

# Enforced Expression of GATA-3 During T Cell Development Inhibits Maturation of CD8 Single-Positive Cells and Induces Thymic Lymphoma in Transgenic Mice<sup>1</sup>

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The zinc finger transcription factor GATA-3 is of critical importance for early T cell development and commitment of Th2 cells. To study the role of GATA-3 in early T cell development, we analyzed and modified GATA-3 expression *in vivo*. In mice carrying a targeted insertion of a *lacZ* reporter on one allele, we found that GATA-3 transcription in CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes correlated with the onset of positive selection events, i.e., TCR $\alpha\beta$  up-regulation and CD69 expression. *LacZ* expression remained high (~80% of cells) during maturation of CD4 single-positive (SP) cells in the thymus, but in developing CD8 SP cells the fraction of *lacZ*-expressing cells decreased to <20%. We modified this pattern by enforced GATA-3 expression driven by the *CD2* locus control region, which provides transcription of GATA-3 throughout T cell development. In two independent *CD2-GATA3*-transgenic lines, ~50% of the mice developed thymic lymphoblastoid tumors that were CD4<sup>+</sup>CD8<sup>+/low</sup> and mostly CD3<sup>+</sup>. In tumor-free *CD2-GATA3*-transgenic mice, the total numbers of CD8 SP cells in the thymus were within normal ranges, but their maturation was hampered, as indicated by increased apoptosis of CD8 SP cells and a selective deficiency of mature CD69<sup>low</sup>HSA<sup>low</sup> CD8 SP cells. In the spleen and lymph nodes, the numbers of CD8<sup>+</sup> T cells were significantly reduced. These findings indicate that GATA-3 supports development of the CD4 lineage and inhibits maturation of CD8 SP cells in the thymus. *The Journal of Immunology*, 2001, 167: 715–723.

In the thymus early CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN)<sup>3</sup> precursors develop into mature CD4 or CD8 single-positive (SP) T cells following a tightly regulated program of cellular differentiation (1–4). The DN population is generally subdivided into four distinct developmental stages, defined by differential expression of the surface markers IL-2R $\alpha$  chain CD25 and phagocyte glycoprotein 1 CD44 (5). Precursor T cells rearrange their *TCR $\beta$*  genes during the CD25<sup>+</sup>CD44<sup>-</sup> DN stage, and only those cells that produce a functional TCR $\beta$  protein proceed via a proliferative phase to the CD25<sup>-</sup>CD44<sup>-</sup> DN stage (3, 6, 7). These cells rapidly up-regulate CD4 and CD8 and start to rearrange their *TCR $\alpha$*  genes. After successful *TCR $\alpha$*  rearrangement, TCR $\alpha\beta$ -bearing immature cells are selected for MHC recognition during the process of positive selection (8–10). Concomitantly, developing T cells will undergo lineage commitment to ensure the correlation of the TCR specificity for MHC class I with the CD8 lineage and for MHC class II with the CD4 lineage (2, 11, 12). In addition, potential

self-reactive T lymphocytes are eliminated by selection against self-recognition within the MHC context (13).

T cell development is regulated by a large number of transcription factors (14, 15). One of the transcription factors critically involved in T cell development is GATA-3, which was originally identified in the T cell lineage as a protein that binds to the *TCR $\alpha$*  gene enhancer (16). GATA-3 is a member of a family of transcription factors that bind a GATA consensus motif through a highly conserved C<sub>4</sub> zinc finger binding domain (17). Mice with a targeted deletion of *GATA-3* display massive internal bleeding and central nervous defects and die between embryonic days 11 and 12 due to noradrenaline deficiency (18, 19). *GATA-3*<sup>-/-</sup> fetuses that were pharmacologically rescued by feeding catechol intermediates to pregnant females displayed severe thymic hypoplasia at fetal day 16.5 (19). GATA-3 expression is abundant in the developing CNS, adrenal gland, and kidney. Within the hemopoietic system, GATA-3 expression is confined to T lymphocytes (18, 20–23). In mature Th cells, GATA-3 has been shown to be essential for Th2 differentiation (24–26) and has been implicated in the regulation of locus accessibility of the *IL-4*, *IL-5*, and *IL-13* genes by chromatin remodeling (27–29).

The *GATA-3* gene is expressed in common lymphoid progenitors and in the earliest CD25<sup>-</sup>CD44<sup>+</sup> DN progenitors in day 12 fetal thymus (23, 30). Antisense *GATA-3* oligonucleotides inhibited T cell development from fetal liver precursors in fetal thymic organ cultures, indicating the critical importance of GATA-3 for early T cell development (30). Moreover, *RAG-2*<sup>-/-</sup> complementation experiments *in vivo* demonstrated that the development of *GATA-3*<sup>-/-</sup> embryonic stem (ES) cell-derived T cell precursors is arrested at or before the DN stage (31). In such *GATA-3*<sup>-/-</sup>/*RAG-2*<sup>-/-</sup> chimeric mice, the GATA-3-deficient ES cells contributed significantly to nonhemopoietic tissues and to the erythroid, myeloid and B cell lineages. In chimeric mice generated by injection

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<sup>3</sup> Abbreviations used in this paper: DN, double negative; DP, double positive; FDG, fluorescein-di- $\beta$ -D-galactopyranoside; LCR, locus control region; SP, single positive; ES, embryonic stem; HSA, heat-stable Ag; CD62L, CD62 L-selectin; HA, hemagglutinin; FSC, forward scatter.

of *GATA-3*-deficient *lacZ*-expressing ES cells in wild-type blastocysts, we previously showed that *GATA-3*<sup>-/-</sup> ES cells did not contribute to the T cell lineage, not even to the earliest subset of CD25<sup>-</sup>CD44<sup>+</sup> DN thymic progenitors (22).

Because *GATA-3*<sup>-/-</sup> cells display a block before the earliest T cell progenitor, few data are available on the role of *GATA-3* during T cell development in the thymus. Using mice with an insertion of a *lacZ* reporter in the *GATA-3* gene on one allele (*GATA-3*<sup>+/*nslacZ*</sup>), we examined the proportion of *GATA-3*-expressing cells as a function of T cell development (22). We found significant *GATA-3* expression at the earliest DN stage in the thymus. The two waves of *TCRβ* and *TCRα* gene recombination were associated with low proportions of *lacZ*<sup>+</sup> cells. The stage of rapidly proliferating CD44<sup>-</sup>CD25<sup>-</sup> DN cells, which insulates these two periods of *TCR* rearrangement, was characterized by a large proportion of *lacZ*-expressing cells. The proportion of *lacZ*<sup>+</sup> cells increased again as double-positive (DP) cells progressed into CD4 or CD8 SP cells. The presence of significant proportions of *lacZ*<sup>+</sup> cells within the CD8 SP T cell subpopulation in the thymus was in strong contrast with the almost complete absence of *lacZ* expression in mature CD8<sup>+</sup> T cells in the periphery (22).

