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Regulation of thymocyte homeostasis by Fliz1

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SUMMARY

Fliz1 (fetal liver zinc finger protein 1) is a novel zinc finger protein preferentially expressed in fetal liver hematopoietic progenitors and in several adult organs, including the thymus. To investigate the *in vivo* function of Fliz1 in regulating the development of T cell lineage, we generated and studied transgenic mice overexpressing human Fliz1 in a T-cell specific and inducible manner. We found that overexpression of human Fliz1 in adult animals resulted in a substantial decrease in total number of thymocytes due to enhanced apoptosis, whereas maturation of thymocytes remained undisturbed. The Fliz1-induced apoptosis is dependent on the N-terminus of Fliz1, which contains an acidic and a serine-rich domains, and might be due to augmented expression of *bad*, a pro-apoptotic gene. Taken together, our data suggest that Fliz1 plays a role in regulating thymocyte homeostasis.

INTRODUCTION

Mouse fetal liver zinc finger protein 1 (mFliz1) is one of a handful of CCCH-type zinc finger proteins that have been identified.[1] Most of the CCCH-type zinc finger proteins contain two repeated YKTEL $CX_8CX_5CX_3H$ motifs and are capable of binding to RNA.[2,3] The prototype of mammalian CCCH zinc finger proteins is TIS11/Nup475/TTP, which was originally cloned as a growth factor-inducible nuclear protein.[4] Subsequently, it was demonstrated that TIS11/Nup475/TTP, by specifically binding to an AU-rich element in the 3′ untranslated region, critically regulated the stability and levels of tumour necrosis factor-α (TNF-α) gene transcripts.[5,6] Fliz1, unlike other CCCH zinc finger proteins, contains three $CX_{7-8}CX_5CX_3H$ zinc finger domains without the conserved YKTEL peptides, suggesting unique roles of Fliz1 among the CCCH family.

During embryogenesis, Fliz1 was preferentially expressed in fetal liver-derived haematopoietic progenitor cells. In adult animals, low levels of Fliz1 transcripts can be detected in several organs, including the thymus[1] suggesting that Fliz1 might play a role in regulating the development/maturation of T cells. To test this hypothesis, we have generated transgenic mice overexpressing human Fliz1 (hFliz1) in a T-cell specific and inducible manner.

Here, we report that overexpression of hFliz1 *in vivo* resulted in significant thymic hypocellularity because of enhanced apoptosis, but not hypoproliferation. The thymic hypocellularity and enhanced apoptosis were dependent on the N-terminus of hFliz1 containing an acidic and a serine-rich domains, and might be partly explained by dysregulation of a pro-apoptotic gene, *bad*.

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Abbreviations: Fliz1, fetal liver zinc finger protein 1; BrdU, bromodeoxyuridine; 7-AAD, 7-actinomycin D; STg, single transgenic; DTg, double transgenic.

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MATERIALS AND METHODS

Generation of T-cell specific and tetracycline-inducible transgenic mice

A full length hFliz1 cDNA was cloned into the *Bam*HI site of pTRE (Clontech, Palo Alto, CA), which contains the TetO and a minimal CMV promoter, to generate the pTRE–hFliz1 transgenic construct. A truncated hFliz1 (hFliz1-ΔN), encoding amino acid residues 103–291, was also thus generated. Both the full length and truncated hFliz1 were tagged with six copies of myc peptide and used separately to generate transgenic mice. The pTRE–hFliz1 or pTRE–hFliz1-ΔN transgenic mice thus generated were bred with CD2-rtTA transgenic (rtTA STg) mice, which express VP16-rtTA under the control of a human CD2 promoter[7] to

create double transgenic mice, henceforth called hFliz1 DTg and hFliz1-ΔN DTg, respectively.

Doxycycline administration
Doxycycline hydrochloride (Dox; Sigma, St Louis, MO) was dissolved in 2% sucrose solution to a concentration of 2 mg/ml and was given to 6–8-week-old mice as drinking water. The Dox-containing water was changed every other day, and mice were routinely fed with Dox water for 4 days prior to killing. Sucrose solution (2%) was given as control.

