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Critical Roles for Transcription Factor GATA-3 in Thymocyte Development

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Summary

The transcription factor GATA-3 is expressed at every stage of thymic development, but its role in thymocyte differentiation is unknown. The fact that RAG chimeric animals lacking GATA-3 cannot generate early thymocytes from common lymphoid progenitors has thus far precluded investigation of the function of GATA-3 in the thymus. To address this, we generated mice deficient in GATA-3 at early and late stages of thymic differentiation. Our studies revealed that GATA-3 is involved in β selection and is indispensable for single-positive CD4 thymocyte development. Thus, our data demonstrate that the coordinated and regulated expression of GATA-3 at each stage of thymic development is critical for the generation of mature T cells.

Introduction

Lymphoid progenitors committed to the T lineage must differentiate in the thymus prior to becoming fully functional peripheral α/β T cells. Maturation of α/β T cells in the thymus ensures that only thymocytes bearing a functional TCR will be selected at critical developmental checkpoints and escape apoptotic death. The first critical checkpoint occurs in very early $CD4^-$ $CD8^-$ double-negative (DN) thymocytes, which are further subdivided into four stages (DN1–4) based on their surface expression of CD44 and CD25. At the DN3 stage ($CD44^-$ $CD25^+$), those cells that have productively rearranged a T cell receptor β (TCRβ) gene assemble the pre-T cell receptor (pTCR), in which TCRβ is paired with the pre-T cell receptor α (pTα) chain. The formation of a functional pTCR is required for DN3 cell survival, a process also termed β selection (reviewed in Borowski et al., 2002; Michie and Zuniga-Pflucker, 2002). Failure of β selection caused by lack of rearrangement, lack of pTα or TCRβ chains, or deficiency of various components of the pTCR signaling complex results in arrest in development at the DN3 stage (Cheng et al., 1997; Clements et al., 1998; Fehling et al., 1995; Malissen et al., 1995; Mombaerts et al., 1992; Pivniouk et al., 1998; Shinkai et al., 1992; van Oers et al., 1996; Wolfer et al., 2002; Zhang et al., 1999). In contrast, cells that successfully pass the β selection checkpoint activate multiple signaling cascades that in turn trigger rapid proliferation, rescue from apoptosis, and differentiation into the DN4 ($CD44^-$ $CD25^-$) stage.

After passing through an intermediate single-positive (ISP) stage ($CD8^{med}$ $CD5^{low}$ TCR^{low}), β-selected thymocytes differentiate into $CD4^+$ $CD8^+$ (double-positive or DP) thymocytes. DP thymocytes then must survive positive and negative selection, the second critical checkpoint (reviewed in Sprent and Kishimoto, 2002; Starr et al., 2003). Only a small fraction of DP thymocytes survive, differentiating into cells singly positive for CD4 or CD8 (SP CD4 or SP CD8) that are then exported to the periphery. Successful generation of mature CD4 T cells requires commitment to the CD4 lineage; postcommitment events then ensure survival in the periphery. The process by which CD4/CD8 lineage commitment occurs has been heavily investigated, and conflicting lines of evidence support stochastic, instructive, and coreceptor reversal models (reviewed in Germain, 2002; Singer, 2002). Some signaling molecules, such as lck and Erk, may act as a lineage switch, based on experiments showing that high kinase activity can drive the generation of CD4 T cells even in animals expressing an MHC class I-restricted transgenic TCR, while low kinase activity promotes the development of $CD8^+$ MHC class II-restricted cells (Hernandez-Hoyos et al., 2000; Legname et al., 2000; Sharp and Hedrick, 1999). The transcription factors downstream of these signals that control the generation and maintenance of the CD4 lineage are currently unknown.

GATA-3 is a zinc finger transcription factor that is essential for the differentiation of common lymphoid progenitors into early DN thymocytes (Ting et al., 1996). GATA-3 is expressed throughout thymic development (Hendriks et al., 1999) but its role in DN, DP, and SP thymocyte differentiation is unknown. In vitro studies implicate GATA-3 in the control of transcription of multiple TCR chains (Henderson et al., 1994; Ho et al., 1991; Joulin et al., 1991) as well as the CD8α molecule (Landry et al., 1993), suggesting that GATA-3 might be essential for thymic development. To test this hypothesis, we generated mice conditionally deficient in GATA-3 at the DN and DP stages, and uncovered novel roles for GATA-3 in thymic differentiation. Specifically, we demonstrate here that GATA-3 is crucial for thymocytes to pass through β selection and that, without GATA-3, thymocytes committed to the CD4 lineage either cannot be generated or fail to survive. The coordinated and continued expression of GATA-3 is thus clearly required at multiple stages of thymocyte differentiation.

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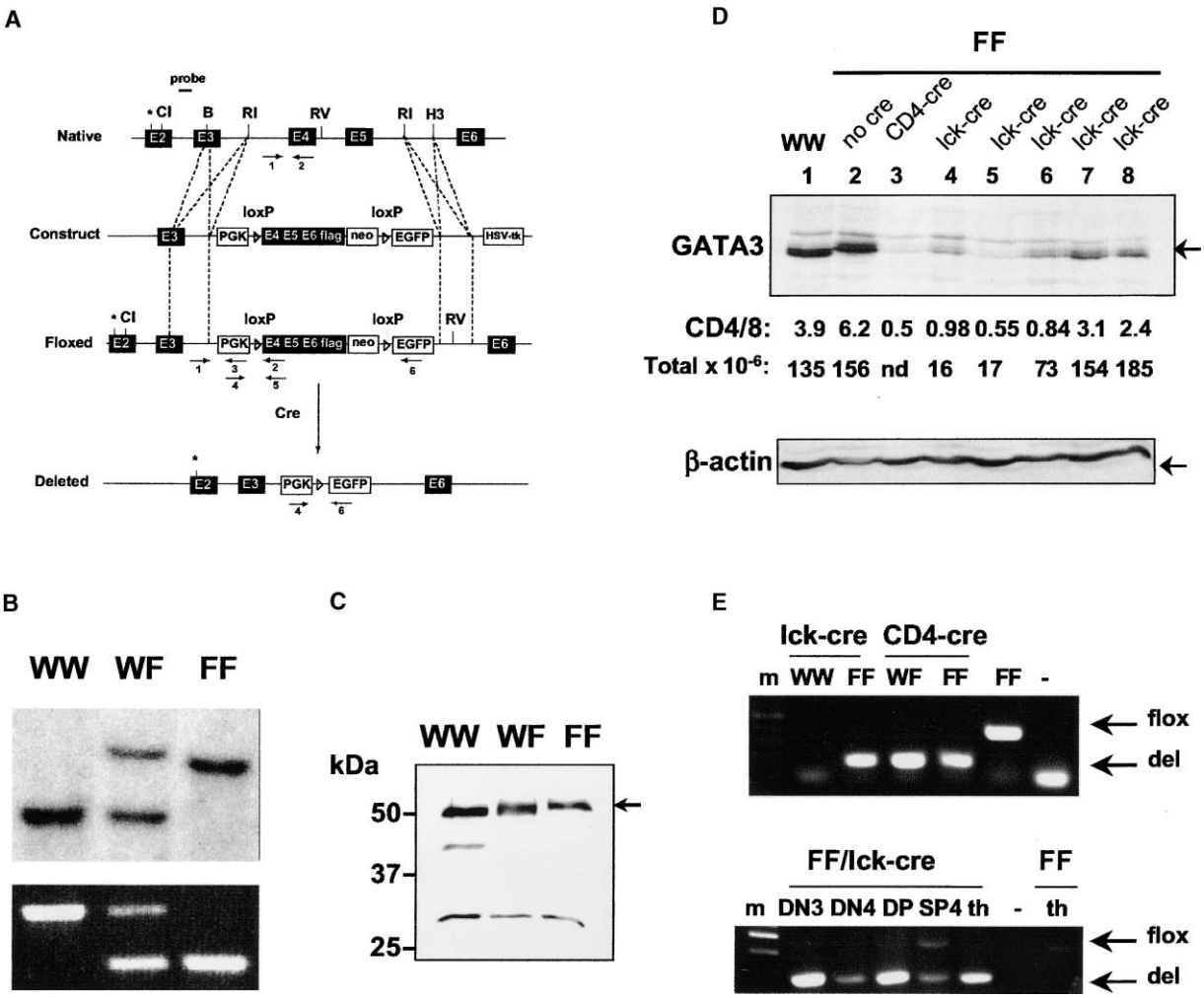

Figure 1. Conditional Deletion of Murine GATA-3 in Thymocytes

(A) Schematic of native murine GATA-3 locus, targeting construct, floxed locus after homologous recombination, and locus after Cre-mediated excision. The locations of probe used in (B), transcriptional start site (*), loxP sites, restriction enzyme sites (CI, ClaI; B, BamHI; RI, EcoRI; RV, EcoRV; H3, HindIII), and primers used for PCR analyses (1–6) are shown. The black boxes stand for exons. PGK denotes a PGK promoter, HSV-tk the HSV thymidine kinase gene, and neo the neomycin resistance gene.

