, which were confirmed using line 4, show that all T cell types express HA-BATF and that the percentages of DN, DP, and SP T cells in transgenic animals are comparable to those in wild-type mice (Fig. 1D and data not shown). Similarly, no differences were noted between transgenic and nontransgenic littermates with respect to the number of B cells populating the spleen and peripheral blood (data not shown).

**Limited expression of T cell cytokines in BATF transgenic mice**

To determine whether the overexpression of BATF influences cytokine gene expression, thymocytes and splenocytes from wild-type and transgenic FVB-lck line 2 littermates were stimulated in vitro with plate-bound anti-CD3e mAb. Total RNA was isolated after 24 h, and RPA was performed. The induction of IL-2, IL-4, IL-5, IL-10, and IL-13 mRNA by transgenic thymocytes is impaired relative to that in wild-type controls, while the expression of IL-6 and IFN-\(\gamma\) mRNA is comparable (Fig. 2A). Transgenic splenocytes showed reduced expression of IL-4 and IL-5 and en-
hanced expression of IL-2 (Fig. 2A). The RPA was repeated with RNA prepared from FVB-lck line 4 animals, and similar results were obtained (data not shown).

To confirm that the results of the RPA reflect cytokine secretion by these cells, thymocytes and splenocytes prepared from transgenic and wild-type FVB-lck line 2 littermates were stimulated with anti-CD3e for 48 h, and the culture supernatants were analyzed for IL-4 and IFN-γ by ELISA. Although IL-4 was secreted by thymocytes and splenocytes from wild-type mice, IL-4 was not detected in samples from transgenic mice (Fig. 2B). In contrast, IFN-γ levels showed a modest increase in the thymocytes of transgenic mice and no change relative to the control in splenocytes (Fig. 2B). These data reveal the selective inhibition of a subset of T cell cytokines in BATF transgenic animals.

Transgenic thymocytes show impaired proliferation in vitro

The role of AP-1 in cell proliferation is well documented (2, 3), and as previously reported by our laboratory, mice expressing the p56<sup>lck</sup>-BATF transgene show a thymus-specific, 30% decrease in AP-1 transcriptional activity (13). To determine whether thymocytes from BATF transgenic animals are impaired in their ability to proliferate in vitro, total thymocytes were isolated from wild-type and transgenic FVB-lck line 2 mice and stimulated in vitro for 48 h with anti-CD3e or anti-CD3e plus anti-CD28. [3H]Thymidine was added to the cultures for 18 h, and proliferation was measured by incorporation of the label into DNA. Compared with wild-type controls, the transgenic thymocytes showed a >50% reduction in proliferation (Fig. 3), suggesting that the observed negative impact of BATF on AP-1 activity in vivo correlates with an inability of these cells to respond to proliferative signals in vitro. The mechanistic basis for this proliferation defect, and whether it is linked to the inability of the transgenic thymocytes to produce and/or to respond to specific cytokines, remain to be determined.

Peripheral CD4<sup>+</sup> T cell functions are normal in BATF transgenic mice

Newly activated CD4<sup>+</sup> Th cells (Tph) in the periphery differentiate into Th1 and Th2 subsets, which display a distinct pattern of cytokine expression following stimulation (29, 30). The lack of IL-4 and IL-5 mRNA and IL-4 protein expression in stimulated transgenic splenocytes (Fig. 2) suggested that the level of BATF transgene expression retained in the spleen (Fig. 1B) may impact cytokine production by Th1 and Th2 cells. To examine this possibility, Tph cells were isolated from the spleens of wild-type and FVB-lck line 2 mice and skewed in vitro toward the Th1 and Th2 subsets. The differentiated cultures were restimulated for 24 h with anti-CD3e, and RNA isolated from these cultures was examined for the expression of the BATF transgene by RT-PCR (data not shown) and for the expression of cytokine genes by RPA. The results show a similar cytokine expression profile for both wild-type and transgenic Th1 and Th2 populations. Th1 cells display the characteristic induction of IFN-γ mRNA following stimulation, and Th2 cells express IL-4, IL-5, IL-10, and IL-13 mRNA (Fig. 4A). The RPA results for IL-4 and IFN-γ were confirmed at the protein level by ELISA (Fig. 4B). The differentiation of transgenic Tph cells in vitro provides strong evidence that the lack of IL-4 production noted in Fig. 2B is not due to the inability of transgenic Th2 cells to produce this cytokine.