The differential regulation of *GATA-3* gene expression in the CD4 vs the CD8 lineage prompted us to investigate its expression during positive selection and CD4/CD8 lineage commitment in the thymus in more detail. We analyzed the *GATA-3*<sup>+/*nslacZ*</sup> mice using additional markers for the maturation stages of DP and SP cells, including CD3, *TCRαβ*, heat-stable Ag (HSA), CD62 L-selectin (CD62L), and particularly CD69, which is typically induced by TCR signaling and therefore marks cells that are in the process of positive selection (32–36). In addition, we investigated the functional role of *GATA-3* during T cell development in vivo by the generation of transgenic mice with enforced *GATA-3* expression driven by the human *CD2* locus control region (LCR), which provides expression of the *GATA-3* transgene throughout T cell development (37).

## Materials and Methods

### Mice

The *GATA-3*<sup>+/*nslacZ*</sup> mice in which one *GATA-3* allele was replaced by a *lacZ* reporter have been described previously (22). For the generation of the *CD2-GATA3* construct, the translation initiation site was mutated (ATG to GTG) in a murine *GATA-3* cDNA clone and three hemagglutinin (HA) epitope tags were added along with a new ATG and Kozak's consensus sequence. Subsequently, the ~2-kb *mGATA-3* was cloned into a human *CD2* mini-gene Bluescript SK vector, with ~5 kb of *CD2* 5' promoter sequence and ~5.5 kb of 3' *CD2* flanking sequences (38). The latter contained the 3' untranslated sequence and poly(A) addition site of the *CD2* gene, as well as the LCR, which was shown to confer T cell-specific, copy-dependent, integration site-independent expression in transgenic mice (37). A 13.2-kb linear fragment was injected into pronuclei of FVB × FVB fertilized oocytes at a concentration of ~2 ng/μl. Founder mice were identified by genomic Southern blotting and crossed onto an FVB background. To determine the genotype of the subsequent generations, tail DNA was analyzed by Southern blotting of either *EcoRI/XbaI* double digests hybridized to a 2-kb *HindIII* *CD2* LCR probe (39) or *EcoRI* digests hybridized to a 800-kb partial *GATA-3* cDNA probe (21).

### Western blotting analyses

Total nuclear protein extracts were prepared according to Andrews and Fallor (40). Protein concentration in the nuclear extracts was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). For Western blotting analysis, 50 μg of total nuclear protein was loaded per lane and separated on 10% SDS-PAGE gels under reducing conditions and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). Blots were blocked with 2% BSA in PBS (pH 7.0)/0.05% Tween 20 and incubated with first- and second-step reagents in 2% nonfat dry milk in PBS (pH 7.0)/0.05% Tween 20. The mouse anti-*GATA-3* mAb Hg-3-31 and the polyclonal rabbit-anti-HA Ab Y11 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Second-step

reagents were HRP-conjugated goat anti-mouse Ig and swine anti-rabbit Ig from Dako (Glostrup, Denmark). Peroxidase activity was visualized by ECL using standard procedures.

### Flow cytometric analyses

The preparation of single-cell suspensions, determination of β-galactosidase activity using fluorescein-di-β-D-galactopyranoside (FDG), mAb incubations, and three- or four-color cytometry have been described previously (41). The following mAb were purchased from BD PharMingen (San Diego, CA): FITC-conjugated anti-CD3ε and anti-*TCRαβ*, PE-conjugated anti-CD4 (L3T4), anti-CD24/HSA, anti-CD25 (clone 3C7), anti-CD62L and anti-CD69, CyChrome-conjugated anti-CD4, anti-CD8 and anti-CD44, biotinylated anti-CD4 and anti-CD8, APC-labeled anti-CD3ε, and anti-CD4. Secondary Abs used were PE-, TriColor-, or APC-conjugated streptavidin (Caltag, Burlingame, CA). FDG and To-Pro3 were purchased from Molecular Probes Europe (Leiden, The Netherlands). FITC-labeled annexin V was obtained from Nexins Research (Hoeven, The Netherlands).

For intracellular detection of *GATA-3* protein, cells were fixed and permeabilized using paraformaldehyde and saponin as described previously (42) and subsequently incubated with the Hg-3-31 anti-*GATA-3* mAb (Santa Cruz Biotechnology) and FITC-labeled anti-mouse IgG1 (BD PharMingen) as a second step.

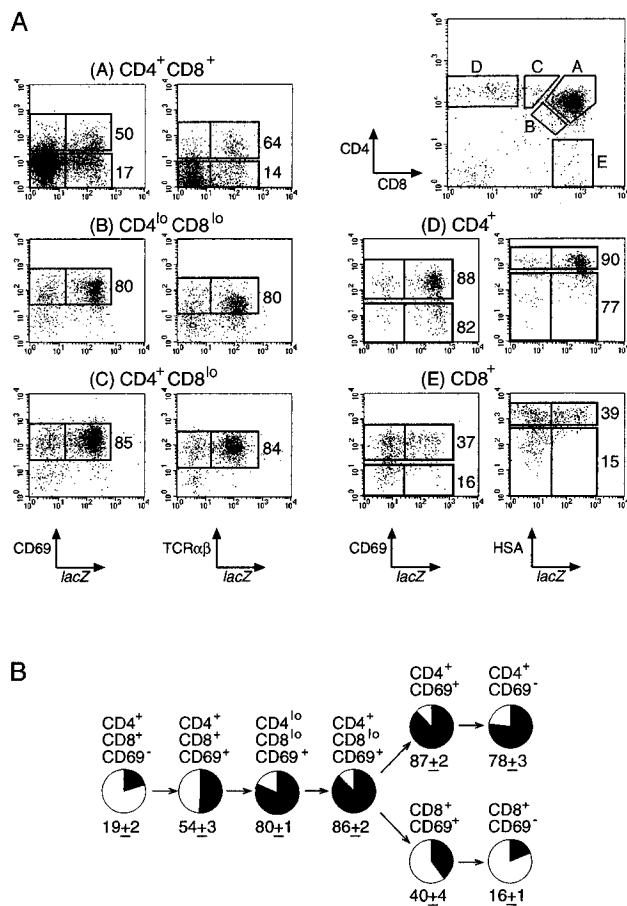
Simultaneous two-color staining of membrane CD4 and CD8, combined with a TUNEL technique to quantify apoptosis, was performed using fluorescein in situ cell death detection (Roche Molecular Biochemicals, Mannheim, Germany) as described elsewhere (43).

## Results

### *GATA-3* expression is strongly induced during positive selection of developing T cells

We have previously quantified the *GATA-3* expression profile during T cell development in vivo by placing a *lacZ* reporter gene, containing a nuclear localization signal, under direct *GATA-3* transcriptional control. In these *GATA-3*<sup>+/*nslacZ*</sup> mice, *GATA-3*-directed *lacZ* expression was analyzed by flow cytometry using FDG as a β-galactosidase substrate, and differential expression of *GATA-3* in DP (~16% *lacZ*<sup>+</sup> cells), CD4 SP (~84%), and CD8 SP (~33%) cells was found (22). Since it has been shown that DP T cells differentiate into mature SP T cells via a series of phenotypically distinct subpopulations, reflecting the multistage process of positive selection and CD4/CD8 lineage commitment (2, 11, 12), we investigated *GATA-3* gene expression in these subpopulations in more detail.