(B) Genomic Southern blot (upper panel) detecting a smaller 10 kb wild-type ClaI-EcoRV fragment and a larger 13 kb flox fragment using the probe indicated in (A) and genomic PCR (lower panel) showing a larger 494 bp wild-type and smaller 334 bp flox product are shown. Results using DNA from wild-type (WW), heterozygous flox (WF), and homozygous flox (FF) animals are shown.

(C) Level of GATA-3 protein in thymocytes from WW, WF, and FF mice seen by Western blot analysis with the expected size of GATA-3 indicated by the arrow. The band between 37 and 50 kilodaltons seen in the WW lane is not reproducible.

(D) Western blot analysis of GATA-3, indicated by the arrow, using whole-cell thymocyte lysates from mice of the indicated genotypes is depicted (upper panel). The CD4/CD8 ratio and total thymocyte number in millions of cells in these mice is indicated below. Equal loading was confirmed by stripping and reprobing the membrane with antibody to actin (lower panel).

(E) PCR showing the relative proportion of flox allele (flox) and deleted (del) alleles in genomic DNA prepared from whole thymus of the indicated genotypes (upper panel). A similar analysis using genomic DNA from sorted thymocyte populations is also shown (lower panel). DN3, DP, and SP CD4 (SP4) thymocytes were obtained from FF/lck-cre animals and compared with whole thymus (th) from FF and FF/lck-cre animals. In both panels, the first lane (m) had molecular weight markers, and the lane marked with − had no input DNA.

Results

Generation of Mice Conditionally Deficient in GATA-3

To study the role of GATA-3 in the development and function of T cells, we generated mice conditionally deficient in GATA-3 using the Cre-lox system (Sauer, 1998). The targeting construct is shown in Figure 1A. Using standard homologous recombination techniques, the region of murine GATA-3 encoding exons 4 and 5, which contain the zinc finger domains, was replaced with a cassette containing exons 4, 5, and 6, flanked by loxP sites, creating a "floxed" allele. A PGK promoter and the sequence encoding enhanced green fluorescent protein (EGFP) were engineered such that deletion of the cassette containing exons 4, 5, and 6 would place the PGK promoter immediately upstream of EGFP, with the intention that the cells would thus be marked after Cre-mediated excision. Germline transmission from two chimeric animals was confirmed by Southern blotting and PCR

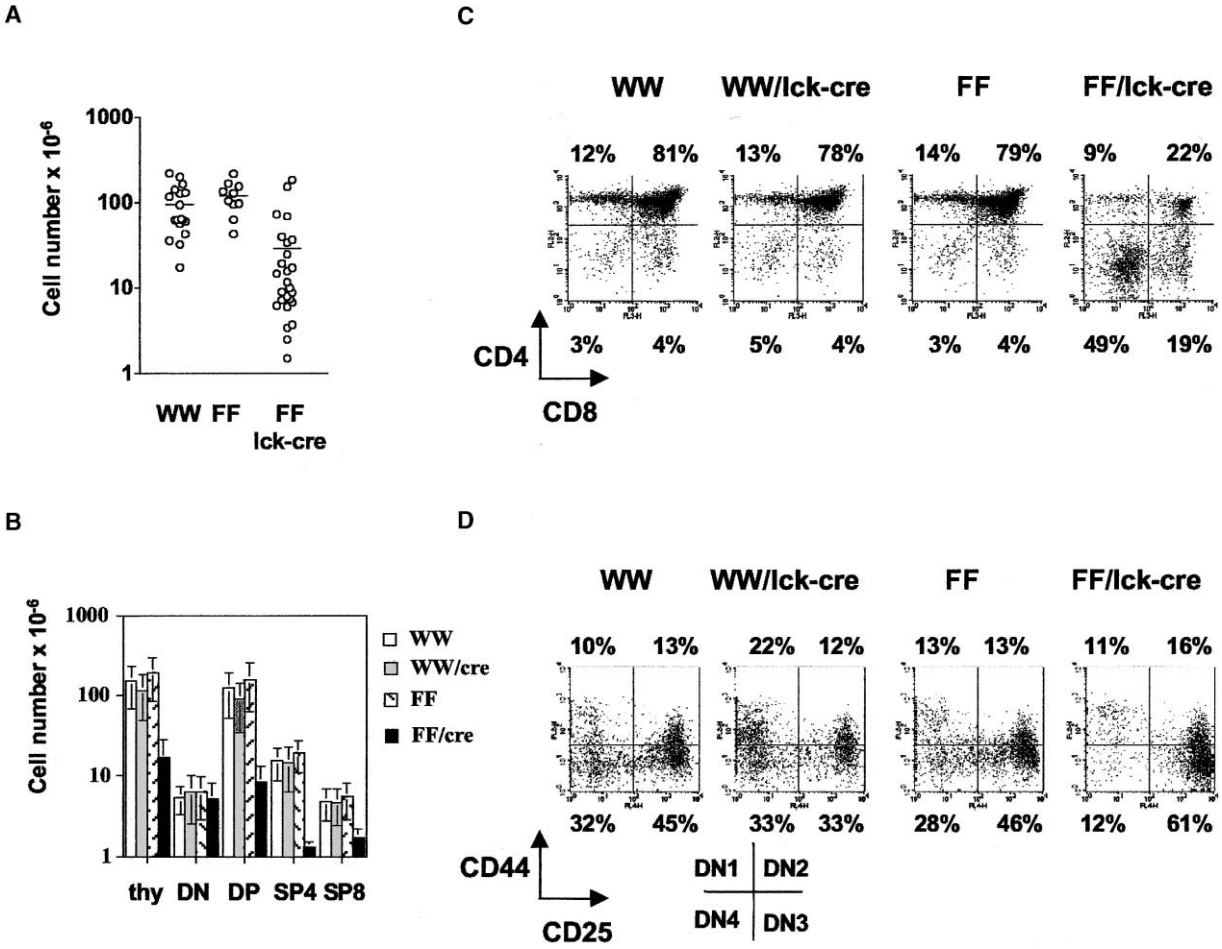

Figure 2. Abnormal Thymocyte Development of FF/lck-cre Mice

(A) Number of total thymocytes from WW (n = 18), FF (n = 10), and FF lck-cre (n = 27) with each circle representing an individual mouse. The average number is indicated by the horizontal line.

(B) Number of total thymocytes and of DN, DP, SP CD4, and SP CD8 subsets from the first six litters of wild-type (WW, WW/lck-cre), homozygous flox (FF), and knockout (FF/lck-cre) mice. Error bars denote standard deviation.

(C) Representative FACS analysis of thymocytes from WW, WW/lck-cre, FF, and FF/lck-cre mice stained with antibodies against CD4 and CD8. The total thymocyte numbers of these mice were 1.66×10^8, 2.02×10^8, 1.50×10^8, and 6.2×10^6, respectively. The percentages of DN, DP, SP CD4, and SP CD8 cells are indicated.

(D) Representative FACS analysis of DN thymocytes from WW, WW/lck-cre, FF, and FF/lck-cre mice, gating on cells negative for CD4 and CD8, then stained with antibodies against CD44 and CD25. The percentages of DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$), and DN4 ($CD44^-$ $CD25^-$) subsets are indicated.

(Figure 1B), and all results presented here were observed in both lines. Animals bearing one floxed allele (WF) were intercrossed to generate homozygous flox (FF) animals, which had near normal amounts of thymic GATA-3 protein (Figure 1C) and normal numbers of thymocytes and thymic subsets (Figure 2 and data not shown); therefore, we considered FF mice to be essentially equivalent to wild-type mice. Because a PGK promoter was inserted upstream of the engineered region encoding exons 4–6 in the recombination cassette, it was possible that a zinc finger only truncated protein could be generated in the FF animals; however, no shorter transcript or protein was detected by Northern or Western analysis (data not shown). In addition, Western blotting did not reveal any truncated protein that could encode exons 2 and 3 in any of the knockout animals (data not shown).