Western blot analysis
Whole cell extract was isolated from thymocytes and liver cells and separated on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. The resolved proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with mouse monoclonal anti-myc (9E10) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 500-fold or anti-VP16 antibody (Clontech) diluted 1000-fold in phosphate-buffered saline (PBS) containing Tween-20, followed by incubation with horseradish peroxidase (HRP)-conjugated specific secondary antibodies and detection with ECL kit (Amersham, Piscataway, NJ).

BrdU incorporation assay
One mg of bromodeoxyuridine (BrdU) supplied in the BrdU Flow kit (PharMingen, San Diego, CA), was injected into mice intraperitoneally. Two hours later, thymocytes were harvested and stained with fluoroscein isothiocyanate (FITC)-conjugated anti-BrdU antibody and 7-actinomycin D (AAD) and analysed by flow cytometry according to the manufacturer's instructions.

FACS analysis and apoptosis assays
For thymocyte subset analysis, thymocytes were suspended in RPMI-1640 medium (Gibco BRL, Grand Island, NY) and stained with FITC-conjugated anti-CD4 (RM4-4) and phycoerythrin (PE)-conjugated anti-CD8a (53-6.7) antibodies (PharMingen). For apoptosis assays, thymocytes were washed twice with PBS and stained with PE-conjugated anti-annexin V and 7-AAD supplied in the Annexin V–PE Apoptosis detection kit I (PharMingen). Cytometric analyses were performed by using CELLQuest™ program (Becton Dickinson, San Jose, CA).

Gene expression array (GEArray) assay
Total thymocyte RNA was prepared by using TRIzol reagent (Gibco BRL, Grand Island, NY). Ten μg of each RNA sample was subjected to reverse transcription by using MuLV reverse transcriptase in the presence of [α-^{32}P]dCTP (NEN Life Science Products Inc., Boston, MA). The resulting products were used as probes. Duplicate GEArray blots, supplied in the Mouse Apoptosis-1 GEArray kit™ (Super Array Inc., Bethesda, MD), were separately hybridized with probes in the presence of 10 μg/ml of salmon sperm DNA (Sigma). The blots were washed twice with 2× standard saline citrate (SSC)/0·1% SDS and 0·2× SSC/0·1% SDS, respectively, and subjected to autoradiograph.

Real-time polymerase chain reaction (PCR)
For real-time PCR, 1 μg of total RNA was used in reverse transcription (RT) and amplification by using a superscript II RT kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). A master mix of TaqMan or SYBR Green reagents was prepared and 10 ng of each RT product was used in the PCR reaction (PE Applied Biosystems, Foster City, CA). The sequences of primers and probes are: *bad*; 5′-GGGAG-CAACATTCATCAGCA-3′, 5′-CCATAGCCCCTGC-GCC-3′, FAM-CGGGCAGCCACCAACAGTCATCAT-TAMRA, GATA-3; 5′-AGAACCGGCCCCTTATCAA-3′, 5′-AGTTCGCGCAGGATGTCC-3′, 6FAM-CCAAGC-GAAGGCTGTCGGCAG-TAMRA, β-actin; 5′-ACCAA-CTGGGACCGATATGGAGAAGA-3′, 5′-TACGACCA-GAGGCATACAGGGACAA-3′, 6FAM-CCCTCTGAA-CCCTAA-TAMRA, Fliz1; 5′-TGGAAAGTAAAAC-GGGACCG-3′, 5′-TTGCAGCGTTTCCAAGGTG-3′. The expression level of each gene was normalized against that of β-actin gene.

RESULTS

Generation of transgenic mice overexpressing human Fliz1 in a T-cell specific and tetracycline-inducible manner

We first obtained a human homologue of mFliz1 from several EST clones (Genbank accession numbers AL530577, AL563786, and AW958306). Sequence analysis revealed that hFliz1 contained 291 amino acid residues and shared 82% homology of nucleotide sequences and 66% identity of amino acid sequences with mFliz1, which encodes 305 amino acid residues (Fig. 1a). Similar to mFliz1, hFliz1 contains an N-terminal acidic domain, followed by a serine-rich domain, two consecutive putative nuclear localization signals, and three CCCH type zinc fingers (Fig. 1b). Of note, the amino acid sequences of the zinc fingers of hFliz1 are highly homologous to those of mFliz1, suggesting conserved functions of the zinc finger domains.

