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Enforced Expression of GATA-3 During T Cell Development Inhibits Maturation of CD8 Single-Positive Cells and Induces Thymic Lymphoma in Transgenic Mice

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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 expression in CD4+CD8+ double-positive thymocytes correlated with the onset of positive selection events, i.e., TCRαβ 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+CD8−/low and mostly CD3+. 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 CD69lowHSAlow CD8 SP cells. In the spleen and lymph nodes, the numbers of CD8+ 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+CD8− double-negative (DN)3 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-2Rα chain CD25 and phagocytosis glycoprotein 1 CD44 (5). Precursor T cells rearrange their TCRβ genes during the CD25+CD44+ DN stage, and only those cells that produce a functional TCRβ protein proceed via a proliferative phase to the CD25−CD44− DN stage (3, 6, 7). These cells rapidly up-regulate CD4 and CD8 and start to rearrange their TCRαα genes. After successful TCRαα rearrangement, TCRαβ-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 TCRα enhancer (16). GATA-3 is a member of a family of transcription factors that bind a GATA consensus motif through a highly conserved C2 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−/− fetuses 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 hematopoietic 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−CD44+ DN progenitors in day 12 fetal thymus (23, 30). Antisense GATA-3 oligonucleotides inhibited T cell development from fetal liver progenitors in fetal thymic organ cultures, indicating the critical importance of GATA-3 for early T cell development (30). Moreover, RAG-2−/− complementation experiments in vivo demonstrated that the development of GATA-3−/− embryonic stem (ES) cell-derived T cell precursors is arrested at or before the DN stage (31). In such GATA-3−/−/RAG-2−/− 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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3 Abbreviations used in this paper: DN, double negative; DP, double positive; PDG, fluorescein-di-b-o-galactopyranoside; LCR, locus control region; SP, single positive; ES, embryonic stem; HSA, heat-stable Ag; CD62L, CD62L-selectin; HA, hemaglutinin; FSC, forward scatter.

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

Because GATA-3−/− 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+−/lacZm1), 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+ cells. The stage of rapidly proliferating CD44+CD25− DN cells, which insulates these two periods of TCR rearrangement, was characterized by a large proportion of lacZ+ cells. The proportion of lacZ+ cells increased again as double-positive (DP) cells progressed into CD4 or CD8 SP cells. The presence of significant proportions of lacZ+ 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+ 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−/−/lacZm1 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−/−/lacZm1 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 promoter; subsequently, the epitope tags were added along with a new ATG and Kozak’s consensus promoter. FVB fertilized oocytes at a concentration of 9×105 were microinjected into the pronuclei of CD2-GATA3 mice. The construct, the translation initiation site was mutated (ATG to GTG), 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).

Western blotting analyses

Total nuclear protein extracts were prepared according to Andrews and Fuller (40). Protein concentration in the nuclear extracts was determined using the bichinchoninic 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 incubated with 2% BSA in PBS (pH 7.0) at 0.05% Tween 20 and incubated with first- and second-step reagents in 2% nonfat dry milk in PBS (pH 7.0) at 0.05% Tween 20. The mouse anti-GATA-3 mAb Hg3-3-31 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-CD3e 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-CD3e, and anti-CD8. 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 Hg3-3-31 anti-GATA-3 mAb (Santa Cruz Biotechnology) and FITC-labeled anti-mouse IgG1 (BD Pharmingen) as a second step.

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 control of the CD4/CD8 transcriptional control. In these GATA-3−/−/lacZm1 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+ cells), CD4 SP (∼84%), and CD8 SP (∼33%) cells was found (22). Since it has been shown that DP T cell subpopulation 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− DP population lacZ was expressed in ∼19% of cells. By contrast, within the CD69+ DP subpopulation, lacZ was expressed in ∼54% of the cells. After subsequent down-regulation of coreceptor expression and transition into the CD4+CD8low subpopulation, ∼80% of the CD69− cells expressed lacZ (Fig. 1). The CD4+CD8−low cells have been shown to subsequently enhance CD4 expression (2), thereby developing into the CD4+CD8low 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+CD8lowCD69− cells (Fig. 1). In addition, we found that in the DP, CD4+CD8low, and CD4+CD8low subpopulations, lacZ expression correlated with the expression levels of TCRαβ on CD3 cells (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.
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GATA-3 gene expression is down-regulated after commitment to the CD8 lineage

Bipotential CD4<sup>+</sup>CD8<sup>+</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 CD25 surface markers, which are instrumental to specify subpopulations of naive, activated, or memory T cells.