These FF mice were then crossed to mice carrying a transgene for Cre, either driven by the proximal lck promoter (lck-cre) or by the CD4 promoter (CD4-cre) to delete the GATA-3 gene at the DN3 or DN4/DP stages, respectively (Lee et al., 2001; Wolfer et al., 2002). The construct design was intended to distinguish deleted from nondeleted cells by GFP expression; indeed, GFP expression was detected when ES cells bearing one floxed allele were transfected with Cre plasmid (data not shown). However, in thymus, lymph node, and spleen of FF mice crossed with Cre transgenic mice, less than 1% of the cells, if any, expressed GFP, which likely results from weak activity of the PGK promoter in these tissues compared to embryonic tissues. Western analysis confirmed that both sets of knockout mice have reduced amounts of GATA-3 protein (Figure 1D), though the degree of GATA-3 deficiency in FF/lck-cre mice was

highly variable. As discussed later, the severity of the phenotype observed varied with the level of remaining GATA-3 protein. In FF/lck-cre animals that had a severe phenotype and in all FF/CD4-cre animals examined, PCR analysis of genomic DNA from whole thymus confirmed near complete deletion of the GATA-3 gene (Figure 1E, upper panel). Whole thymus would include DN1 and DN2 cells that had not yet expressed lck-cre and DN cells that had not yet expressed CD4-cre; thus, the trace amount of remaining floxed allele and GATA-3 protein is consistent with the proportion of nondeleted cells expected. These PCR findings correlated well with Southern blotting analysis (data not shown). In addition, PCR analysis of DNA from sorted thymocyte subsets in severely affected FF/lck-cre mice confirmed that deletion was practically complete in DN3, DN4, and DP stages (Figure 1E, lower panel). SP CD4 cells in contrast had a mixture of deleted and nondeleted DNA (Figure 1E, lower panel).

Mice Lacking GATA-3 in the DN Stage Have Abnormal Thymic Development

FF animals carrying the lck-cre transgene (FF/lck-cre mice) were normal in appearance and size, and showed no gross abnormalities. Among the first 124 animals born from WF animals crossed with WF/lck-cre animals, only 6.5% were FF/lck-cre, versus 12.5% as expected, suggesting that there might have been misexpression of Cre in a nonspecific manner during embryonic development, causing embryonic lethality. Mice were analyzed between 4–6 weeks of age, and the number of total thymocytes is shown in Figure 2A. The average total thymocyte number was 9.5, 12.2, and 2.9 $\times$ 10^7 in wild-type mice, FF mice, and FF/lck-cre mice, respectively. In most experiments, FF/lck-cre mice had approximately a 10-fold reduction in the number of α/β TCR^+ thymocytes compared to littermate controls. There was considerable variability in the phenotype with a few mice having normal thymus size (Figure 2A), likely due to incomplete excision of the GATA-3 gene by lck-driven Cre. Indeed, Western blotting confirmed that those animals with near normal thymocyte number (70–200 million total) in general had a substantial amount of GATA-3 protein remaining while those with small thymus size (less than 20 million total) had much less (Figure 1D). FACS analysis of the first six litters of mice after staining for CD4 and CD8 showed that the absolute number of DN cells was preserved but that the DP and SP CD4 cell numbers and percentages were markedly decreased (Figures 2B and 2C). The total SP CD8 cell number was also decreased, though in some cases the percentage was higher than in wild-type mice (Figure 2C). Overall, about two-thirds of FF/lck-cre mice had less than 20 million thymocytes, of which approximately 30%–60% were DN cells and 20%–60% were DP cells, suggesting a partial developmental arrest from the DN to the DP stage. To further characterize the defect, thymocytes were stained with antibodies against CD4, CD8, CD44, and CD25 to separate the DN1–4 stages. This revealed a relative accumulation of cells in the $CD44^-$ $CD25^+$ DN3 stage with a deficiency of $CD44^-$ $CD25^-$ DN4 cells (Figure 2D). Thus, FF/lck-cre mice had markedly decreased numbers of mature α/β T cells due to partial arrest at the DN3 to DN4 transition.

We then investigated the mechanism of the DN3 arrest. DN3 cells typically manifest a characteristic change in cell size as they undergo β selection. The smaller, less mature cells, termed E cells, have not yet fully rearranged TCRβ, whereas the larger L cells have completed VDJ recombination, begin to assemble the pTCR, and go on to differentiate into DN4 and later DP cells (Hoffman et al., 1996). We thus examined the percentage of L cells as a surrogate marker of whether events downstream of pTCR assembly had occurred. On average, 14%–30% of DN3 cells in wild-type or FF animals were larger by analysis of forward scatter. In contrast, FF/lck-cre DN3 cells had less than half as many L cells, ranging from 2%–10%. A representative experiment is shown in Figure 3A. Thus, FF/lck-cre DN3 thymocytes failed to undergo the changes in cell size that precede their differentiation into DN4 cells, presumably due to insufficient signals via the pTCR.

In addition to changes in cell size, thymocytes undergoing proper β selection should be rescued from apoptosis. We therefore examined the degree of apoptosis in FF/lck-cre mice by Annexin V staining. FF/lck-cre animals had increased levels of Annexin V staining in most thymic subsets, 2- to 8-fold higher than WW or FF littermates, except in the total DN population (Table 1). We also examined the percent of Annexin V-stained cells within the DN1–4 populations and found that DN4 cells in FF/lck-cre mice had more apoptosis than DN4 cells in control animals (Figure 3B). In contrast, DN3 cells from FF and FF/lck-cre mice had equivalent amounts of apoptosis (Figure 3B). Thus, those DN3 cells that successfully developed into DN4 cells in the absence of GATA-3 were more prone to apoptosis at the DN4 stage.

FF/lck-cre DN3 Cells Are Deficient in T Cell Receptor β Protein

On the basis of our findings that FF/lck-cre DN3 thymocytes failed to develop into L cells and that there was increased apoptosis in all stages following DN3, we suspected that pTCR signaling was deficient in these animals. As stated in the Introduction, GATA-3 is known to bind the TCRβ enhancer, and TCRβ is required to form the pTCR. We found that FF/lck-cre DN3 thymocytes had lower amounts of intracellular TCRβ protein by FACS staining (Figure 3C). In addition, only about 40% of FF/lck-cre DN4 cells, in comparison to 85%–90% of wild-type DN4 cells, contain a detectable amount of intracellular TCRβ protein (Figure 3C). Thus, not only were there few DN4 cells, but half of those that matured did so in the absence of pTCR expression. We showed that these TCRβ-negative DN4 cells were not contaminants by confirming that these cells were CD2 positive (data not shown). One possible explanation for these findings is that those DN4 cells with adequate intracellular TCRβ protein could have failed to delete GATA-3. However, PCR analysis confirmed that the majority of DN4 and DP cells in these animals had indeed deleted the GATA-3 gene (Figure 1E, lower panel).

DN3 cells in Notch-1 conditionally deficient animals also have reduced intracellular TCRβ protein, and DN4 cells in these animals can mature in the absence of pTCR; these mice have been shown to have a defect in TCRβ gene rearrangement (Wolfer et al., 2002). We