To examine the *in vivo* function of Fliz1 in regulating the development of T cells, we chose to use the Tet-on system to generate transgenic mice overexpressing hFliz1 in a T-cell specific and tetracycline-inducible fashion. We first obtained transgenic mice (rtTA STg) overexpressing a tetracycline-dependent artificial transactivator, VP16-rtTA, in a T-cell specific fashion.[7] We then generated mice carrying a myc-tagged full length hFliz1 or a truncated hFliz1 (hFliz1-ΔN), encoding amino acid residues 103–291 and lacking the N-terminal acidic and serine-rich domains, under the control of a VP16-rtTA responsive promoter (Fig. 1b). Three independent hFliz1 and one hFliz1-ΔN founders were thus generated, which were then separately bred with the rtTA STg mice to create VP16-rtTA/hFliz1 (hFliz1 DTg) and VP16-rtTA/hFliz1-ΔN (hFliz1-ΔN DTg) double transgenic mice. To induce the expression of hFliz1 or

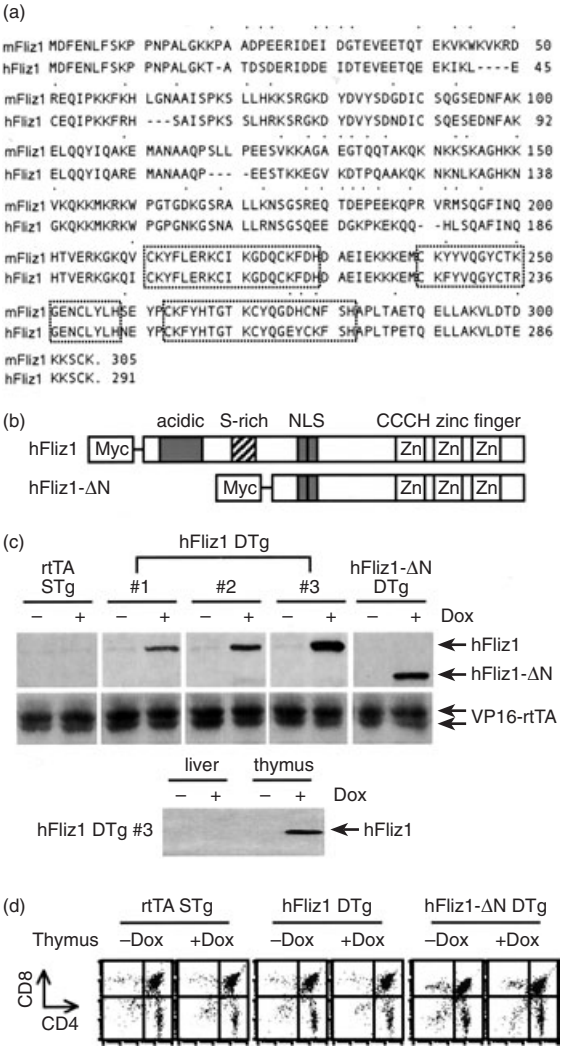

Figure 1. (a) Comparison of human (hFliz1) and mouse Fliz1 (mFliz1) amino acid sequences. The hyphens and dots indicate non-conserved amino acid residues. The zinc finger domains are boxed. (b) Schematic diagrams of hFliz1 and hFliz1-ΔN transgenic constructs. The acidic, serine (S)-rich, nuclear localization signal (NLS), and three CCCH type zinc finger (Zn) domains are indicated. (c) Expression of hFliz1 and hFliz1-ΔN in transgenic thymocytes. Six to 8-week-old-mice derived from the rtTA STg, three independent hFliz1 DTg, and one hFliz1-ΔN DTg founder lines were fed with sucrose (−) or Dox water (+). Whole thymocyte or liver cell extract was prepared and resolved on 10% SDS–PAGE. The myc-tagged hFliz1 or hFliz1-ΔN was detected by using anti-myc monoclonal antibody. The expression of VP16-rtTA was detected by anti-VP16 antibody. (d) Thymocytes obtained from mice described in (c) were stained with anti-CD4–FITC and anti-CD8-PE antibodies, and analysed by fluorescence-activated cell sorting.

