Enforced Expression of GATA-3 Severely Reduces Human Thymic Cellularity

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Tom Taghon, Magda De Smedt, Frank Stolz, Maggy Cnockaert, Jean Plum, and Georges Leclercq

Following bone marrow transplantation, patients often suffer from immune incompetence by reduced or late T cell development. Moreover, adult bone marrow stem cells have a lower capacity to generate T cells compared with fetal liver- and umbilical cord blood-derived progenitors. Therefore, enhancing thymic-dependent T cell generation might hold great therapeutic potential. GATA-3 is a transcription factor that is essential in T cell development. In this study we examined the therapeutic potential of GATA-3 to enhance T cell generation by overexpressing GATA-3 in T cell progenitors followed by fetal thymic organ culture (FTOC). We observed that early during FTOC, there was an enhanced differentiation toward the double positive stage of T cell development. From day 10 of FTOC, however, overexpression of GATA-3 induced a severe reduction in thymic cellularity, which probably correlates with the absence of a functional TCR-β chain. We further show that the frequency of apoptosis was increased in GATA-3-transduced thymocytes. Despite the absence of a functional TCR-β chain, GATA-3 transduced progenitors were able to differentiate into CD8β+ double positive thymocytes. This study shows that a strictly regulated expression of GATA-3 is essential for normal T cell development and this puts severe restrictions on the potential therapeutic use of continuously overexpressed GATA-3. The Journal of Immunology, 2001, 167: 4468–4475.

A competent immune system requires the generation and maintenance of a diverse repertoire of T cells. This repertoire is primarily generated in prenatal and early postnatal life by the processing of progenitor cells in the thymus. From young adulthood onward, however, thymic involution occurs, and this causes a severe decline in thymic output of mature T cells (1–3). Despite the fact that a recent study showed that thymic output still remains substantial until late adulthood (4), T cell numbers in adults are primarily maintained by the expansion of dividing mature T cells and the survival of memory T cells (5–7). As a result, after myeloablative chemoradiotherapy and bone marrow transplantation (BMT), the T cell repertoire is often characterized by a reduction of the CD4+ T cell compartment (8, 9) and a loss of TCR diversity (10, 11). Moreover, several studies have shown that adult bone marrow stem cells have a lower capacity to generate T cells compared with fetal liver or umbilical cord blood stem cells (12–14). Therefore, enhancing thymic-dependent T cell generation following BMT might hold great therapeutic potential.

Several transcription factors have been shown to be essential for T cell development (15–18). One of these is GATA-3, a member of the GATA family of transcription factors that bind to a GATA consensus motif through a highly conserved Zn-finger domain (19–21). Several studies have shown that GATA-3 is only expressed in hematopoietic cells belonging to the T cell lineage, except for one report where expression was detected in NK cells (22). Moreover, using chimeric mice, it was clearly shown that GATA-3 is only required for the development of T cells and not for any other hematopoietic lineage (23). Expression of GATA-3 is detected in the most immature subset of fetal day 12 thymocytes (24) and several studies showed that GATA-3 is essential in the earliest stages of T cell development (23–25). In light of this, we hypothesized that GATA-3 might be able to stimulate thymic-dependent T cell generation.

T cell development is characterized by a well-defined order of differentiation stages (26). Human CD34+CD1+ hematopoietic progenitor cells seed the thymus and differentiate into CD34+CD1− and subsequently mainly into CD34−CD4−CD3+CD8− (immature single positive, ISP4+) (27) and CD4+CD8α+ thymocytes. These early double positive (DP) cells undergo β selection in which they are checked for the intracellular expression of a functional, rearranged TCR-β chain (28). If successful, these thymocytes will start to express the TCR-β chain in combination with the surrogate TCR-α chain (29), called pre-TCR, and they will further differentiate to become CD4+CD8α+CD8β+ DP thymocytes, which initiate TCR-α rearrangement and subsequently express the full TCR complex. After positive and negative selection, selected DP cells acquire expression of CD69 (30) and differentiate into CD3+CD4+CD8− or CD3−CD8+CD4− SP thymocytes that further mature and migrate to the periphery (31).

Overexpression of a certain gene might cause oncogenic side effects or other abnormal differentiation processes, as recently shown for several members of the GATA family of transcription factors in various differentiation processes (32–34). In vitro and in vivo systems that allow the differentiation of human cells provide a useful tool to analyze this. Therefore, in this report we examined the effect of GATA-3 overexpression on human T cell differentiation in fetal thymic organ culture (FTOC) by retroviral transduction of CD34+ thymocytes with GATA-3. We show that early in
FTOC there was indeed an enhanced differentiation toward the DP stage of T cell development. However, at later time points, there was a severe reduction in thymic cellularity that was probably associated with an inability to express the TCR-β chain. These results show that a strictly regulated expression of GATA-3 is necessary for normal T cell development, and they demonstrate that attempts to enhance a certain differentiation process by continuous expression of a relevant gene can have opposite results. Therefore, the search for regulated expression systems for human therapeutic use remains essential.