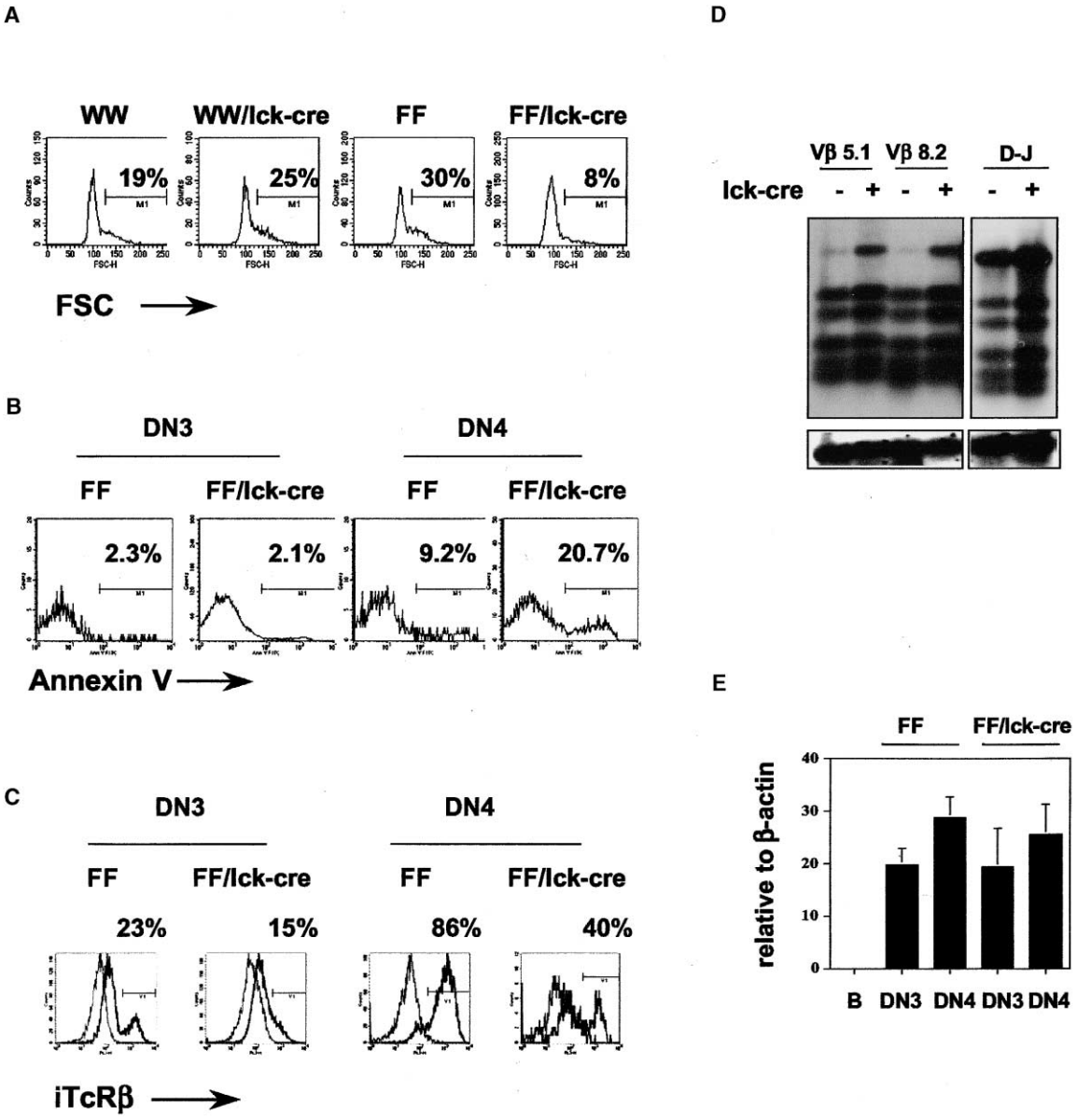

Figure 3. FF/lck-cre DN3 Thymocytes Have Deficient β Selection and Have Low Intracellular TCRβ Levels

(A) The percentage of L cells within the DN3 population by FACS analysis, gating on $CD4^-$ $CD8^-$ $CD44^-$ $CD25^+$ thymocytes, then analyzing forward scatter of thymocytes from mice of the indicated genotypes is shown.

(B) Thymocytes negative for CD4, CD8, and CD44 were divided into DN3 and DN4 populations by CD25 staining, and the percentage of cells positive for Annexin V staining was measured by FACS analysis.

(C) DN3 and DN4 thymocytes of indicated genotypes were permeabilized, stained with antibody against TCRβ (bold histogram) or isotype control antibody (light histogram), and analyzed by FACS. The percentage of intracellular TCRβ-positive cells is indicated.

(D) Genomic DNA from DN3 thymocytes of FF or FF/lck-cre mice was purified and subjected to PCR amplication using primers derived from Vβ5.1, Vβ8.2, and D-J regions of TCRβ. The presence or absence of lck-cre transgene is noted by + and − symbols. The PCR products were then analyzed by Southern blotting using the D-J unrearranged PCR product as probe (upper panels). Reprobing membranes with probe to Thy-1 confirmed equal loading (lower panels).

(E) Real-time PCR analysis showing levels of TCRβ transcript relative to β-actin control detected in cDNA prepared from FF or FF/lck-cre DN3 and DN4 thymocytes. Results using cDNA prepared from B cells (B) served as a negative control. All reactions were run in triplicate, and error bars denote the standard deviation.

therefore examined TCRβ rearrangement in our animals by purifying DN3 thymocyte genomic DNA, amplifying across V-J and D-J segments, and detecting the various sized products by Southern blotting, but were unable to detect any deficiency in TCRβ gene rearrangement (Figure 3D). This observation is in agreement with our finding that the TCRβ repertoire in mature SP cells from these animals was normal (data not shown). Given that rearrangement was normal, we also examined transcription. Real-time PCR analysis of RNA from purified DN3

Table 1. Fold Change in Percentage of Annexin V-Positive Cells Comparing Control Thymocytes to FF/lck-cre Thymocytes

	DN	DP	SP4	SP8	All Subsets
Experiment 1	1.7	8.7	2.5	3.5	7.4
Experiment 2	1.5	5.7	6.5	5.6	3.4
Experiment 3	1.2	3.0	2.4	6.3	3.3

Thymocytes from control and FF/lck-cre littermates were stained with antibodies to CD4 and CD8, and then stained with Annexin V reagent. The percentage of Annexin V-positive cells was measured by FACS analysis in total thymocytes and in each thymocyte subset, and then expressed as fold change compared to control animals. The control animal was a WW animal in Experiment 2 and a FF animal in Experiments 1 and 3.

and DN4 thymocytes surprisingly showed that the amount of TCRβ message was also normal (Figure 3E). It is possible, therefore, that GATA-3 controls the expression of TCRβ in DN3 cells by an as yet uncharacterized posttranscriptional mechanism.

TCR Transgene Expression Failed to Rescue the DN3 Arrest in FF/lck-cre Mice

Our findings suggested that the thymus in FF/lck-cre mice was small due to partial DN3 arrest, which in turn was caused by deficient TCRβ protein and insufficient pTCR signaling. If deficient pTCR expression were the only cause of the DN3 arrest, and if DN3 arrest were the only barrier to the generation of DP cells, the provision of a functional, rearranged TCR transgene would be expected to overcome the DN3 arrest and potentially restore thymocyte numbers. To test this hypothesis, FF/lck-cre mice were crossed to DO11.10 (DO11) TCR transgenic animals. The DO11 transgene encodes an α/β TCR that is fully rearranged, MHC class II restricted, and specific for ovalbumin peptide (Murphy et al., 1990). Approximately 30%–60% of FF/lck-cre/DO11 DN3 ($CD4^-$ $CD8^-$ $CD44^-$ $CD25^+$) cells were positive for the DO11 transgene, confirming appropriate expression (Figure 4A). In Figure 4B we show that FF/lck-cre/DO11 DN3 cells expressed the DO11 transgene at high levels; FF/DO11 DN3 cells were mostly DO11 negative. In addition, as shown in Figure 4A, the percentage of $DO11^+$ L cells was increased to 21%, as compared to 12% of $DO11^-$ population, indicating that at least some TCR signals in DN3 cells were restored by the DO11 transgene. However, the thymus of FF/lck-cre/DO11 animals remained small, with preservation of total DN cell number and more than 10-fold reduction in DP and SP CD4 number (Figure 4C). The SP CD8 number was also decreased though less dramatically. Subset analysis of total thymocytes (data not shown) or gating only on $DO11^+$ cells (Figure 4D) revealed persistent abnormalities. Thus, $DO11^+$ FF/lck-cre cells were partially deficient in the generation of DP cells, similar to nontransgenic knockout cells.

In wild-type DO11 mice, $DO11^+$ DN3 cells rapidly progressed to the DN4 stage, and approximately 70%–80% of the $CD4^-$ $CD8^-$ $CD44^-$ (DN3 and DN4) population was $CD25^-$ DN4 cells (Figure 4E). In contrast, $DO11^-$ knockout cells accumulated at the DN3 ($CD25^+$) stage, demonstrating persistence of the DN3 arrest even in the presence of a functional TCR (Figure 4E). Thus, while TCR expression and signaling were restored, the partial DN3 arrest seen in FF/lck-cre mice was not corrected by provision of a functional TCR. These observations argue that, in addition to regulating TCRβ protein levels, GATA-3 also may play a critical role in events downstream of the pTCR that are required for the progression from the DN3 to the DN4 stage.

Deletion of GATA-3 after β Selection Results in a Profound Deficiency of SP CD4 Cells

FF/lck-cre mice have a reduced CD4/CD8 ratio in the thymus, suggesting that GATA-3 may affect the CD4/CD8 lineage determination (Figures 2B and 2C). This was especially striking in the mice with small thymus size, with an average CD4/CD8 ratio of 1.33 in mice with less than 20 million total thymocytes compared to a normal ratio of 3 or 4. As discussed above, there was clearly incomplete excision of the GATA-3 gene in individual FF/lck-cre mice; Figure 1D shows that those FF/lck-cre mice with a more striking reversal in CD4 to CD8 ratio had much less GATA-3 protein remaining than those with a normal ratio. These data implied that GATA-3 is necessary to generate or maintain SP CD4 cells and that the amount of GATA-3 protein determines the SP CD4 cell number. To confirm this observation and to eliminate the confounding effect of the DN3 arrest seen in FF/lck-cre mice, we examined thymocyte development in FF mice crossed with CD4-cre transgenic animals (FF/CD4-cre).