hFliz1-ΔN, adult mice were fed with doxycycline (Dox)-containing water for 4 days prior to killing. We found that hFliz1 was not detected in the absence of Dox, whereas high levels of hFliz1 or hFliz1-ΔN were present in thymocyte extract, but not in liver cell extract, derived from all double transgenic mice fed with Dox water (Fig. 1c). In contrast, VP16-rtTA was comparably expressed in all thymocyte extracts independent of Dox. These results firmly establish that T-cell specific and tetracycline-inducible expression of hFliz1 can be achieved by the Tet-on system.

Overexpression of hFliz1 *in vivo* caused thymic hypocellularity

All double transgenic mice thus generated were developmentally normal. The distribution of thymocyte subsets, peripheral T/B and CD4/CD8 ratios, and the levels of T- and B-cell surface markers were all comparable to those of rtTA STg or wild type mice (Fig. 1d and data not shown). However, all hFliz1 DTg mice, derived from three independent hFliz1 double transgenic founder lines, had substantial reductions, 28–63%, of total thymocyte numbers when exposed to Dox (Table 1). The thymic hypocellularity could be readily appreciated even only after 4-day long exposure to Dox, but was not further aggravated by longer periods of exposure (data not shown). In addition, the hypocellularity was not caused by non-specific effects of Dox and was dependent on the N-terminus of hFliz1, because it was not observed in rtTA STg or hFliz1-ΔN DTg mice exposed to Dox (Table 1). In subsequent analyses, all three independent hFliz1 DTg lines yielded similar results; and only the data obtained from the #3 founder line of hFliz1 DTg mice will be shown and discussed.

The hFliz1-induced thymic hypocellularity was caused by enhanced thymocyte apoptosis

The thymic hypocellularity seen in hFliz1 DTg mice might be caused by increased apoptosis and/or decreased proliferation. To distinguish these two possibilities, we performed *in vivo* BrdU incorporation assay. Mice were fed with sucrose or Dox water for 4 days and injected with sterile BrdU via intraperitoneal routes 2 hr prior to killing. Single-cell suspension was isolated from the thymus, stained with FITC-conjugated anti-BrdU antibody and 7-AAD, and analysed by flow cytometry. As shown in Fig. 2(a), the transition of thymocytes through the cell cycle was not disturbed by overexpression of hFliz1, whereas the percentage of apoptotic cells was dramatically increased by approximately threefold (from 6·2 to 18·4%) in Dox-fed hFliz1 DTg thymus. In contrast, no such increase in apoptotic population was observed in Dox-fed rtTA STg or hFliz1-ΔN DTg thymus. The increased apoptosis was further confirmed by staining with annexin V and 7-AAD, and was mainly due to an increase (from 4 to 18%) in annexin V^+/7-AAD^+ population, which represents end stage apoptotic cells (Fig. 2b, middle panels).

Bad was induced by overexpression of hFliz1

Several pro- and antiapoptotic molecules, such as FasL, Bcl-2, and Fas-associated death domain protein, have been shown to affect thymocyte apoptosis and homeostasis.[8–10]

Table 1. Total thymocyte numbers in transgenic mice

Transgenic mice		Sex	Total thymocytes ($\times 10^6$) −Dox	Total thymocytes ($\times 10^6$) +Dox	Reduction (%)
rtTA STg		Female	120·1 ± 9·8	132·0 ± 11·2	−9·9
		Male	105·6 ± 6·4	110·2 ± 3·7	−4·4
hFliz1 DTg	#1	Female	106·4 ± 6·8	75·4 ± 2·5	29·1 (28·5%)
		Male	86·7 ± 10·3	62·3 ± 6·9	28·1
	#2	Female	125·4 ± 5·3	83·2 ± 2·7	33·7 (35·6%)
		Male	87·9 ± 8·2	54·9 ± 2·9	37·5
	#3	Female	136·8 ± 11·2	46·4 ± 3·4	66·1 (63·2%)
		Male	74·8 ± 7·5	29·8 ± 2·9	60·2
hFliz1-ΔNDTg		Female	125·4 ± 4·7	132·5 ± 5·8	−5·7
		Male	116·8 ± 7·5	109·9 ± 9·4	5·9