The phenotype of BATF transgenic mice is consistent with a defect in NKT cells

It is known that CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the thymus do not secrete large amounts of cytokines. Instead, NKT cells, a population of mature DN or CD4<sup>+</sup> T cells representing ~1% of total thymocytes, are responsible for much of the cytokine production in this tissue (15, 16). Since IL-4, IL-5, IL-10, and IL-13 mRNAs are not induced following stimulation of thymocytes isolated from BATF transgenic mice, we investigated whether the NKT cell compartment was altered in these animals. Total thymocytes from wild-type and transgenic mice were enriched for NKT cells by complement lysis of immature, HSA<sup>high</sup> cells (23, 24). Enrichment

| FIGURE 2. Cytokine gene expression in the thymus and spleen of BATF transgenic mice. A, RPA were used to establish the pattern of cytokine gene expression in the spleen and thymus of BATF transgenic and wild-type mice. Protected fragments were resolved by PAGE and identified by size. Expression levels of L32 and GAPDH mRNA served as controls. B, Cytokine ELISA were used to compare the levels of IL-4 and IFN-γ secreted by wild-type and transgenic thymocytes or splenocytes following stimulation with anti-CD3e for 48 h. The data are from one of three independent experiments using four mice of each genotype per group. Bars denote the SEM.

| FIGURE 3. Proliferation of thymocytes isolated from wild-type and BATF transgenic mice. Thymocytes were stimulated in vitro with anti-CD3e or anti-CD3e and anti-CD28 for 48 h before labeling replicating DNA with [3H]thymidine for 18 h. The data are from one of four independent experiments using four mice of each genotype per group. Bars denote the SEM.
was monitored using flow cytometry to detect anti-CD3ε- and anti-CD44-reactive cells before and after lysis (data not shown). The HSA<sup>low</sup> cells were analyzed for IL-4 production by ELISA. As expected, in the NKT cell-enriched population from wild-type animals, IL-4 production was increased 10-fold over that routinely produced by total thymocytes (compare Fig. 5A, left panel, with Fig. 2B). In contrast, IL-4 remained virtually undetectable in the HSA<sup>low</sup> cells from BATF transgenic mice. As a control, the production of IFN-γ, which is not an exclusive property of NKT cells (16), was equivalent for transgenic and nontransgenic cultures (Fig. 5A, right panel). These data provide strong evidence that the BATF transgene negatively impacts the development and/or function of thymic NKT cells.

If NKT cell development and/or function are compromised in the thymus by the overexpression of BATF, we would expect this phenotype to extend into the periphery. One approach for testing NKT cell function in vivo is to challenge animals with an i.v. injection of anti-CD3ε and measure cytokine production within 90 min. Previous studies have shown that NKT cells respond within this time-frame to express IL-4 and IL-13 (21, 22). Tail vein injection of anti-CD3ε, or PBS as a control, was performed on three FVB-<i>lck</i> line 2 littermates from the F2 generation for analysis. Single-cell suspensions prepared from thymus, spleen, and liver were examined by flow cytometry for reactivity with anti-TCR<sup>fi</sup> and anti-NK1.1 (16, 21, 27). However, direct immunodetection of NK1.1 was not possible in certain mouse strains, including BALB/C and FVB (33) (K. Williams and E. Taparowsky, unpublished observations). Therefore, we crossed FVB-<i>lck</i> line 2 to C57BL/6 mice (H-2<sup>b</sup>) were used as stimulators for splenocytes isolated from wild-type or FVB-<i>lck</i> line 2 mice (H-2<sup>d</sup>). After 5 days, the primed CTL were tested for activity using a <sup>51</sup>Cr release cytotoxicity assay with MC57G fibroblast cells (H-2<sup>b</sup>). The results averaged from three independent assays reveal a 22% decrease in the cytotoxic response of CD8<sup>+</sup> T cells derived from the BATF transgenic mice (Fig. 5C). This result is consistent with the findings of others (31) and shows that diminished NKT cell function in BATF transgenic animals correlates with a decrease in the overall level of CTL activity.