Because CD4-cre mediated excision occurs after β selection (Lee et al., 2001; Wolfer et al., 2002), FF/CD4-cre animals should not suffer from DN3 arrest and thus should have normal numbers of thymocytes. Indeed, total thymocyte number was preserved in these animals (Figure 5A); however, the percentage of SP CD4 thymocytes was dramatically reduced from 10%–15% in control animals to 3% or less in FF/CD4-cre animals (Figure 5B). This was also reflected in the absolute numbers, where SP CD4 cell number was at least one log lower, while the number of SP CD8 cells was normal and not increased above control (Figure 5A). The CD4 promoter used to drive the cre transgene does not contain the intronic silencer (Sawada et al., 1994) and is therefore active in both SP CD4 and SP CD8 populations. Indeed, nearly 100% of DP and SP CD8 cells of FF/CD4-cre mice have undergone Cre-mediated deletion (Figure 5C). The preservation of SP CD8 cell number argued against a general defect in positive selection. Indeed, $CD4^{high}$ $CD8^{med}$ cells gated as shown in Figure 5B were mostly $CD69^{high}$ in both FF and FF/CD4-cre animals, confirming that positive selection was occurring (Figure 5D). Furthermore, SP CD8 thymocytes in FF/CD4-cre mice were mature with high levels of CD5 and had high levels of surface TCRβ, though slightly lower than wild-type levels (Figure 5D). Thus, the reversal in CD4 to CD8 ratio was even more striking in the FF/CD4-cre mice than in FF/lck-cre mice, supporting a dose dependence of CD4 number on the amount of GATA-3 protein. These observations indicate that, once thymocytes reach the DP stage, GATA-3 is essential for further development of SP CD4 cells but dispensable for the maturation of SP CD8 cells.

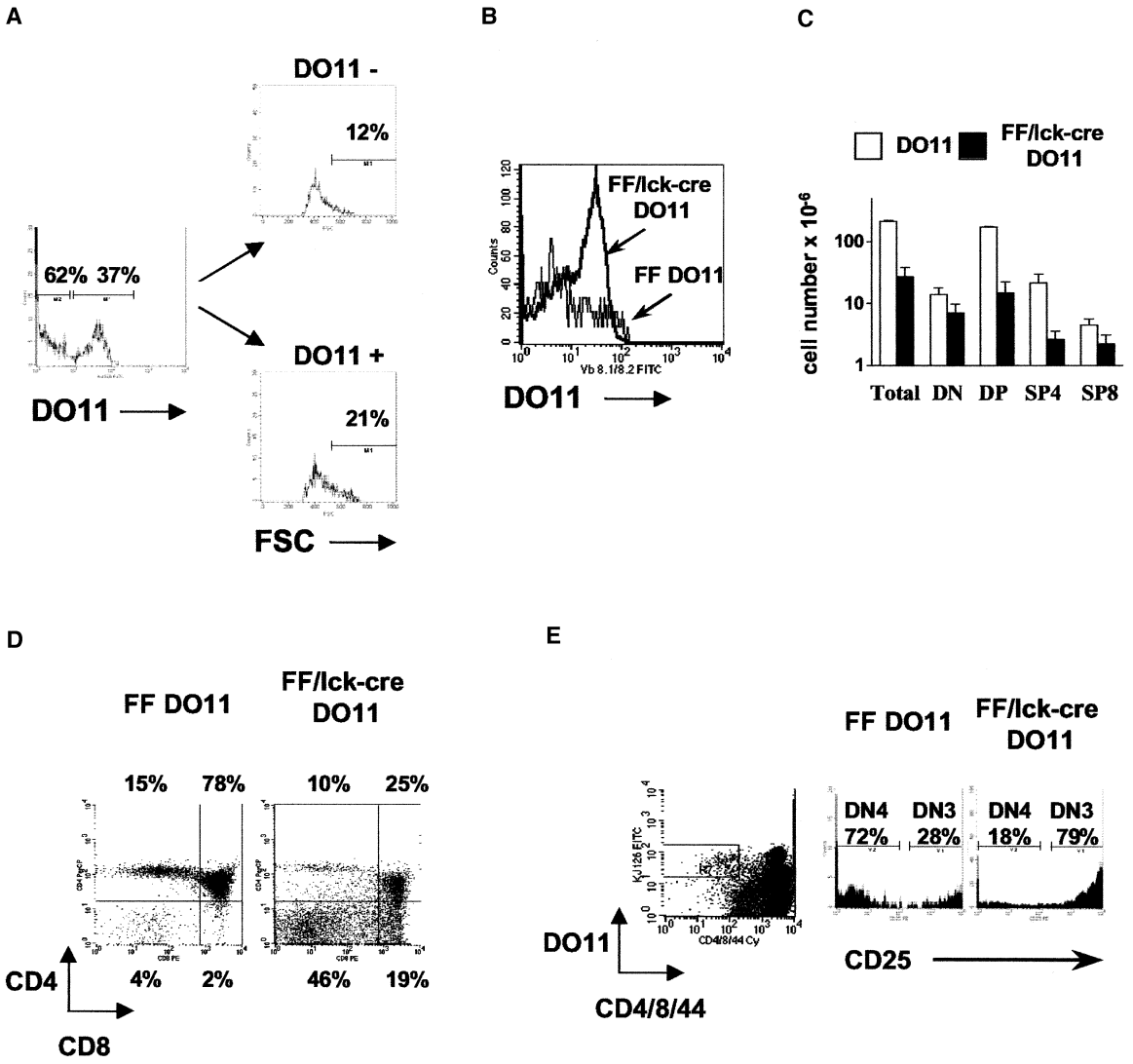

Figure 4. Analysis of Thymocytes from FF/lck-cre DO11 Mice

(A) DN3 thymocytes from FF/lck-cre/DO11 mice were gated as in Figure 3A and analyzed for staining for DO11 transgene as shown in the left panel, with the percentages of DO11$^+$ and DO11$^-$ DN3 cells as indicated. Analysis of these cells by forward scatter and the percentage of L cells in each population are shown in the right two panels.

(B) DN3 cells from FF DO11 (light histogram) and FF/lck-cre/DO11 mice (bold histogram) were analyzed by gating on CD4$^-$ CD8$^-$ CD44$^-$ CD25$^-$ cells, and the level of DO11 transgene expression was compared.

(C) Total thymocyte number and numbers of DN, DP, SP CD4, and SP CD8 subsets from control DO11 transgene-positive and FF/lck-cre DO11 transgene-positive (FF/lck-cre DO11) mice. Control DO11 mice were one each of WW, WF, and FF genotypes. Standard errors are indicated by error bars.

(D) FACS analysis showing CD4 and CD8 staining of thymocytes gating on DO11 transgene-positive cells from mice of the indicated genotypes. The percentages in each quadrant are shown.

(E) DN thymocytes that were CD4$^-$ CD8$^-$ CD44$^-$ and DO11 transgene positive were gated as indicated in the left panel. The percentage of cells that were CD25$^+$ (DN3) and CD25$^-$ (DN4) within this population in control DO11 and FF/lck-cre DO11 mice are shown.

GATA-3-Deficient Mice Have a Postcommitment Defect in CD4 Thymocyte Generation

Having found that mice lacking GATA-3 after β selection were highly deficient in CD4 cells, we questioned whether GATA-3 is necessary for commitment to the CD4 lineage or alternatively for CD4 survival or maintenance. As discussed in the Introduction, strength of TCR signaling, in particular the levels of phospho-lck and phospho-Erk, has also been shown to affect the CD4/CD8 lineage decision. Western blotting for tyrosine-phosphorylated proteins in thymocytes left unstimulated, stimulated with antibodies to CD3 alone, or stimulated with CD3 and CD4 combined resulted in an increase in tyrosine-phosphorylated ZAP70 and lck in response to stimulation. However, no consistent differences in the amount of phosphorylated lck in WW, FF, or FF/CD4-cre animals were seen (Figure 6A). In specific immunoprecipitation assays for lck, the amount of activated lck in FF and FF/CD4-cre thymocytes appeared equivalent when compared to loading controls (two representative exper-