Upon MHC-*TCRαβ* interaction in DP cells, the surface expression of the CD69 marker is up-regulated (32–35), followed by a down-regulation of the CD4/CD8 coreceptor surface expression (2). Therefore, we analyzed *lacZ* activity in conjunction with surface expression of CD4, CD8, and CD69 (Fig. 1). The majority of DP cells (~85%) did not express CD69 on the cell surface and in this CD69<sup>-</sup> DP population *lacZ* was expressed in ~19% of cells. By contrast, within the CD69<sup>+</sup> DP subpopulation, *lacZ* was expressed in ~54% of the cells. After subsequent down-regulation of coreceptor expression and transition into the CD4<sup>low</sup>CD8<sup>low</sup> subpopulation, ~80% of the CD69<sup>+</sup> cells expressed *lacZ* (Fig. 1). The CD4<sup>low</sup>CD8<sup>low</sup> cells have been shown to subsequently enhance CD4 expression (2), thereby developing into the CD4<sup>+</sup>CD8<sup>low</sup> subset, which still contains precursors for both CD4 and CD8 SP T cells (2, 44–46). *LacZ* expression was present in ~86% of these CD4<sup>+</sup>CD8<sup>low</sup>CD69<sup>+</sup> cells (Fig. 1). In addition, we found that in the DP, CD4<sup>low</sup>CD8<sup>low</sup>, and CD4<sup>+</sup>CD8<sup>low</sup> subpopulations, *lacZ* expression correlated with the expression levels of *TCRαβ* or CD3 on the cell surface (shown for *TCRαβ* in Fig. 1A). These results indicated that the induction of *GATA-3* transcription coincides with CD3 and *TCRαβ* up-regulation and CD69 expression in DP cells.



**FIGURE 1.** Analysis of *lacZ* expression in thymocytes from *GATA-3<sup>+/nlslacZ</sup>* mice by four-color flow cytometry. **A**, Thymus cell suspensions were loaded with the  $\beta$ -galactosidase substrate FDG and subsequently stained with anti-CD4 and anti-CD8 in combination with anti-CD69, anti-TCR $\alpha\beta$ , or anti-HSA Abs. Cells were analyzed for the expression of CD4 and CD8, the indicated subpopulations A–E were gated and analyzed for *lacZ* expression and the fourth surface marker (CD69, TCR $\alpha\beta$ , or HSA). The numbers indicate the percentage of *lacZ*-expressing cells in the subpopulations analyzed. All samples are lymphocyte gated by FSC and side scatter. **B**, Overview of the proportions of *lacZ*-expressing cells in the indicated thymocyte subpopulations in *GATA-3<sup>+/nlslacZ</sup>* mice. The numbers are mean values  $\pm$  SD ( $n = 3$ ). The background percentages of  $\beta$ -galactosidase-positive cells, as determined in wild-type control mice, was  $<1\%$  in all subpopulations.

#### *GATA-3* gene expression is down-regulated after commitment to the CD8 lineage

Bipotential CD4<sup>+</sup>CD8<sup>low</sup>CD69<sup>+</sup> T cells differentiate into either CD4 or CD8 SP cells by shutting down expression of the reciprocal coreceptor gene (2, 44–46). Final maturation of SP thymocytes is accompanied by down-regulation of CD69 and HSA on the cell surface and induction of high-level expression of CD62L and CD44 (36, 47, 48).

As shown in Fig. 1, *lacZ* expression was present in  $\sim 87\%$  of the cells committed to the CD4 lineage (CD4<sup>+</sup>CD8<sup>-</sup>CD69<sup>+</sup>TCR $\alpha\beta$ <sup>high</sup> cells) and in  $\sim 78\%$  of the more mature CD4<sup>+</sup> cells with a CD69<sup>-</sup>HSA<sup>low</sup> surface profile. By contrast, during the maturation of CD8 lineage cells,  $\sim 40\%$  of the CD69<sup>+</sup>HSA<sup>+</sup> and only  $\sim 16\%$  of the mature CD69<sup>-</sup>HSA<sup>low</sup>CD8<sup>+</sup>TCR $\alpha\beta$ <sup>high</sup> cells expressed *lacZ*. Likewise, *lacZ* expression was found to be significantly down-regulated in mature CD44<sup>+</sup> and CD62L<sup>+</sup>CD8<sup>+</sup> cells (data not shown). The intensities of the fluorescence signals show that the *lacZ* expression levels per cell increased slightly during the maturation process of CD4

SP cells, whereas CD8 SP cells displayed lower and more heterogeneous *lacZ* expression levels (Fig. 1A).

When the T cells leave the thymus, the proportions of GATA-3<sup>+</sup> cells decrease to  $\sim 20\%$  of the CD4<sup>+</sup> and to  $<1\%$  of the CD8<sup>+</sup> T cell populations in the spleen and lymph nodes (22). For the CD4<sup>+</sup> lineage cells in the spleen, we did not observe a clear correlation between GATA-3 and the expression of the HSA, CD69, CD44, CD62L, or CD25 surface markers, which are instrumental to specify subpopulations of naive, activated, or memory T cells (49, 50).

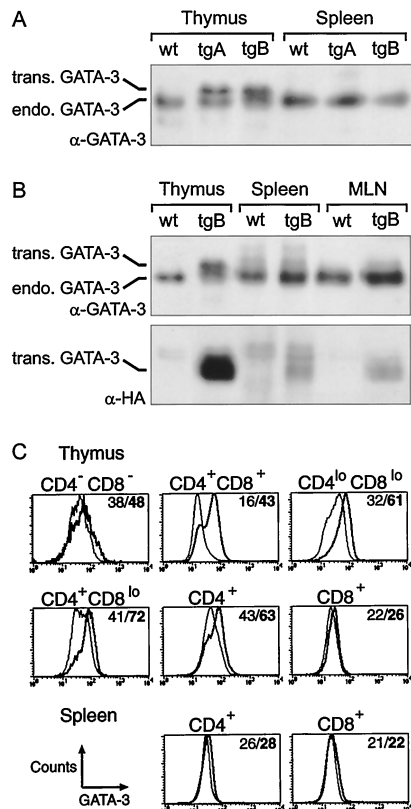
As summarized in Fig. 1B, the proportions of GATA-3-expressing cells were low in CD3<sup>-</sup>TCR $\alpha\beta$ <sup>-</sup>CD69<sup>-</sup>DP cells ( $\sim 19\%$ ) and increased at the onset of positive selection events characterized by up-regulation of CD3 and TCR $\alpha\beta$  surface expression and induction of CD69. The proportions increased to  $\sim 86\%$  at the stage of the last uncommitted subset of CD4<sup>+</sup>CD8<sup>low</sup>CD69<sup>+</sup> cells and remained high for the most mature thymic CD69<sup>-</sup>CD4<sup>+</sup> subpopulation. By contrast, commitment to the CD8 lineage was associated with down-regulation of GATA-3 expression, resulting in  $<20\%$  GATA-3<sup>+</sup> cells within the mature population of CD69<sup>-</sup>CD8<sup>+</sup> cells.