As shown in Fig. 1A, the proportions of CD3<sup>-</sup>TCR<sup>β</sup> CD69<sup>+</sup> DP cells (~19%) and 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<sup>β</sup> CD69<sup>+</sup> DP cells (~19%) and increased at the onset of positive selection events characterized by up-regulation of CD3 and TCR<sup>β</sup> surface expression and induction of CD69. The proportions increased to ~86% at the stage of the last uncommitted subset of CD4<sup>+</sup>CD8<sup>+</sup>CD69<sup>+</sup> 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 ~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 ~51-kDa band in the thymus samples from mice of both transgenic lines. Comparison of the intensities of the ~47- and ~51-kDa GATA-3-specific bands in these nuclear protein extracts from thymus samples showed that the expression level of the 3XHA-GATA3 transgene-encoded protein was similar to that of the endogenous GATA-3 protein. In contrast, in the spleen samples, the ~51-kDa GATA-3-specific band was very weak or absent (Fig. 2A). The ~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 1. Analysis of lacZ expression in thymocytes from GATA-3<sup>+/+</sup>lacZ mice by four-color flow cytometry. A. Thymus cell suspensions were loaded with the β-galactosidase substrate FDG and subsequently stained with anti-CD4 and anti-CD8 in combination with anti-CD69, anti-TCRβ, 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, TCRβ, 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>+/+</sup> mice. The numbers are mean values ± SD (n = 3). The background percentages of β-galactosidase-positive cells, as determined in wild-type control mice, was <1% in all subpopulations.
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-GATA-3 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. CD4lowCD8low cells and CD4+CD8low 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 CD4lowCD8low cells, and were high in CD4 SP cells (Fig. 2C), consistent with our findings in the GATA-3+/+β2m−/−lacZ mouse (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, CD4lowCD8low, CD4+CD8low, 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 thymic 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 ± 34 × 106, n = 21) and nontransgenic littersmates (103 ± 37 × 106, n = 14). No significant differences were detected between CD2-GATA3-transgenic mice and normal littersmates 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.
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 J\_\text{\textit{b}}\_\text{\textit{b}} gene segments (Fig. 4B). In a fraction of the tumors analyzed, we observed J\_\text{\textit{a}}\_\text{\textit{a}} restriction fragment patterns that would be consistent with bclonality (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\_\text{\textit{+}} lymphoblasts with variable levels of CD8 coexpression. Fig. 5A illustrates four examples of thymic lymphomas (CD4\_\text{\textit{+}}CD8\_\text{\textit{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\_\text{\textit{+}}CD8\_\text{\textit{low}} lymphoblasts. Most of the tumors contained areas that had lost expression of CD8 and sometimes coexpression. Fig. 5B, compare thymus and lymph node of TL7).

**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 rearrangement patterns in various lymphoblastoid tumor samples. EcoRI digests were hybridized to a TCR J\_\text{\textit{b}}\_\text{\textit{b}} probe. T, Thymus; S, spleen; L, lymph node. *, Position of the germline 2.4-kb EcoRI fragment; on the left the positions of a X BstII 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\_\text{\textit{+}}CD8\_\text{\textit{low}} thymic lymphoma primary tumor samples as well as metastases present in lymph node and spleen. B, Identification of an atypical GATA-3\_\text{\textit{low}}CD3\_\text{\textit{+}}CD4\_\text{\textit{+}}CD8\_\text{\textit{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\_\text{\textit{+}}CD8\_\text{\textit{low}} populations are of all thymocytes. C, Analysis of FSC, CD3, and intracellular GATA-3 expression in the gated CD4\_\text{\textit{+}}CD8\_\text{\textit{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\textsuperscript{CD8\textsuperscript{+}/low} subsets contained atypical fractions of CD\textsuperscript{+} 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 CD\textsuperscript{3+} CD4\textsuperscript{-} CD8\textsuperscript{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\textsuperscript{+} CD8\textsuperscript{low} thymocyte subpopulation.

**GATA-3 enhances TCRαβ up-regulation during positive selection**

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

**FIGURE 6.** The CD2-GATA3 transgene enhances TCRαβ up-regulation during positive selection. A. Cell suspensions were stained for CD4, CD8, and CD69 expression along with either CD3 or TCRβ. The indicated T cell subpopulations (see also Fig. 1A) were gated and analyzed for TCRβ 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\textsuperscript{+} CD4\textsuperscript{low}/CD8\textsuperscript{low} cells that are in the indicated TCRαβ\textsuperscript{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. 6B). The increased size of CD2-GATA3-transgenic 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 thymus 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/TCRαβ\textsuperscript{low} DP into CD3/TCRαβ\textsuperscript{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 TCRαβ 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\textsuperscript{+} 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 (Figs. 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\textsuperscript{+} cells, as a selective deficiency of CD69\textsuperscript{low}/HSA\textsuperscript{low} cells was observed, when CD2-GATA3-transgenic and wild-type littermates were compared (Fig. 7C). For CD4\textsuperscript{+} 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. 7C). This phenomenon was also seen in the mature CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the spleen (see accompanying paper). Finally, the expression of L-selectin (CD62L), a marker which is expressed at high levels on naïve 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\textsuperscript{+} 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\textsuperscript{low}/HSA\textsuperscript{low} cells in the thymic CD8 SP subpopulation, as well as reduced numbers of CD8\textsuperscript{+} T cells in the peripheral organs. These findings indicate that enforced GATA-3 expression resulted in a partial differentiation arrest of CD8\textsuperscript{+} 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\textsuperscript{+}/\textsuperscript{lacZ} mice.
FIGURE 7. Enforced expression of GATA-3 induces apoptosis and inhibits the maturation of CD69lowHSAlow CD8 SP cells in the thymus. Thynduces apoptosis and inhibits the maturation of GATA-3 transgenic control mice (thin lines). transgenic mice (bold lines) along with those of non-Bymphocytes 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, 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+CD8low 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 CD69lowHSAlow 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 manifest functional defects in IL-2 and IFN-γ 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-12Rβ expression and, as a result, IL-12 induced IFN-γ 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 TCRαβ 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 CD8α 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
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TCRαβ up-regulation in developing CD69<sup>+</sup> thymocytes that progress from the DP to the CD4<sup>+</sup>CD8<sup>+</sup> stage, we did not see any effects on the surface expression of presumed GATA-3 target loci such as TCRα, β, and δ or CD8α.

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 TCRα 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 retinol-dehyde 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 (22).

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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References

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