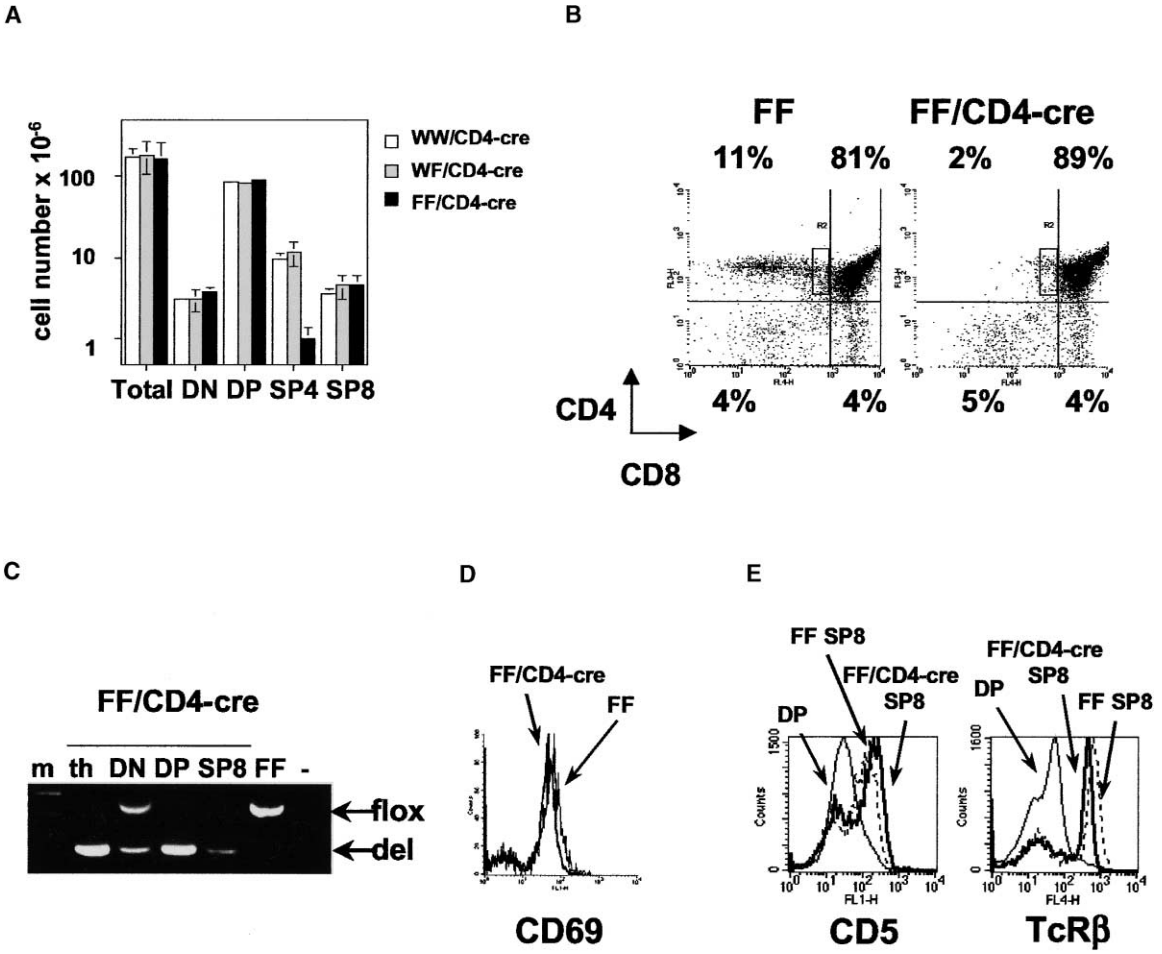

Figure 5. FF/CD4-cre Mice Are Severely Deficient in CD4 SP Thymocytes

(A) Total number of thymocytes and absolute numbers of DN, DP, SP CD4, and SP CD8 cells from WW/CD4-cre, WF/CD4-cre, and FF/CD4-cre mice. Error bars indicate standard deviation.

(B) FACS analysis of thymocytes from FF and FF/CD4-cre mice stained with antibodies against CD4 and CD8. The percentages of DN, DP, SP CD4, and SP CD8 cells are indicated.

(C) PCR detecting floxed and deleted alleles in sorted thymocyte populations from FF/CD4-cre mice. DN3, DP, and SP CD8 (SP8) cells were obtained from FF/lck-cre animals and compared with whole thymus (th) from FF and FF/lck-cre animals. The first lane (m) had molecular weight markers and the lane marked with − had no input DNA. The locations of floxed (flox) and deleted (del) alleles are indicated by the arrows.

(D) FACS analysis of CD69 surface expression in FF/CD4-cre mice. $CD4^{high}$ $CD8^{med}$ cells were gated as shown by the box in (B), and the level of CD69 expression in FF (light histogram) and FF/CD4-cre mice (bold histogram) is indicated by the arrows.

(E) FACS analysis of CD5 and TCRβ surface expression in FF/CD4-cre mice. The levels of the indicated molecules are shown after gating on DP cells (light histogram), FF SP CD8 cells (dashed histogram), and FF/CD4-cre SP CD8 cells (heavy histogram).

iments shown in Figure 6B). We also did not detect a consistent difference in the level of phosphorylated Erk, a further downstream target of TCR signaling, in sorted DP thymocytes (Figure 6C). Thus, we could not ascribe the lack of CD4 cells in FF/CD4-cre mice to weaker TCR signaling, leading to a failure of commitment to the CD4 lineage. The kinetics of signaling or the activation of other molecules not examined could possibly be affected, or, alternatively, GATA-3 could be required at a level of signaling downstream from the relatively proximal events examined.

As a more definitive test of whether GATA-3 determines the CD4 lineage, FF/CD4-cre mice were crossed with DO11 mice. If the absence of GATA-3 were sufficient to drive cells into the CD8 lineage, we would expect to see an enrichment of transgene-positive mature SP CD8 cells. This has been seen in mice expressing a catalytically inactive form of lck, which develop MHC class II-restricted CD8 cells (Hernandez-Hoyos et al., 2000). Subset analysis of the FF/CD4-cre mice crossed with DO11 was performed, gating on cells expressing DO11 transgene. Single-positive thymocytes in control DO11 animals were mostly SP CD4 cells; in contrast, FF/CD4-cre DO11 animals had a severe lack of SP CD4 cells and no enrichment of SP CD8 cells (Figure 6D). The FF/CD4-cre DO11 SP CD8 cells that were generated, though transgene-positive, were $CD5^{low}$, $CD69^{low}$, and HSA^{high}, characteristic of ISP cells rather than mature SP CD8 cells (data not shown). As a positive control, FF/CD4-cre mice crossed with OT-1 mice, which carry an MHC class I-restricted TCR transgene, were fully capable of generating SP CD8 cells (Figure 6E). Thus, GATA-3

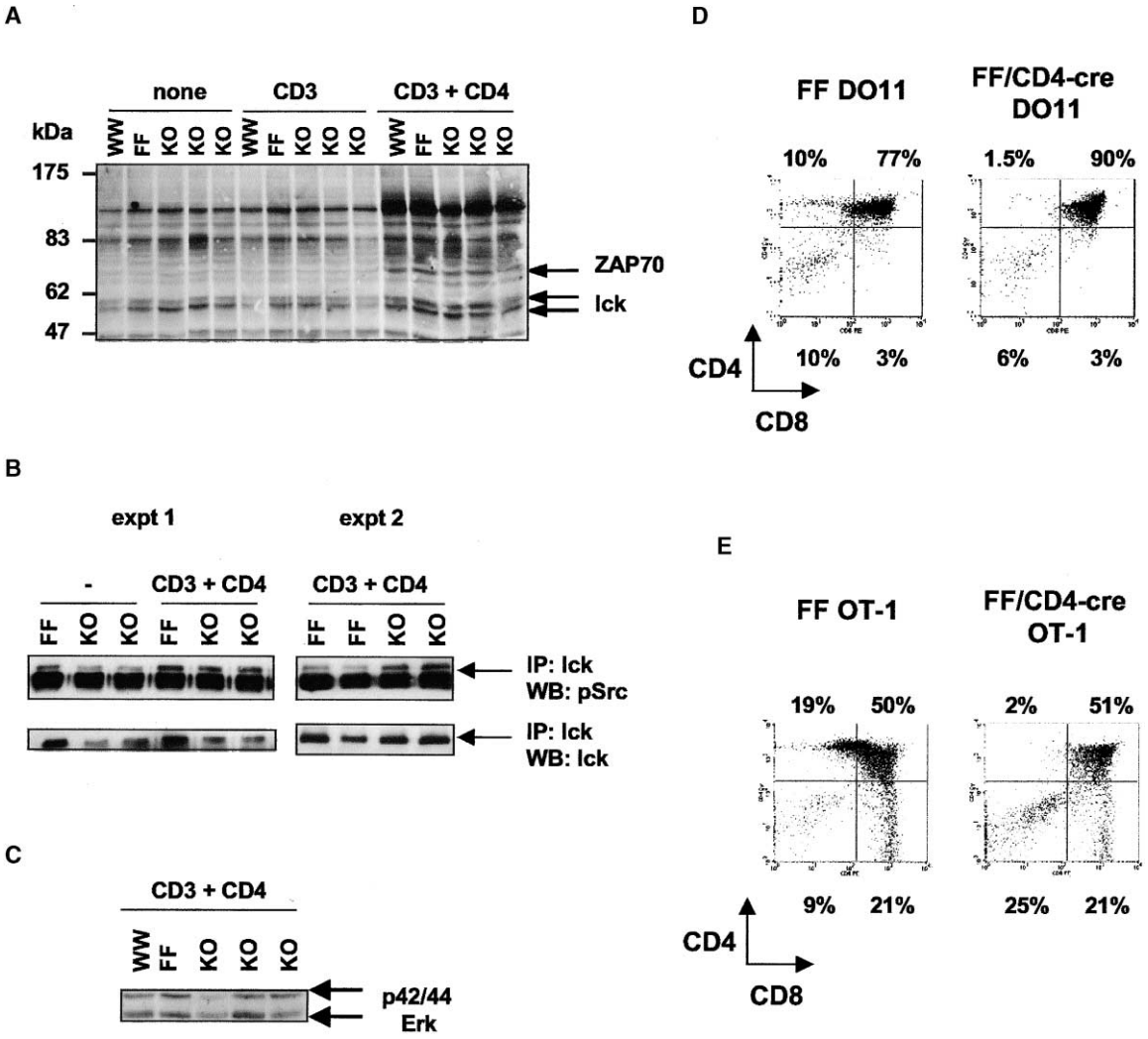

Figure 6. FF/CD4-cre Mice Signal Normally and Have a Postcommitment Defect in CD4 T Cell Generation

(A) Whole-cell lysates from thymocytes left unstimulated (none), stimulated by crosslinking CD3 (CD3), or crosslinking CD3 and CD4 (CD3 + CD4) were subjected to Western blotting analysis using anti-phosphotyrosine antibody. The areas representing phospho-ZAP70 and phospho-lck are indicated by arrows. Thymocytes from WW, FF, and FF/CD4-cre mice (KO) were analyzed.