#### Transgenic expression of GATA-3 driven by the human CD2 LCR

To modify GATA-3 expression in vivo, transgenic mice were generated in which the murine GATA-3 gene, 5' tagged with three HA epitopes, was expressed under the control of the human CD2 LCR (38). Two independent CD2-GATA3-transgenic lines, TgA and TgB, were established that appeared to contain comparable numbers of transgene copies (data not shown). No differences were found between the two lines in any of the performed analyses. The offspring did not manifest developmental defects or any increased susceptibilities to infectious disease or malignancies for over 9 mo of age, with the exception of the observed thymic lymphomas discussed below.

Expression of the CD2-GATA3 transgene was analyzed in various lymphoid tissues by comparing transgenic and nontransgenic littermates. Western blotting experiments were performed on nuclear protein extracts from 2- to 3-mo-old mice using a mouse mAb specific for GATA-3 and a polyclonal Ab specific for the HA tag. The endogenous GATA-3 gene encodes a  $\sim 47$ -kDa protein that was detected in nuclear extracts from thymus and spleen of wild-type as well as CD2-GATA3-transgenic mice (Fig. 2A). The anti-GATA-3 Ab also detected a slightly larger  $\sim 51$ -kDa band in the thymus samples from mice of both transgenic lines. Comparison of the intensities of the  $\sim 47$ - and  $\sim 51$ -kDa GATA-3-specific bands in these nuclear protein extracts from thymus samples showed that the expression level of the 3XHA-GATA-3 transgene-encoded protein was similar to that of the endogenous GATA-3 protein. In contrast, in the spleen samples, the  $\sim 51$ -kDa GATA-3-specific band was very weak or absent (Fig. 2A). The  $\sim 51$ -kDa band in the thymus extracts of transgenic mice was also recognized by Ab against the HA tag, but the expression levels of transgene-encoded GATA-3 protein in the spleen and lymph node extracts were very low and often almost undetectable by Western blotting analyses (Fig. 2B).

To further investigate differential expression of GATA-3 in the individual stages of T cell development, intracellular flow cytometry experiments were performed using the mouse monoclonal antiserum specific for GATA-3. Although this technique is limited by a background signal of the GATA-3 Ab, it allows a comparison of GATA-3 expression levels in nontransgenic and CD2-GATA3-transgenic mice in separate T cell subpopulations. In the wild-type



**FIGURE 2.** Expression of GATA-3 in lymphoid organs of wild-type and *CD2-GATA3*-transgenic mice. **A**, Western blotting analyses of GATA-3 protein expression in total nuclear protein extracts from thymus and spleen from wild-type (wt) and *CD2-GATA3*-transgenic mouse lines (tgA and tgB) as detected by anti-GATA-3 Abs (~47-kDa band, endogenous (endo.) GATA-3; ~51-kDa band, transgenic (trans.) GATA-3). **B**, Western blotting analyses of GATA-3 protein expression in nuclear extracts from the indicated tissues from wild-type and *CD2-GATA3* tgA mice. GATA-3 is detected by anti-GATA3 Abs (upper half) or anti-HA Abs (lower half). In the anti-HA blot, thymus and spleen cell extracts from both nontransgenic and *CD2-GATA3* transgenic mice displayed two weak background bands, just above the ~51-kDa HA-GATA-3 band. **C**, Cell suspensions were stained for surface CD3, CD4, and CD8 expression, and subsequently for intracellular GATA-3 protein. The indicated T cell subpopulations were gated and analyzed for GATA-3 expression. The results are displayed as histograms of *CD2-GATA3*-transgenic mice (bold lines) along with those of nontransgenic control mice (thin lines).  $CD4^+CD8^-$  populations were gated on  $CD3^+$  cells.  $CD4^{low}CD8^{low}$  cells and  $CD4^+CD8^{low}$  were gated on  $CD3^+$  cells. The numbers indicate the mean fluorescence intensities in nontransgenic (normal type) and *CD2-GATA3*-transgenic mice (bold type). Data shown are representative of six mice examined within each group.

animals, the GATA-3 levels were low in DP cells, increased during positive selection in  $CD4^{low}CD8^{low}$  cells, and were high in  $CD4$  SP cells (Fig. 2C), consistent with our findings in the *GATA-3<sup>+/nlslacZ</sup>* mice (Fig. 1). Expression of the *CD2-GATA3* transgene was determined by comparison of the mean fluorescence intensities of intracellular GATA-3 staining in histogram overlays of transgenic and nontransgenic mice, revealing substantial GATA-3 overexpression in most thymocyte subpopulations (Fig. 2C). GATA-3 protein levels were uniformly higher in DP,  $CD4^{low}CD8^{low}$ ,  $CD4^+CD8^{low}$ , and  $CD4$  SP thymic subpopulations from *CD2-GATA3*-transgenic mice as compared with wild-type mice. By contrast, for the  $CD8$  SP cells in the thymus and the  $CD4^+$  or  $CD8^+$  T cells in the spleen, GATA-3 levels in the *CD2-GATA3*-transgenic mice were close to those observed in wild-type

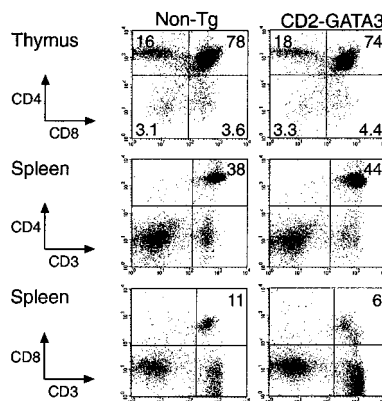
littermates. Therefore, these findings confirm the very low expression levels of the transgene-encoded GATA-3 protein in peripheral T cells that were observed in the Western blotting experiments. Since  $CD2$  surface expression in the individual T cell subpopulations in thymus and spleen was comparable (data not shown), the observed modulated GATA-3 protein expression profile in the transgenic mice does not appear to reflect the activity of the *CD2* LCR.

Collectively, these data show that the presence of the *CD2-GATA3* transgene resulted in a modification of the expression pattern of GATA-3 during T cell development, without extreme overexpression of GATA-3 protein in any of the thymic subpopulations. Especially in the DP population, which normally show little GATA-3 expression, the presence of the *CD2-GATA3* transgene strongly increased the GATA-3 protein levels.