Mice of indicated genotype and gender were fed with sucrose (−Dox) or Dox water (+Dox). Thymocyte numbers were counted by trypan blue exclusion assay. The data shown are the averages and standard deviations of total thymocyte numbers obtained from at least 20 mice from each genotype.

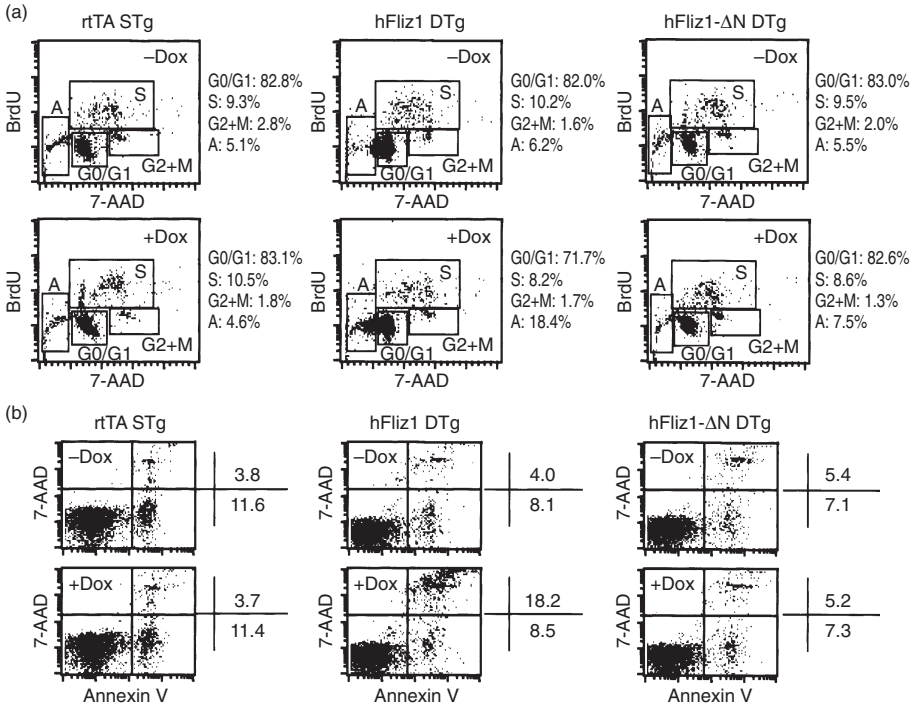

Figure 2. Enhanced apoptosis by overexpression of hFliz1. (a) Mice of indicated genotypes were fed with sucrose (−Dox) or Dox water (+Dox) for 4 days prior to intraperitoneal injections with 1 mg of BrdU. Thymocytes were harvested 2 hr post injection, stained with anti-BrdU antibody and 7-AAD, and analysed by flow cytometry. Various stages of the cell cycle are boxed and indicated. A stands for apoptosis. Percentages of cells in each stage of the cell cycle are also indicated. (b) Mice fed with sucrose (−Dox) or Dox water (+Dox), as described in (a), were sacrificed without injection of BrdU. Thymocytes were prepared, stained with annexin V–PE in a buffer containing 7-AAD, and analysed by flow cytometry. The numbers stand for the percentages of cells at the indicated populations.

To identify the pathway that might mediate the hFliz1-induced thymocyte apoptosis, we performed gene expression array analysis by comparing total thymocyte RNA samples obtained from sucrose or Dox water-fed hFliz1 DTg mice. We found that, among various pro- and antiapoptotic genes included in the array, only the levels of the *bad* gene transcripts were significantly affected by overexpression of hFliz1 (Fig. 3a and Fig. 1a, b), an approximately threefold induction by hFliz1 as determined by densitometry (Fig. 3b). The increase in the levels of *bad* transcripts was further confirmed by real-time PCR by using independently prepared RNA samples. In contrast, the

levels of *bad* gene transcripts were not affect by over-expression of hFliz1-ΔN (Fig. 3c).