Molecular analyses demonstrate under-representation of NKT cells in BATF transgenic mice

The identification and/or isolation of NKT cells is commonly achieved using mAbs reactive with TCR and the NK cell marker NK1.1 (16, 21, 27). However, direct immunodetection of NK1.1 is not possible in certain mouse strains, including BALB/C and FVB (33) (K. Williams and E. Taparowsky, unpublished observations). Therefore, we crossed FVB-<i>lck</i> line 2 to C57BL/6 mice (NK1.1<sup>-</sup>) and selected age- and sex-matched transgenic and nontransgenic littermates from the F<sub>2</sub> generation for analysis. Single-cell suspensions prepared from thymus, spleen, and liver were examined by flow cytometry for reactivity with anti-TCR<sup>β</sup> and anti-NK1.1 mAbs. Total cell populations as well as populations gated as HSA<sup>low</sup> cells were examined. While the profiles of TCR<sup>β+</sup> NK1.1<sup>+</sup> cells from the nontransgenic F<sub>2</sub> animals were as expected

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**FIGURE 4.** Transgenic CD4<sup>+</sup> T cells display the expected pattern of cytokine expression. CD4<sup>+</sup> T cells from the spleens of four wild-type or four BATF transgenic mice were pooled and cultured for 5 days under conditions skewing their development toward the Th1 or Th2 subset. A. The groups were restimulated during the last 24 h of differentiation, and cytokine mRNA expression levels were analyzed by RPA. Expression levels of L32 and GAPDH mRNA served as controls. B. Th1 and Th2 cultures derived in A were restimulated for 48 h, and supernatants were analyzed by ELISA for IL-4 and IFN-γ. The data are from one of three independent experiments. For both genotypes, results show the expected production of IL-4 by stimulated Th2 cells and of IFN-γ by stimulated Th1 cells.
Wild-type and transgenic thymocytes enriched for NKT cells (HSA low) by cytokine mRNA by sequential Northern blot hybridization. Hybridization, spleen, and 84% in the liver (Fig. 6). Transgenic tissues were reduced by 75% in the thymus, 56% in the spleen, and 84% in the liver (Fig. 6). Analysis of the Vβ/H9252 (16, 33), the numbers of TCRβ+ NK1.1+ cells detected in transgenic tissues were reduced by 75% in the thymus, 56% in the spleen, and 84% in the liver (Fig. 6A, upper panels). Detection of TCRβ+ NK1.1+ cells in the HSA low population from thymus tissue revealed the same pronounced deficiency (Fig. 6A, lower panel). These results clearly show that transmission of the BATF transcript in FVB × C57BL/6 hybrids is associated with the loss of NK1.1+ T cells (NKT) from the thymus and periphery.

A second approach for detecting NKT cells uses a molecular analysis of the Vα14-Jα281 TCR α-chain, which is expressed by the vast majority of CD1d-dependent NKT cells (15–17). Genomic DNA from the thymus, spleen, and liver of wild-type and transgenic FVB-1ck line 2 littersmates was amplified with primers specific for the Vα14-Jα281 rearrangement. Genomic DNA from the spleens of CD1d null mice, which lack NKT cells, and from the NKT cell line, DN32.D3, provided negative and positive controls, respectively. Based on the relative intensity of the amplified bands in the control vs the transgenic samples, we estimate that the frequency of Vα14-Jα281 rearrangement is decreased 3- to 5-fold in BATF transgenic mice (Fig. 6B).