#### *CD2-GATA3*-transgenic mice have decreased $CD8^+$ T cell numbers in the periphery

To analyze the effect of the *CD2-GATA3* transgene on T cell development, we examined the T cell populations in thymus, spleen, and mesenteric lymph nodes from 2- to 3-mo-old *CD2-GATA3*-transgenic mice and nontransgenic littermates by flow cytometry (Fig. 3). In the *CD2-GATA3* mice, the sizes of the main thymocyte subpopulations, the DN, DP, and SP cells, were within the normal ranges, indicating that the enforced GATA-3 expression did not dramatically impede thymocyte development (Fig. 3). Moreover, thymus cellularity was not significantly different between transgenic mice ( $99 \pm 34 \times 10^6$ ,  $n = 21$ ) and nontransgenic littermates ( $103 \pm 37 \times 10^6$ ,  $n = 14$ ). No significant differences were detected between *CD2-GATA3*-transgenic mice and normal littermates within the DN subpopulations as defined by differential  $CD44$  and  $CD25$  expression (data not shown). In contrast, the *CD2-GATA3*-transgenic mice had fewer  $CD8^+$  T cells (~50% of control) in spleen and lymph nodes (shown for spleen in Fig. 3). The residual transgenic  $CD8^+$  T cells present exhibited a more heterogeneous  $CD8$  expression and higher  $CD3$  expression on the cell surface. The numbers of  $CD4^+$  T cells in the periphery were comparable between the two groups of mice.

Taken together, these results indicated that enforced expression of GATA-3 did not result in detectable adverse effects on  $CD4^+$  T cell development in 2- to 3-mo-old *CD2-GATA3*-transgenic mice.



**FIGURE 3.** Phenotype of *CD2-GATA3*-transgenic mice. T cell development in the presence of the *CD2-GATA3* transgene results in reduced numbers of peripheral  $CD8^+$  cells. Flow cytometric analyses of the thymus and spleen of 2-mo-old wild-type and *CD2-GATA3* mice. Single-cell suspensions were stained with anti- $CD3$ , anti- $CD4$ , and anti- $CD8$  Abs. Results are displayed as dot plots of lymphocyte gate cells; percentages of total cells within the indicated quadrants are given. Data shown are representative of >20 mice examined within each group.

In contrast, mature CD8 SP cells manifested decreased survival, either within the thymus or shortly after leaving the thymus.

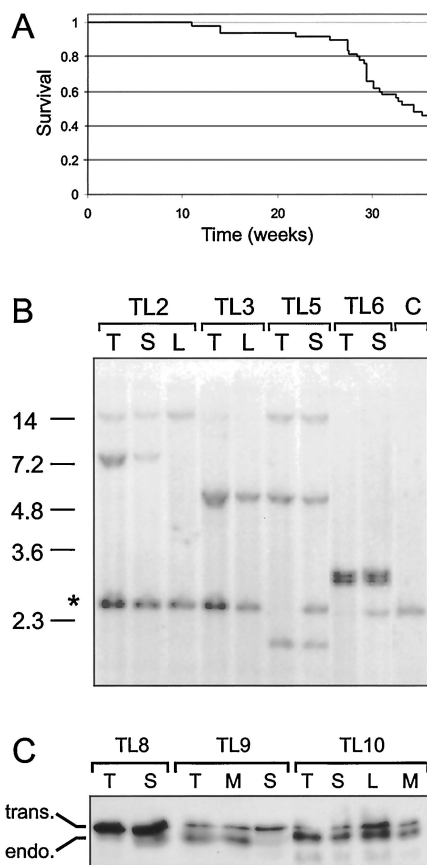
*CD2-GATA3-transgenic mice develop thymic lymphomas*

When the *CD2-GATA3*-transgenic mice were followed up to 9 mo of age, ~50% (26 of 51) developed thymic lymphomas (Fig. 4A). Typically, these lymphomas were noticed as mice displayed respiratory distress at the age of 6–8 mo, but in three cases such animals were observed at ~3 mo of age. Tumor frequencies in the two independent transgenic lines were similar, whereas tumors were not seen in nontransgenic littermates. Several animals with a thymic lymphoma exhibited enlargement of spleen or lymph nodes. Lymphoma cells were found to be present in the spleen, liver, lymph nodes, and kidney, indicating that the thymic lymphomas metastasized to the periphery. This was confirmed by the presence of identical clonal *TCRβ* rearrangement patterns in Southern blotting analyses using probes specific for *Jβ1* or *Jβ2* gene segments (Fig. 4B). In a fraction of the tumors analyzed, we observed *Jβ2* restriction fragment patterns that would be consistent with biclonality (see Fig. 4B, TL5). Often particular restriction

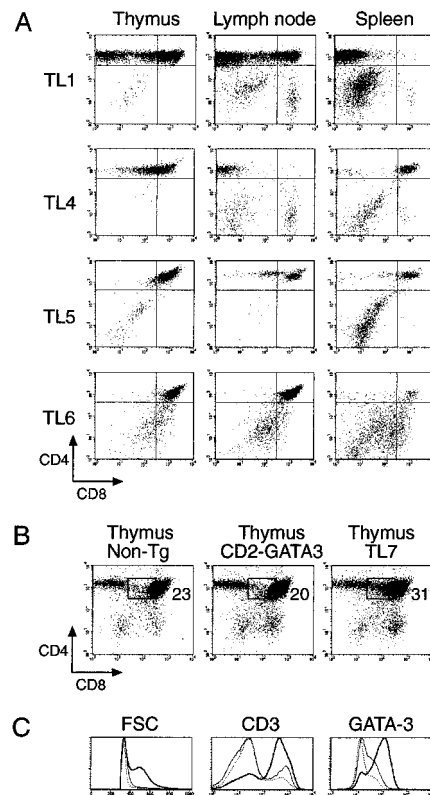
fragments were lost in metastases, suggesting ongoing *TCRβ* rearrangement or deletion (see Fig. 4B, compare thymus and lymph node of TL2).

When tumor cell samples from thymus, spleen, or lymph node were analyzed for the expression of GATA-3 in Western blotting experiments, high levels of transgenic HA-tagged GATA-3 were observed, often accompanied by high endogenous GATA-3 expression (Fig. 4C). The ratio between transgenic and endogenous GATA-3 varied, not only between individual tumors but also between different metastases of a single tumor (see Fig. 4C, compare mesenteric lymph node and spleen of TL 9).

Flow cytometric analyses demonstrated that the thymic lymphomas consisted of  $CD4^+$  lymphoblasts with variable levels of CD8 coexpression. Fig. 5A illustrates four examples of thymic lymphomas ( $CD4^+CD8^{+/low}$  cells), with different metastases in lymph nodes and spleen, showing the variability of surface CD4 and CD8 expression on the malignant cells. Immunohistochemical examination of thymic tumor tissue sections confirmed that the tumors mainly consisted of  $CD4^+CD8^+$  lymphoblasts. Most of the tumors contained areas that had lost expression of CD8 and sometimes



**FIGURE 4.** Characteristics of thymic lymphomas in *CD2-GATA3*-transgenic mice. *A*, Survival of wild-type mice (gray line,  $n = 42$ ) and *CD2-GATA3*-transgenic mice (black line,  $n = 51$ ), followed for 38 wk in a Kaplan-Meier curve as fraction of the total numbers of mice. *B*, Southern blotting analysis of *TCRβ* rearrangements in various lymphoblastoid tumor samples. *EcoRI* digests were hybridized to a *TCR Jβ2* probe. T, Thymus; S, spleen; L, lymph node. \*, Position of the germline 2.4-kb *EcoRI* fragment; on the left the positions of  $\lambda$  X *BsteII* restriction fragments are indicated in kb. *C*, Western blotting analyses of GATA-3 protein expression in total nuclear extracts from the indicated tumor tissues as detected by anti-GATA-3 Abs. trans., ~51-kDa transgenic GATA-3; endo., ~47-kDa endogenous GATA-3; T, thymus; S, spleen; M, mesenteric lymph node; L, axillary lymph node.