Fliz1 is expressed at low levels in all subsets of thymocytes

Given the effects of Fliz1 on thymocyte homeostasis, we examined the expression of Fliz1 in subsets of thymocytes. We found that the levels of Fliz1 transcripts were below the detectable threshold of Northern blot analysis (data not shown). We then repeated the experiment by using real-time PCR. Among thymocyte subsets, Fliz1 transcripts were relatively enriched in double negative and CD8 single positive populations, whereas the levels of Fliz1 transcripts were reduced by approximately 50% in double positive and CD4 single-positive populations (Fig. 4a). Despite the enrichment, the levels of Fliz1 transcripts in the double-positive population were only approximately $7{\cdot}5 \times 10^{-4}$-fold to those of β-actin. As comparisons, the levels of GATA-binding protein3 (GATA-3), a T-cell specific transcription factor, were approximately 5×10^{-2} -fold to those of β-actin (Fig. 4b). This observation is in agreement with a previous report showing low level expression of Fliz1 in adult organs.[1]

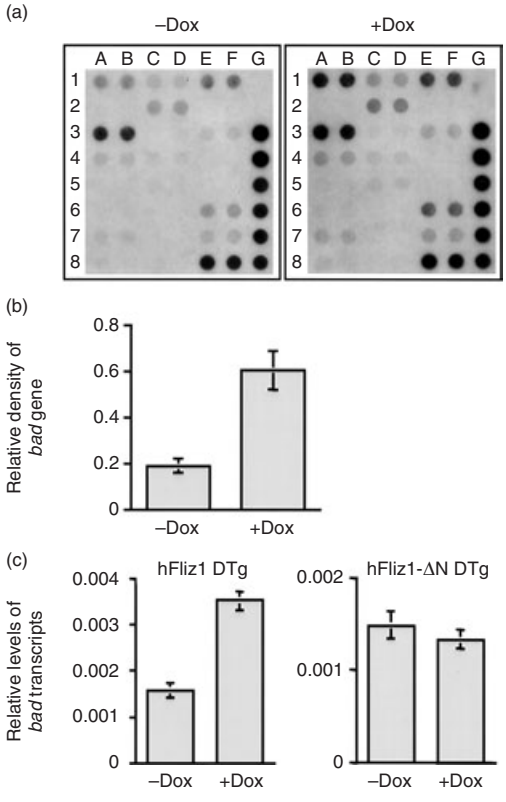

Figure 3. (a) Gene expression array assay. The radiolabeled cDNA probes were prepared from thymocytes, derived from hFliz1 DTg mice fed with sucrose (−Dox) or Dox water (+Dox). The genes included in the mouse apoptosis blot are as follows: *bad* (1A, 1B), *bax* (1C, 1D), *bcl-2* (1E, 1F), *bcl-w* (2A, 2B), *bcl-x* (2C, 2D), *caspase-1* (2E, 2F), *caspase-2* (3A, 3B), *caspase-3* (3C, 3D), *caspase-7* (3E, 3F), *caspase-8* (4A, 4B), *c-myc* (4C, 4D), DR5 (4E, 4F), E2F1 (5A, 5B), E124 (5C, 5D), FasL (5E, 5F), growth arrest and DNA damage-inducible gene family-45 (6A, 6B), inducible nitric oxide synthase (6C, 6D), mdm2 (6E, 6F), nuclear factor κB1 (7A, 7B), $p21^{waf1}$ (7C, 7D), p53 (7E, 7F), Rb (8A, 8B), TNF-related apoptosis-inducing ligand (8C, 8D), β-actin (3G, 4G), reduced glyceraldehyde-3-phosphate dehydrogenase (5G, 6G, 7G, 8E, 8F, 8G), and pUC18 (1G, 2G). (b) The autoradiograph of the hybridized mouse apoptosis blots was subjected to densitometric analysis. Data shown is the relative autoradiography intensity of *bad* as compared to that of β-actin. (c) Real-time PCR analysis of *bad* expression. Total thymocyte RNA was isolated from the mice of indicated genotypes fed with sucrose (−Dox) or Dox water (+Dox). The levels of *bad* transcripts were normalized against those of β-actin.