To determine whether the decreased frequency of Vα14-Jα281 gene rearrangements in BATF transgenic mice leads to a decrease in Vα14-Jα281 transcript levels, RNA from the spleen and thymus tissue of wild-type and transgenic mice was analyzed by RT-PCR. DN32.D3 RNA was used as a positive control, and the amplification of HPRT mRNA controlled for sample integrity and amount. Transcripts representing the Vα14-Jα281 TCR were observed in wild-type thymus and spleen and, in agreement with the genomic DNA analysis, were barely detectable in the samples from BATF transgenic mice (Fig. 6C).

The reduced number of NKT cells in BATF transgenic mice could be due to a defect in the environmental signals required for NKT precursor maturation or to the inability of developing NKT cells to respond to these signals. NKT cells develop in response to interaction with cells bearing the nonclassical MHC class I-like molecule, CD1d (19, 23, 24, 27, 34). Therefore, as a first step toward identifying the molecular basis for the NKT cell-deficient phenotype described here, single-cell suspensions from the thymus, spleen, and liver of wild-type and BATF transgenic mice were analyzed for CD1d expression by flow cytometry. No significant differences in the overall levels of cell surface CD1d expression were observed (Fig. 7). While this experiment does not address the functionality of CD1d, it demonstrates that BATF does not alter expression of a major stimulus for NKT cell lineage expansion in these animals.

Discussion

The negative regulatory activity of BATF toward AP-1-mediated transcription was used to investigate the importance of AP-1 component expression and activity across key transitions in T cell development. Of paramount interest was the effect of maintaining BATF expression in DP thymocytes, since these cells dramatically down-regulate AP-1 activity, as defined by the absence of AP-1 DNA binding complexes (4, 6) and the lack of expression of Jun dimerization partners such as c-Fos, FosB, and BATF (7, 13). Positive selection of DP thymocytes results in the emergence of SP thymocytes and is accompanied by the reactivation of AP-1 binding and component expression (4, 6, 7, 13). In fact, many of the genes encoding proteins that define the functional capabilities of SP cells as they differentiate further in the periphery rely on AP-1-mediated transcription for their expression (2, 35). Another T cell population that emerges coincident with selection of DP cells in the thymus are the NKT cells (Fig. 8A) (17, 36, 37). This small, but functionally significant, population can be detected as CD4+ or DN cells biased for the expression of the Vα14-Jα281 TCR and the NK cell marker, NK1.1 (15–17). Although the status of AP-1 component expression in NKT cells has not been described, the fact that these cells are the major producers of T cell cytokines in the thymus (15, 16) indicates that AP-1 is probably present to positively regulate the expression of these well-characterized AP-1 target genes (2, 35).

Our characterization of lymphocyte development in BATF transgenic animals revealed the appropriate numbers and relative percentages of the major T and B cell subsets. However, most striking with regard to this normal profile was the observation that transgenic thymocytes are deficient in the induction of IL-2, IL-4,
IL-5, IL-10, and IL-13 mRNAs and proliferate poorly in response to in vitro stimulation. Initially, we suspected that BATF overexpression was exerting an overall negative effect on TCR signaling and/or the activation of NFAT/AP-1 target genes such as IL-4 and CD25 (35). However, not all AP-1 target genes induced following stimulation were adversely affected by the transgene (e.g., IFN-γ), and the robust induction of IL-4 gene expression in Th2 cells expressing both endogenous (13) and transgene-derived BATF (data not shown) indicated otherwise. Thus, a more likely explanation was that the phenotype of the transgenic animals was reflecting the targeted effect of BATF on a specific cell type. The obvious candidate was the NKT cell.