**FIGURE 5.** Surface profile of lymphoblastoid tumor cells in *CD2-GATA3*-transgenic mice. *A*, Flow cytometric analyses of four different  $CD4^+CD8^{low}$  thymic lymphoma primary tumor samples as well as metastases present in lymph node and spleen. *B*, Identification of an atypical  $GATA3^{high}CD3^+CD4^+CD8^{low}$  lymphoblastoid cell population in the thymus of a *CD2-GATA3*-transgenic mouse, indicative for a thymic lymphoma (TL7). Nontransgenic and tumor-free *CD2-GATA3*-transgenic mice are shown as controls. Results are displayed as dot plots for CD4 and CD8. The given percentages of the gated  $CD4^+CD8^{low}$  populations are of all thymocytes. *C*, Analysis of FSC, CD3, and intracellular GATA-3 expression in the gated  $CD4^+CD8^{low}$  thymocyte subpopulation shown in *B*. The results are displayed as histograms of the *CD2-GATA3*-transgenic TL7 mouse (bold lines) along with those of a nontransgenic (dashed lines) and a tumor-free *CD2-GATA3*-transgenic mouse (thin lines). Cell suspensions were stained for CD3, CD4, and CD8 and subsequently for intracellular GATA-3. All samples are lymphocyte/lymphoblast gated by FSC and side scatter.

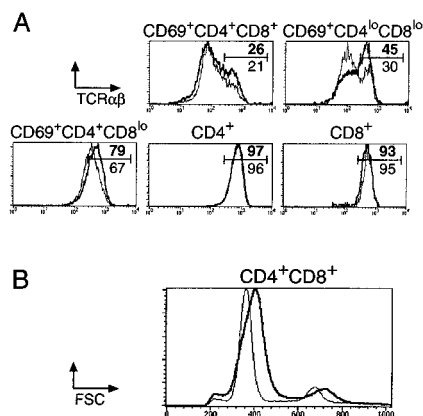
also CD4. A network of MHC class II-negative fibroblasts supported these lymphoblasts, whereas characteristic structures of epithelial cells expressing cortical or medullar cell markers were absent (data not shown).

Among *CD2-GATA3*-transgenic mice that did not exhibit outward signs of illness nor manifested a macroscopically visible thymic tumor at ~3 mo of age, we found evidence for early stages of tumor development in 6 of 32 cases (~19%). In flow cytometric analyses of thymus cell suspensions, the  $CD4^+CD8^{+/low}$  subsets contained atypical fractions of  $CD3^+$  lymphoblastoid cells with high forward scatter (FSC) characteristics suggestive of tumor growth. In the example shown in Fig. 5, *B* and *C*, the lymphoblastoid cells had a  $CD3^+CD4^+CD8^{low}$  phenotype and expressed high levels of GATA-3 protein, as determined by intracellular flow cytometry. In these lymphoblastoid cells, CD69 expression was variable (data not shown).

These findings indicate that dysregulation of GATA-3 expression results in the formation of lymphoblastoid tumors at a specific stage of thymic development, i.e., the  $CD4^+CD8^{+/low}$  thymocyte subpopulation.

#### *GATA-3 enhances TCR $\alpha\beta$ up-regulation during positive selection*

As we observed a correlation between *GATA-3* expression and TCR $\alpha\beta$  or CD3 surface levels in *GATA-3<sup>+nlslacZ</sup>* mice, we investigated these parameters in the thymocyte subpopulations of the *CD2-GATA3* mice (Fig. 6*A*). The expression of CD69 in the DP,  $CD4^{low}CD8^{low}$ , and  $CD4^+CD8^{low}$  subpopulations was similar in *CD2-GATA3* and wild-type mice. In contrast, the proportions of TCR $\alpha\beta^{high}$  or  $CD3^{high}$  cells were significantly increased in *CD2-GATA3* mice, particularly in the  $CD69^+CD4^{low}CD8^{low}$  subpopulation (shown for TCR $\alpha\beta$  expression in Fig. 6*A*). In the more mature fractions of  $CD4^{low}CD8^+$  and SP cells, the expression levels of CD3 and TCR $\alpha\beta$  were similar in transgenic animals and wild-type littermates.



**FIGURE 6.** The *CD2-GATA3* transgene enhances TCR $\alpha\beta$  up-regulation during positive selection. *A*, Cell suspensions were stained for CD4, CD8, and CD69 expression along with either CD3 or TCR $\alpha\beta$ . The indicated T cell subpopulations (see also Fig. 1*A*) were gated and analyzed for TCR $\alpha\beta$  expression. The results are displayed as histograms of *CD2-GATA3*-transgenic mice (bold lines) along with those of nontransgenic control mice (thin lines). The percentages shown are the fractions of the  $CD69^+CD4^{low}CD8^{low}$  cells that are in the indicated TCR $\alpha\beta^{high}$  gate in wild-type mice (below marker) and *CD2-GATA3*-transgenic mice (above marker, bold type). *B*, The effect of the *CD2-GATA3* transgene on the cell sizes of the DP thymocyte subpopulation. Cell suspensions were stained for CD4 and CD8. DP cells were gated and analyzed for FSC; the results are displayed as histogram overlays of a *CD2-GATA3*-transgenic (bold line) and nontransgenic control (thin line) mouse.

We noticed that in the *CD2-GATA3*-transgenic mice, the cells within the DP subpopulations had increased average FSC values closer to those of normal SP cells (Fig. 6*B*). The increased size of *CD2-GATA3*-transgenic DP cells did not reflect an enhanced activation status of these cells, as we failed to detect activated cells with high Th2 cytokine production (26) in immunohistochemical analyses of the thymi of *CD2-GATA3* mice. We also did not find evidence for a direct effect of transgenic GATA-3 on the cell cycle in DP cells, as flow cytometric analyses, using anti-CD4, anti-CD8, and To-Pro3, did not reveal differences in the cell cycle between *CD2-GATA3*-transgenic animals and their wild-type littermates (data not shown). The development of  $CD3/TCR\alpha\beta^{low}$  DP into  $CD3/TCR\alpha\beta^{high}$  SP cells is normally accompanied by an increase in the average cell size. Therefore, the findings of the small increase in DP cell size and the slightly accelerated up-regulation of surface TCR $\alpha\beta$  and CD3 expression in *CD2-GATA3*-transgenic mice suggest that enforced GATA-3 expression may influence the kinetics of positive selection.