(B) Western blotting of lck immunoprecipitates with anti-phospho-Src antibody from two experiments is shown with lck loading controls below. Thymocytes from FF or FF/CD4-cre mice (KO) were left unstimulated or stimulated by crosslinking CD3 and CD4. The arrow indicates the expected position of lck, and the band below the arrow represents heavy chain of immunoglobulin.

(C) The level of phospho-Erk by direct Western blotting of stimulated sorted DP thymocyte lysate from mice of the indicated genotypes is shown.

(D) FACS analysis of thymocytes stained with antibodies against CD4 and CD8 from FF DO11 mice and FF/CD4-cre DO11 mice, gating on transgene-positive cells. Percentages of DO11$^+$ DN, DP, SP CD4, and SP CD8 cells are indicated.

(E) FACS analysis as in (D) using thymocytes from FF OT-1 and FF/CD4-cre OT-1 mice.

deficiency did not result in an inability to generate SP CD8 cells in the appropriate context. Taken together, our data demonstrate that GATA-3 deficiency results in a severe postcommitment defect in CD4 T cell generation and/or survival.

Discussion

The precise function of GATA-3 in DN thymocytes has yet to be studied. Prior to these experiments, a role for GATA-3 in thymocyte development was predicted based on the presence of GATA-3 binding sites in the TCRα, TCRβ, and TCRδ enhancers. Indeed, a profound block at the DN3 stage or at the DP stage could have been expected if GATA-3 were critical to the function of these enhancers. Our analysis of mice conditionally deficient in GATA-3 at the DN3 stage revealed instead a partial DN3 arrest and reduced numbers of DN4 and DP cells. Given that GATA-3 is thought to regulate multiple TCR genes, this result could easily have been explained by deficient expression of pTCR components. That TCRβ transcription appeared normal and that mature SP CD8 cells in FF/CD4-cre mice expressed a near normal level of functional α/β TCR with a diverse repertoire (Figure 5E and data not shown) calls into question the importance of GATA-3 for transcriptional regulation

of TCR genes in vivo. Our findings argue that GATA-3 deficiency may lead to low levels of TCRβ in DN3 cells because of defects in translational efficiency or post-translational processing, which have not been previously described.

Not only did we find that the low levels of TCRβ protein in GATA-3-deficient DN3 cells occurred in the face of normal transcription, a surprising result, but our TCR transgenic experiments further demonstrate that TCRβ-sufficient thymocytes deficient in GATA-3 were still significantly arrested. Thus, we propose that GATA-3, independent of its role in regulating the expression of pTCR components, may be required for optimal pTCR signaling and function. One key function of the pTCR is to rescue DN3 thymocytes from death by apoptosis (reviewed in Marsden and Strasser, 2003; Michie and Zuniga-Pflucker, 2002), and indeed we found that GATA-3-deficient DN4 cells were more apoptotic (Table 1). Alternatively, it is also possible that GATA-3 may be required for promotion of DN3 differentiation through pathways separate from pTCR, such as the Wnt signaling pathway, a possibility that we have yet to explore (reviewed in Staal and Clevers, 2003). Recently it has been shown that thymic expression of constitutively active β-catenin induces differentiation of DP thymocytes in the absence of intracellular TCRβ protein and pTCR expression (Gounari et al., 2001). Whether GATA-3 is sufficient to drive β selection in the absence of the pTCR could be formally addressed by expressing GATA-3 in RAG-deficient thymocytes.

GATA-3 is expressed throughout thymic development, as is another important factor, Notch1. When Notch1 is deleted in bone marrow precursors, B cells develop in the thymus, demonstrating that Notch1 is required to suppress B cell lineage development from common lymphoid progenitors (Radtke et al., 1999; Wilson et al., 2001). Deletion of Notch1 by the lck-cre promoter, on the other hand, results in a partial DN3 arrest strikingly similar to that seen in our mice, due to deficient TCRβ gene rearrangement (Wolfer et al., 2002). The fact that Notch1 acts at two stages of early thymocyte development that also require GATA-3, the CLP stage and the DN3 stage, raises the possibility that these pathways intersect. In vitro studies have shown that GATA-2 is required for Notch1 to mediate granulocytic differentiation of a myeloid cell line (Kumano et al., 2001), but studies of GATA-3 and Notch1 are lacking. Notch1 deficiency in the DP stage using CD4-cre resulted in normal thymic development (Wolfer et al., 2001); if GATA-3's role in maintaining or generating CD4 SP thymocytes involves the Notch signaling pathway, the interaction may involve other Notch factors such as Notch2 or Notch3, or may take place at a point downstream of Notch action.

We also demonstrate that GATA-3 is indispensable for the survival or maintenance of SP CD4 thymocytes, which were nearly absent in animals in which GATA-3 was deleted before the DP stage. The phenotype observed in these mice is very similar to that of two mice described in the literature, the HD mutant mice (Keefe et al., 1999) and mice deficient in CD83 (Fujimoto et al., 2002). The HD mice, deficient in an unknown gene product, have a profound deficiency in $CD4^+$ T cells. When HD mice were crossed with MHC class II-restricted AND TCR transgenic mice, T cells were diverted into the CD8 lineage, similar to mice expressing inactive forms of lck or Erk. Thus, the HD gene product, as yet unknown, is necessary for commitment to the CD4 lineage (Keefe et al., 1999). CD83, on the other hand, is expressed on thymic epithelium and interacts with an ill-defined ligand, CD83L, on thymocytes. Mice deficient in CD83 lack CD4 T cells, but in contrast to HD mice, fail to develop either mature CD4 or CD8 cells when crossed to the AND TCR transgenic (Fujimoto et al., 2002). GATA-3-deficient TCR transgenic mice more closely resembled CD83-deficient animals in our experiments, suggesting that GATA-3 is necessary for post-commitment CD4 generation or survival, rather than for commitment to the CD4 lineage. That we did not see alterations in lck or Erk signaling further is consistent with our conclusion that lineage commitment is intact. Whether GATA-3 and CD83L directly regulate each other or whether signals from both pathways converge to generate SP CD4 thymocytes is unclear.

That GATA-3 is required for postcommitment survival of SP CD4 cells is in agreement with the work of others. CD2-driven GATA-3 expression in thymocytes did not alter CD4/8 lineage commitment in the thymus but rather inhibited the maturation and survival of committed CD8 peripheral T cells, inducing apoptosis (Nawijn et al., 2001), results that were confirmed, more recently, using in vitro reaggregate fetal thymic organ culture (Rg FTOC) (Hernandez-Hoyos et al., 2003). These latter authors also used overexpression of ROG to repress GATA-3 function and performed knockdown experiments using siRNA in fetal thymocytes. While we found that the SP CD4 cell number in our animals was drastically reduced, these authors report a more modest decrease in SP CD4 cell percentage, which might be explained by residual GATA-3 protein due to incomplete suppression by ROG or siRNA (Hernandez-Hoyos et al., 2003). Our results also differ in that we observed preservation of SP CD8 cell number, while in the latter study enhancement was seen (Hernandez-Hoyos et al., 2003). The relative proportion of SP CD4 and CD8 cells seen in Rg FTOC is known to vary widely with the stage of embryonic development and the number of days in culture; we thus attribute the disparity between our results and those of Hernandez-Hoyos and colleagues to the fact that in vitro Rg FTOC does not precisely recapitulate in vivo adult thymic development. Alternatively, nonspecific effects of ROG expression or of siRNA on molecules other than GATA-3 in the in vitro setting could be responsible. Taken together, our findings show that while positive selection, lineage commitment, and the maturation of SP CD8 cells remain intact in GATA-3-deficient mice, the survival or maintenance of SP CD4 thymocytes is critically dependent on GATA-3.