The inability to use NK1.1 immunoreactivity for the direct identification of NKT cells in FVB mice required that we begin our analysis using several functional approaches to characterize the NKT cell compartment. Stimulation of HSA^low^ T cells from the thymus of wild-type and transgenic FVB-ck line 2 × C57BL/6 F2 littermates were analyzed by flow cytometry. Lymphocytes were costained with PE-anti-NK1.1 and FITC-anti-TCRβ Abs (upper panels) or first selected as HSA^low^ by staining with FITC-anti-HSA Ab before analysis for PE-anti-NK1.1^+^ and CyChrome-anti-TCRβ reactivity (lower panel). For both total and HSA^low^ cells, the population of cells positive for both NK1.1 and TCRβ appears in the circled gate. The experiment was performed on two female mice per group, and the average percentage of dual positive cells is indicated in the upper right corner of each representative plot. B, Vα14-Jα281 DNA rearrangements in wild-type and BATF transgenic mice were detected by PCR. DNA from DN32.D3 cells and from CD1d null mice were used as positive and negative controls, respectively. Amplification of the 43K gene and the BATF transgene (lower panel) was performed in parallel. The migration of DNA standards (marker) was used to size the PCR products. C, Expression of the Vα14-Jα281 DNA rearrangement was detected by RT-PCR of RNA from the thymus and spleen of wild-type and BATF transgenic mice. RNA from DN32.D3 cells was used as a positive control, and the amplification of HPRT cDNA (lower panel) provided a control for sample integrity. The migration of DNA standards (marker) was used to size the PCR products.

FIGURE 6. Molecular detection of NKT cells in BATF transgenic mice. A, Cell suspensions from the thymus, spleen, and liver tissue of wild-type and transgenic FVB-ck line 2 × C57BL/6 F2 littermates were analyzed by flow cytometry. Lymphocytes were costained with PE-anti-NK1.1 and FITC-anti-TCRβ Abs (upper panels) or first selected as HSA^low^ by staining with FITC-anti-HSA Ab before analysis for PE-anti-NK1.1^+^ and CyChrome-anti-TCRβ reactivity (lower panel). For both total and HSA^low^ cells, the population of cells positive for both NK1.1 and TCRβ appears in the circled gate. The experiment was performed on two female mice per group, and the average percentage of dual positive cells is indicated in the upper right corner of each representative plot. B, Vα14-Jα281 DNA rearrangements in wild-type and BATF transgenic mice were detected by PCR. DNA from DN32.D3 cells and from CD1d null mice were used as positive and negative controls, respectively. Amplification of the 43K gene and the BATF transgene (lower panel) was performed in parallel. The migration of DNA standards (marker) was used to size the PCR products. C, Expression of the Vα14-Jα281 DNA rearrangement was detected by RT-PCR of RNA from the thymus and spleen of wild-type and BATF transgenic mice. RNA from DN32.D3 cells was used as a positive control, and the amplification of HPRT cDNA (lower panel) provided a control for sample integrity. The migration of DNA standards (marker) was used to size the PCR products.
BATF as an inhibitor of AP-1-dependent signaling (12) suggests that BATF most likely is blocking the intrinsic ability of NKT cell precursors to develop.

Two previous studies have employed molecules with similar properties as BATF to disrupt AP-1 activity during thymic T cell development. The first study used FosB2, a truncated dominant negative version of FosB that is not normally expressed in the thymus (39). FosB2 transgenic mice display progressive aberrant thymic cellularity that is reflected in elevated levels of CD4+ thymocytes by 5–10 mo of age. No effect on NKT cells was examined or suggested by the data presented. More recently, expression of a dominant negative version of the c-Jun protein, TAM-67, via the p56lck promoter produced mice with reduced numbers of DP and SP thymocytes due to a developmental block at the DN stage (10). Once again, no effect on NKT cell development was investigated. The striking differences between these studies and our own reflect the complexity of AP-1 factor function and regulation and perhaps can be reconciled by considering the unique properties of the AP-1 inhibitors used. FosB2 and TAM-67 are missing not only the transcription activation domains of their respective parent proteins, but potential regulatory sequences as well. FosB2 functions primarily to inhibit Jun proteins, while the dimerization properties of TAM-67 target Fos proteins and, in the absence of Fos, Jun proteins as well. In contrast, BATF is expressed by most T cells, and our transgene expresses a native form of this Jun-binding AP-1 inhibitor. We are reasonably confident that the BATF transgene impacts the T cell types where endogenous BATF is normally not expressed (i.e., DP cells) since we have identified a post-translational modification of BATF in T cells that can regulate DNA binding by Jun:BATF heterodimers (C. Deppmann and E. Taparowsky, manuscript in preparation). While ongoing studies are addressing whether this modification influences the amount of functional BATF being expressed by the transgene, the proper