#### *GATA-3 inhibits maturation of CD8 SP T cells*

As the reduction of peripheral  $CD8^+$  T cell numbers in the *CD2-GATA3* transgenic mice suggested increased cell death or hampered maturation of CD8 SP cells in the thymus, we analyzed the thymic CD8 SP compartment in more detail and specifically evaluated the final maturation steps of CD8 SP cells.

To analyze the extent of apoptosis in the SP subpopulations, we determined the fraction of cells that were annexin V-positive in *CD2-GATA3*-transgenic mice and their nontransgenic littermates. In addition, we performed TUNEL assays in conjunction with surface CD4/CD8 staining. Using these techniques, we found that the thymi of *CD2-GATA3*-transgenic mice contained higher numbers of apoptotic cells, not only in the CD8 SP but to some extent also in the CD4 SP subpopulations (Fig. 7, *A* and *B*).

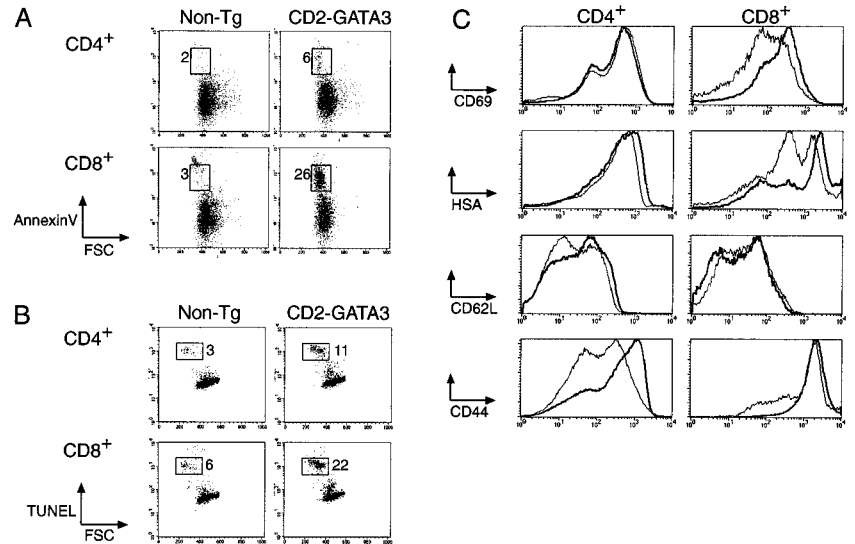
It has been reported that final maturation of SP T cells is accompanied by a down-regulation of CD69 and HSA expression (36, 48). The enforced *GATA-3* expression appeared to inhibit the final maturation of  $CD8^+$  cells, as a selective deficiency of  $CD69^{low}HSA^{low}$  cells was observed, when *CD2-GATA3*-transgenic and wild-type littermates were compared (Fig. 7*C*). For  $CD4^+$  cells, the enforced *GATA-3* expression only mildly affected the final thymic maturation steps. In addition, in the *CD2-GATA3*-transgenic mice an increase in the surface expression of CD44, a marker for activated or memory T cells, was observed both in the CD4 and the CD8 SP population (Fig. 7*C*). This phenomenon was also seen in the mature  $CD4^+$  and  $CD8^+$  T cells in the spleen (see accompanying paper). Finally, the expression of L-selectin (CD62L), a marker which is expressed at high levels on naive T cells and which is essential for homing to peripheral lymphoid organs (51), was comparable in transgenic and nontransgenic animals. Therefore, the decrease in peripheral  $CD8^+$  T cell numbers in *CD2-GATA3*-transgenic mice cannot be explained by a reduced capacity of the mature CD8 SP T cells to leave the thymus.

In summary, we observed a substantial increase of apoptotic CD8 SP cells and a decrease of mature  $CD69^{low}HSA^{low}$  cells in the thymic CD8 SP subpopulation, as well as reduced numbers of  $CD8^+$  T cells in the peripheral organs. These findings indicate that enforced *GATA-3* expression resulted in a partial differentiation arrest of  $CD8^+$  cells associated with significant cell death in the thymus.

## Discussion

In this report, we have used two different mouse models to study the role of GATA-3 in early T cell development in vivo. We evaluated *GATA-3*-directed *lacZ* expression in *GATA-3<sup>+nlslacZ</sup>* mice

**FIGURE 7.** Enforced expression of *GATA-3* induces apoptosis and inhibits the maturation of  $CD69^{\text{low}}HSA^{\text{low}}$  CD8 SP cells in the thymus. Thymus cell suspensions were stained for CD4, CD8, and annexin V (A) or TUNEL (B). Thymocytes were analyzed for the expression for CD4 and CD8; the indicated SP subpopulations were gated and analyzed for FSC and annexin V or TUNEL. The numbers indicate the percentage of annexin V-positive (A) or TUNEL-positive (B) cells in the subpopulations analyzed. C, In four-color flow cytometry experiments, thymus cell suspensions were stained for CD4 and CD8 expression along with anti-HSA and anti-CD69 or with anti-CD62L and anti-CD44. The CD4 and CD8 SP T cells were gated and analyzed for the expression of the indicated markers. The results are displayed as histograms of *CD2-GATA3*-transgenic mice (bold lines) along with those of non-transgenic control mice (thin lines).



and examined the effects of enforced *GATA-3* expression throughout T cell development in *CD2-GATA3*-transgenic mice in which *GATA-3* transcription is driven by the *CD2* LCR.

Our findings implicate *GATA-3* as a participant in the commitment process to the CD4 vs the CD8 lineage. First, we found that commitment to the CD8 T cell lineage coincided with down-regulation of *GATA-3* expression. The most mature subpopulation of uncommitted thymocytes, the  $CD4^+CD8^{\text{low}}$  subset, contained high numbers of *GATA-3*-expressing cells. During the maturation of  $CD8^+$  cells in the thymus, *GATA-3* expression was gradually lost. By contrast, *GATA-3* expression remained high during differentiation of  $CD4^+$  cells in the thymus. Second, enforced *GATA-3* expression inhibited the maturation of  $CD8^+$  cells. The CD8 SP fraction in the thymus contained increased numbers of apoptotic cells and exhibited a selective deficiency of mature  $CD69^{\text{low}}HSA^{\text{low}}$  cells. In the spleen and lymph nodes, the numbers of  $CD8^+$  T cells were significantly reduced.

Enforced expression of *GATA-3* did not appear to directly influence the CD4 vs CD8 lineage cell fate decision, as in the *CD2-GATA3* mice the percentages of CD4 and CD8 SP cells in the thymus were in the normal ranges. Although the molecular mechanisms underlying the developmental choice between CD4 and CD8 T cell fates are not known, they are thought to depend on differences in signal strengths of the MHC class I-CD8 and MHC class II-CD4 interactions. The influence of signaling molecules on lineage commitment is supported by the finding of differentiation toward the CD4 lineage in a gain-of-function extracellular signal-related kinase 2 mutant and in *Csk*- or *C-Cbl*-deficient mice (52–54). Activated Notch transmembrane receptor or *Bcl-2* overexpression was shown to promote differentiation to the CD8 lineage, probably by rescue from apoptosis and development along the CD8 lineage of cells that have a very low-affinity MHC interaction, which would normally die by neglect (55–57).