The manifestations of GATA-3 deficiency that we report here reflect the pluripotency of this important transcription factor, and yet the direct gene targets responsible for these phenotypes are unknown. Given that GATA-3 is required at multiple steps of thymocyte development, the downstream targets may differ at each stage of differentiation, and stage-specific cofactors may be required to elicit the expression of appropriate genes at each step. The dissection of these effects should prove fruitful in furthering our understanding of

thymic development and the generation of a complete and fully responsive immune system.

Experimental Procedures

Generation of Flox-Flox GATA-3 Mice and Other Transgenic Animals

The targeting construct is shown in Figure 1A. The backbone was derived from pPNT (Tybulewicz et al., 1991). Sequences encoding a loxP site flanked by XbaI and BamHI and encoding an EcoRI site, loxP site, PGK promoter, and splice site between exon 3 and 4 were inserted to generate the 3′ and 5′ loxP sites, respectively. The 5′ homology region was derived from a BamHI-EcoRI 5.8 kb fragment from 129 genomic DNA that encompasses part of exon 3 and the following intronic region. The 3′ homology region was derived from a 2.4 kb EcoRI-HindIII fragment from the intron between exons 5 and 6. The sequence encoding EGFP was obtained from a BamHI-AflIII 1 kb fragment from pEGFP1 (Clontech, Palo Alto, CA). One hundred twenty-nine murine embryonic stem cells and murine embryonic fibroblasts were purchased from Incyte Genomics (Palo Alto, CA). A 1.1 kb NcoI-KpnI probe from exon 6 detecting 6.5 kb wild-type and 3.9 kb and 1.5 kb floxed KpnI fragments was used to confirm recombination at the 3′ end. A 0.3 kb BglII-EcoRI probe from intron 2 detecting 10 kb wild-type and 13 kb floxed ClaI-EcoRV fragments was used to confirm recombination at the 5′ end. The sequences of the primers depicted in Figure 1A are as follows: primer 1, CCCCTTTCCCGGCTCTATCTT; primer 2, GGGCCGGT TCTGCCCATT; primer 3, CCTCCCCTACCCGGTAGAATT; primer 4, GGCATTCTCGCACGCTTCAAA; primer 5, GGGGCCGGTTCTGCCC ATTCATTTTATTGGTAGAGTCCGC; primer 6, GGATGGGCACCACC CCGGTGAA. Primers 1, 2, and 3 in Figure 1A were used to simultaneously detect wild-type and floxed alleles after 30 cycles. Alternatively, wild-type bands were detected individually using oligos 1 and 2 and floxed alleles using the primers 4 and 5.

lck-cre and CD4-cre transgenic animals were obtained from Dr. Christopher Wilson and screened as described (Lee et al., 2001). DO11.10 transgenic animals (Murphy et al., 1990) were purchased from Taconic (Germantown, NY). Mice were housed in a specific pathogen-free facility, and experiments were done in accordance with the institutional guidelines for animal care at Dana-Farber Cancer Institute and Harvard School of Public Health under approved protocols. FF/lck-cre animals were backcrossed at least five to six generations onto the BALB/c background while FF/CD4-cre animals were on the C57BL/6 background. FF/CD4-cre animals crossed with DO11.10 transgenic animals were backcrossed to BALB/c background at least three generations, and appropriate MHC typing was confirmed. All animals were sacrificed for analysis between 4 and 6 weeks of age.

Flow Cytometry

Staining was performed with fluorescent-tagged antibodies to CD4 (RM4-5), CD8α (53−6.7), CD44 (IM7), CD25 (PC61) (BD Pharmingen, San Diego, CA), and FITC-Annexin V (Oncogene Research Products, La Jolla, CA). Expression of the DO11.10 transgene was detected either using anti-idiotype antibody KJ1-26 (Caltag, Burlingame, CA) or by anti-Vβ 8.1/8.2 antibody (MR5-2) (BD Pharmingen). Analysis was performed on either a FACSort or a FACSCalibur machine (BD Biosciences, San Jose, CA) using FSC and SSC to gate on live cells. Intracellular staining of TCRβ (H57-597, BD Pharmingen) (Wolfer et al., 2002) and analysis of E cells and L cells (Hoffman et al., 1996) were performed as described. Cell sorting was performed on a MoFlo cell sorting machine (Cytomation, Fort Collins, CO). For some preparations DN thymocytes were enriched by magnetic depletion using CD4 or CD8α beads (Miltenyi Biotec, Auburn, CA).

PCR to Detect GATA-3 Zinc Finger Region Deletion

Primers 4, 5, and 6 as depicted in Figure 1A were used to simultaneously detect floxed and deleted alleles using genomic DNA isolated from thymocytes as template. After 35 cycles of amplification (45 s each at 94°C, 55°C, and 72°C), PCR products were run on a 2% agarose gel, and flox band (432 bp) and deleted band (174 bp) were visualized by ethidium bromide staining.

Detection of TCRβ Gene Rearrangement

Detection of TCRβ gene rearrangement was performed according to the method described in Wolfer et. al. (2002). In brief, oligonucleotides were used to amplify Vβ8.1, Vβ5.2, and DJβ segments. Primers to amplify Thy-1 were also included in each mixture. PCR products were separated on a 1.2% agarose gel and transferred onto Hybond N membrane (Amersham Biosciences, Piscataway, NJ). Southern blotting was performed with gel purified DJβ PCR amplification product. Membranes were then stripped and reprobed with a Thy-1 probe to confirm equal loading.

Real-Time PCR Analysis of TCRβ Constant Region

Thymocyte RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. First-strand cDNA was prepared after DNase I treatment (Invitrogen), and primers were used to detect TCRβ constant region transcripts (forward, TCAGTGCAGAGGCCTGGG; reverse, GACAGAACCCCC TGATGATAGG) and β-actin (forward, GCTCTGGCTCCTAGCAC CAT; reverse, GCCACCGATCCACACCGCGT). A FAM-labeled probe (Operon Technologies, Alameda, CA) was used for detection of β-actin message (TCAAGATCATTGCTCCTCCTGAGCGC). Because of the rarity of the DN3 and DN4 populations, thymocytes from six to nine mice were pooled for the analysis. Reactions were run in triplicate on a 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using 10 pmol of each primer, 0.2 ul of cDNA, and either 2× Taqman or 2× SYBR green reagent (Applied Biosystems) in a total reaction of 25 ul.

Thymocyte Stimulation and Immunoprecipitation

For phosphotyrosine analysis, stimulation of thymocytes and preparation of lysates were performed according to the method of Thien et al. (2003) with modifications. Single-cell suspensions were made, and 5×10^6 cells were chilled on ice in RPMI containing 10% fetal calf serum (Hyclone, Logan, UT) at 50×10^6/ml. Biotinylated antibodies against CD3ϵ (500A2) and CD4 (GK1.5) obtained from BD Pharmingen (San Diego, CA) were added at 10 μg/ml. Cells were warmed at 37°C for 2 min in a shaking water bath, and streptavidin (Sigma-Aldrich, St. Louis, MO) was added at 40 μg/ml for 5 min. Reactions were quenched with ice-cold PBS, and the cell pellet was lysed in ice-cold RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 1 mM sodium vanadate, 0.5 mM PMSF, and protease inhibitors.

For specific lck immunoprecipitation, cells were stimulated as above in the absence of serum, and the pellet was lysed in ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 1% Triton X-100, 20 mM NaF, 1 mM sodium vanadate, 10 mM β-glycerophosphate, 1 mM PMSF, and protease inhibitor cocktail (Mini-Complete, Roche). Clarified lysates were incubated with anti-lck antibody (Cell Signaling Technology, Beverly, MA) and then with protein G Sepharose beads (Amersham Biosciences, Piscataway, NJ). The beads were washed extensively with lysis buffer, boiled in sample buffer, and loaded onto SDS-PAGE.

Western Blotting

For Western blotting of GATA-3, thymocytes were lysed in Laemmli buffer, separated by SDS-PAGE, and transferred onto PVDF (Perkin Elmer, Boston, MA) by wet transfer. Membranes were incubated either with rabbit polyclonal antiserum (laboratory of J.M. Leiden) or with mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). GATA-3 was then detected using HRP-conjugated goat anti-rabbit or rabbit anti-mouse secondary antibody (Zymed, South San Francisco, CA) and ECL (Perkin Elmer, Boston, MA). For phosphotyrosine analysis, membranes were blocked in 1% BSA and incubated with mouse anti-phosphotyrosine antibody (4G10, gift of Dr. Hamid Band), protein A-conjugated HRP secondary (Zymed), and subjected to ECL (Perkin Elmer). For detection of phosphorylated active lck, lck immunoprecipitates were subjected to Western blotting using anti-phospho-Src antibody, and membranes were stripped and reprobed with anti-lck antibody. Anti-pErk was detected by direct Western analysis. Anti-lck (#2752), anti-phospho-Src (#2101), and anti-pErk (#9101) antibodies were purchased from Cell Signaling Technology (Beverly, MA).

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