DISCUSSION

During thymic ontogeny, massive apoptosis has to take place during the double positive stage to delete autoreactive T cells and maintain thymic homeostasis. Our data presented in this report demonstrate, for the first time, that Fliz1 might be a critical regulator of thymocyte homeostasis by inducing thymocyte apoptosis and that the effects of Fliz1 might be mediated by Bad. Bad was first isolated on the basis of its interaction with Bcl-2 and acts to promote cell death by selectively interacting with Bcl-x_L.[11–14] It was recently demonstrated that Bad might be a key regulator of T-cell apoptosis and development.[15] Thymocytes derived from Bad transgenic mice were very prone to apoptosis, either spontaneous or induced. Interestingly, Bad transgenic mice also displayed dramatic thymic hypocellularity similar to, albeit more severe than, that observed in hFliz1 DTg mice. The discrepancy in severity might be explained by the higher levels of Bad in Bad transgenic mice.

While our data suggest that up-regulation of *bad* might be an attractive explanation for the enhanced apoptosis

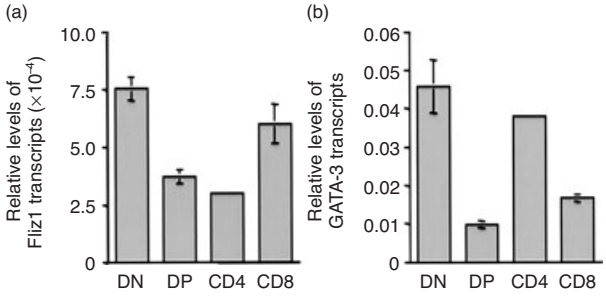

Figure 4. Expression of Fliz1 in thymocyte subsets. Thymocytes obtained from wild type C57BL/6 mice were sorted into $CD4^-$ $CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+$ $CD8^-$ single positive (CD4), and $CD4^-$ $CD8^+$ single positive (CD8) populations by a MoFlo cell sorter (Becton-Dickinson). Total RNA was prepared from each subset and subjected to real time PCR analyses to measure the transcript levels of Fliz1 (a) and GATA-3 (b), which were normalized against those of β-actin.

seen in hFliz1 DTg thymus, it remains unclear how Fliz1 induces the expression of *bad*. It is also very likely that the increased Bad level is consequent to, but not the cause of, enhanced apoptosis, because the levels of Bad could be greatly induced in thymocytes exposed to apoptotic stimuli.[15] In this case, the mechanism mediating the hFliz1-induced apoptosis remains unanswered. It will be informative to study if overexpression of hFliz1 can still induce thymocyte apoptosis in the absence of Bad.

The expression kinetics of Fliz1, which is relatively enriched in double negative population, is somewhat contradictory to its potential role in promoting thymocyte apoptosis. However, there are many examples that biological function of a given protein do not correlate with its transcript levels. Degradation, post-translational modification, and interacting partners can significantly affect the functions of any given protein. Unfortunately, Fliz1-specific antibody is not yet available, precluding us to directly measure the protein levels of Fliz1 among thymocyte subsets; and very little is know about the degradation and post-translational modification of Fliz1 protein. Therefore, the significance of the data presented in Fig. 4 remains to be determined. Finally, while our data imply that Fliz1 might regulate thymocyte homeostasis, we would like to cautiously point out that this conclusion was based on experiments overexpressing Fliz1, which are subjective to non-physiological effects. In addition, the process of thymocyte apoptosis is very complicated and is regulated by multiple mechanisms.[16–18] Thus, the actual contribution of Fliz1 in controlling this complicated process remains to be determined. Generation and studying of Fliz1-deficient mice will be very informative and are currently underway.

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