Our data point at a role for *GATA-3* in the maturation of the cells once commitment has occurred. There is a progressive decline of *GATA-3* expression during CD8 lineage maturation, and the enforced *GATA-3* expression impaired cell survival in the most mature CD8 lineage cells. Furthermore, peripheral  $CD8^+$  T cells from *CD2-GATA3*-transgenic mice manifested functional defects in IL-2 and IFN- $\gamma$  production (see accompanying paper). In this context, there is a striking parallel with Th1/Th2 differentiation, where *GATA-3* is expressed in naive peripheral T cells, followed by a substantial increase during Th2 development and a gradual

down-regulation during Th1 development (24, 25). The Th2 phenotype is initiated by IL-4 signaling, and by the action of *GATA-3* becomes stable over time and independent of extrinsic factors, such as IL-4 (27, 29, 58). Retroviral tagging of naive progenitors with *GATA-3* provided direct evidence for instructive differentiation, rather than selective outgrowth of committed Th1 or Th2 cells (26). It was further shown that *GATA-3* generates stability of Th2 commitment by chromatin remodeling of Th2-specific cytokine loci, associated with a positive autoactivation pathway, which is a recognized mechanism contributing to cell fate determination (29). Concomitantly, *GATA-3* inhibits Th1 development by repressing IL-12R $\beta$  expression and, as a result, IL-12 induced IFN- $\gamma$  production (59). Assuming a parallel role for *GATA-3* in CD4/CD8 and Th1/Th2 development, we propose that *GATA-3* is involved in the stabilization of the distinct gene expression profiles in committed CD4 cells, whereas for the full maturation of CD8 T cells, *GATA-3* expression needs to be down-regulated. Alternatively, *GATA-3* may affect lineage commitment indirectly by inducing higher TCR $\alpha\beta$  expression levels (Fig. 6A), thereby increasing the intensity of the signal delivered to DP cells, which has been shown to skew development toward the CD4 lineage (60, 61). A mechanism by which the influence of enforced expression of *GATA-3* on CD4/CD8 commitment is directly related to the presence of *GATA-3* recognition sites in the *CD8 $\alpha$*  promoter (62) can also not be excluded. In that case, *GATA-3* would have to directly repress CD8 expression in mature cytotoxic T cells.

It is at present not clear why in *CD2-GATA3* mice the levels of transgene-encoded *GATA-3* protein are down-regulated in CD8 SP cells and peripheral T cells. *GATA-3* levels may be subject to posttranslational regulation, as indicated by the presence of caspase-mediated degradation of the closely related transcription factor *GATA-1* in immature erythroid cells (63). However, such a mechanism should apply to both endogenous and transgene-encoded *GATA-3*. Therefore, the presence of the HA tag would then affect posttranscriptional regulation.

Additional experiments will be needed to identify the critical target genes for *GATA-3* in early T cell development. Intriguingly, *GATA* recognition sequences are present in the *Notch4* promoter region (64). If *Notch* genes would be regulated by *GATA-3*, this could explain the parallels that exist between the in vivo function of *GATA-3* and Notch. Both genes are essential for the development of the first stage of T cell development and not for any other hemopoietic lineage (22, 31, 65). Apart from the accelerated

TCR $\alpha\beta$  up-regulation in developing CD69<sup>+</sup> thymocytes that progress from the DP to the CD4<sup>+</sup>CD8<sup>low</sup> stage, we did not see any effects on the surface expression of presumed GATA-3 target loci such as TCR $\alpha$ ,  $\beta$ , and  $\delta$  or CD8 $\alpha$ .

Our previous finding of low GATA-3 expression during the two waves of TCR gene rearrangement, separated by a stage of high GATA-3 expression, suggested a role of GATA-3 in the regulation of proliferation events associated with the essential coupling of V(D)J recombination activity to cell cycle (22). However, the absence of any detectable effects of the CD2-GATA3 transgene on the cell cycle would argue against such an essential role for GATA-3. Nevertheless, all thymic lymphomas in the CD2-GATA3 mice characterized so far appeared to have originated at the DP stage, in which all TCR $\alpha$  locus gene rearrangements occur. Therefore, it remains possible that, in the presence of high levels of GATA-3, oncogenic events, such as translocations, are mediated by aberrant use of the V(D)J recombination machinery, as has been found in V(D)J recombination-driven thymic lymphoma in mice deficient for the *ataxia telangiectasia* gene (66).

Alternatively, the oncogenic potential of GATA-3 could be related to the ability of GATA-3 to form a complex with the TAL-1 and LMO transcription factors, which are implicated in a large fraction of human T cell acute lymphoblastic leukemias (67). Normally TAL-1 and LMO are not expressed in the T cell lineage, but expression is induced by translocation events. It was recently shown that forced expression of GATA-3 in vitro potentiated the induction by the TAL-1 and LMO transcription factors of retinaldehyde dehydrogenase 2, which inhibits apoptosis of T cells by generating retinoic acid (67).

Also, enforced GATA-3 expression probably leads to increased basal transcription of the *RAD50* gene, which is involved in chromosomal double-stranded break repair. Because of the localization of the *RAD50* gene within the *IL-4/IL-5/IL-13* Th2 cytokine gene cluster, an increase of basal *RAD50* transcription is observed in Th2 cells (27). It is possible that in the CD2-GATA3-transgenic T cells the increase might be more extreme, thereby resulting in destabilization of the MRE11-RAD50-NBS1 protein complex, which is essential for chromosome stability (68).

Finally, a more general mechanism might be responsible for the oncogenic effect of GATA-3, since GATA factors have a key role in the regulation of development toward cell division and differentiation via the cell cycle machinery (69). Recently several other GATA family factors have been implicated in various human tumors, e.g., GATA-2 in acute promyelocytic leukemia, acute myeloid leukemia, and myelodysplastic syndrome and GATA-4 in esophageal adenocarcinomas and malignancies of the gonads (70–72). Further characterization of the tumor cells should identify the possible involvement of any of these oncogenic pathways in the origin of the thymic lymphomas in the CD2-GATA3 mice.

In conclusion, this study adds to our knowledge of the function of GATA-3 in early T cell development because we have established a correlation between GATA-3 expression and maturation toward the CD4 vs the CD8 lineage. We propose that in early T cell development, expression of GATA-3 is essential for the maintenance of CD4 cell lineage fate commitment, but inhibits CD8 differentiation. Inferred from the recent findings that GATA-3 acts a key regulator of Th2 development by stabilizing patterns of gene expression, it is attractive to hypothesize that in early T cell development GATA-3 would stabilize, by chromatin remodeling, the unique gene expression profiles that are characteristic for the CD4 lineage.

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