**FIGURE 7.** CD1d expression in wild-type and BATF transgenic mice. Single-cell suspensions from the thymus, spleen, and liver tissue of wild-type and transgenic FVB-lck line 2 × C57BL/6 F2 sex-matched littermates were stained with FITC-anti-CD1d Ab and analyzed by flow cytometry (unshaded). Superimposed are the profiles of cells from the same tissues stained with an isotype control Ab (shaded). The plots shown are representative of the data obtained from two independent experiments using two animals per group.

**FIGURE 8.** Role of AP-1 in NKT cell development in the thymus. The under-representation of NKT cells in transgenic mice overexpressing the AP-1 inhibitor BATF suggests that the positive action of AP-1 transcription complexes is required for the expansion and differentiation of NKT cells in vivo. A, There is now strong evidence to support the derivation of thymic DN and SP NKT cells from AP-1-depleted, DP thymocytes in response to stimulation from the MHC class-I like molecule CD1d (17, 36, 37, 48). B, In our model, blocking reactivation of AP-1 in DP cells with BATF interferes with signaling triggered by the interaction of the TCR with CD1d-expressing cells and results in an NKT cell precursor population incapable of further expansion and destined for elimination. The NKT cell-depleted phenotypes displayed by Fyn and Ets1 knockout animals can be incorporated into our model to propose the existence of a novel signaling pathway relying on Fyn and the downstream collaboration between the Ets1 and AP-1 transcription factors to initiate the gene expression changes required for NKT cell expansion in vivo.
development of transgenic Thp cells to differentiated Th1 and Th2 cells in vitro would indicate that the negative effects of BATF overexpression on AP-1-mediated transcription can be controlled in some cell types.

These results provide novel and important information regarding the role of AP-1 in T cell development (Fig. 8). Maintaining high levels of BATF expression in thymic T cell populations does not influence the emergence of the conventional T cell subsets, but severely impairs the development and function of NKT cells. Since it is now recognized that CD1d-restricted NKT cells originate from DP cells within the thymus (36), and we have speculated that the p56^LA-BATF transgene will have the greatest impact where endogenous BATF is down-regulated (i.e., DP cells), our data are the first to implicate AP-1 in the molecular events driving NKT cell selection, expansion, or survival. Expression of the Vo14-Jo281 TCR and interaction with CD1d are essential for thymic NKT cell development. Mice engineered to overexpress Vo14-Jo281 have increased numbers of NKT cells (40), while mice deficient in CD1d have no NKT cells (19, 24, 27). The expression of the IL-2Rβ/IL-15Rβ-chain in NKT cells (41) suggests a role for IL-15 signaling in the development of this lineage, an observation confirmed by the NKT-depleted phenotype of both IL-15 (42) and IL-2Rβ/IL-15Rβ (41) null mice. As far as cytoplasmic signaling molecules are concerned, targeted disruption of the Fyn protein kinase or the Tec family kinase, Itk, in mice impacts the NKT cell compartment more than other T cell types (43–45), suggesting that signaling downstream of the IL-15Rβ and/or Vo14-Jo281 TCR relies on these molecules. At the nuclear level, mice null for the Ets1 transcription factor have a phenotype similar to that of BATF transgenic mice (46), suggesting that the collaborative actions of Ets1 and AP-1 (47) are required to affect changes in gene expression necessary for NKT precursor maturation and expansion. As investigations into the origin and function of NKT cells continue, future studies on BATF knockout animals and BATF transgenic mice generated in the NK1.1/C57BL/6 strain should provide additional information on the intriguing connection between the regulation of AP-1 and the development of this unique T cell subset in mice.

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References