Materials and Methods

**mAbs and reagents**
The mAbs used were rat anti-mouse CD45 (CD45-CyChrome, 30F1.1.1; BD Pharmingen, San Diego, CA) and the following mouse anti-human mAbs from BD Immunocytometry Systems (Mountain View, CA): CD3 (Leu-4 FITC or APC), CD4 (Leu-3a FITC or APC), CD8α (Leu-2a FITC), and CD43 (HPCA-2 PE). The CDBβ (2ST8.5H7 PE) and TCR panαβ (BMA031 PE) mouse anti-human mAbs were from Coulter (Miami, FL). The mAb used for intracellular TCR-β chain (β-FI; Endogen, Woburn, MA) staining was revealed with rat anti-mouse-IG-PE (BD Biosciences). For annexin V staining, cells were stained with annexin V-biotin (Boehringer). The supernatant containing the depleted cells was recovered.

**Mice**
NOD-LtSz-scid/scid (NOD-SCID) mice, originally purchased from The Jackson Laboratory (Bar Harbor, ME), were obtained from our own pathogen-free breeding facility. For timed pregnancies, females were housed separately from the males until mating. The appearance of vaginal plugs after overnight mating was noted as day 0 of pregnancy. Fourteen- to 15-day pregnant mice were killed by cervical dislocation to obtain the embryos for isolation of the fetal thymic lobes. Animals were treated according to the guidelines of the Laboratory Animal Ethical Commission of the University Hospital of Ghent.

**Preparation of human thymocytes**
Human thymus tissue from children undergoing cardiac surgery was obtained and used following the guidelines of the Medical Ethical Commission of the University Hospital Ghent. For the preparation of thymocytes, the thymic tissue was cut into small pieces of 0.5 × 0.5 cm, which were then extensively teased apart with cataract knives in serum-free RPMI 1640 medium (Life Technologies, Paisley, Scotland) at 4°C, and the freed cells were either used immediately for further purification or were cryopreserved in liquid N2 until use.

**Purification of human CD3+ CD4− CD8− thymocytes**
Thymocytes, either freshly prepared or thawed and layered over Lymphoprep (Nycomed, Oslo, Norway) gradient at 4°C to remove most of the dead cells, were labeled with FITC-labeled CD3 and CD8. For immuno-magnetic depletion, the cells were resuspended in 1 ml of cold PBS/2% FCS and were mixed with 1 ml of prewashed (to remove the preservative) sheep anti-mouse Ig-coated Dynabeads (Dynal Biotech, Oslo, Norway) to obtain a 1:4 ratio of cells:Dynabeads. After 30 min at 4°C, the suspension was subjected to a magnetic field in a magnetic particle concentrator (Dynal Biotech). The supernatant containing the depleted cells was recovered.
The cells were labeled with CD34-PE and CD4-FITC to allow sorting of CD34+CD3−CD4+CD8− thymocytes. Cells were sorted on a FACSVantage (BD Biosciences) equipped with an argon-ion laser tuned at 488 nm. Data acquisition and analysis was done using CellQuest software (BD Biosciences). Sorted cells were checked for purity, which was always at least 99.0%.

**Cell culture and cell lines**
All cultures were performed at 37°C in a humidified atmosphere containing 7.5% (v/v) CO2 in air. The medium used was IMDM, supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FCS (complete IMDM; all products from Life Technologies). Sorted human CD34+CD3−CD4+CD8− thymocytes were cultured in complete IMDM supplemented with human stem cell factor (SCF, 10 ng/ml) and IL-7 (10 ng/ml; all cytokines from R&D Systems Europe, Abingdon, UK). FTOC was performed in complete IMDM containing 10% heat-inactivated human serum (AB blood type; BioWhittaker, Walkersville, MD) instead of 10% FCS. HL-60 cells (American Type Culture Collection, Rockville, MD) were cultured in complete IMDM.

**Cloning of the human GATA-3 cDNA**
To amplify and clone the coding region of GATA-3, RNA was isolated from a freshly prepared thymus cell suspension using TRizol (Life Technologies) according to the instructions of the supplier, and cDNA was prepared using Superscript (Life Technologies) following the guidelines of the manufacturer. An aliquot of this cDNA was used to amplify the GATA-3 gene using Platinum Pfx DNA Polymerase (Life Technologies) with the following primers: 5’-ATCTCTGAAATTCTCCATGGAATCGACGGCGAC-3’ (sense) and 5’-ATGGTACTCGAGCTAAACCTGATGGCCTGAAC-3’ (antisense) (Life Technologies). This PCR product was then cloned using the EcoRI/XhoI restriction sites of Lambda Zap II (Stratagene). The clone used contained the published coding region of the human GATA-3 gene (37) (GenBank accession no. X55122).

**Generation of GATA-3-encoding retroviruses**
The Phoenix-A-based amphotrophic packaging cell line (a kind gift from Dr. P. Achacoso and Dr. G. P. Nolan, Stanford University School of Medicine, Stanford, CA) was transfectioned with the LZRS-EGFP-IRE and LZRS-GATA-3-IRE-EGFP plasmids using calcium-phosphate precipitation (Life Technologies) to generate both retroviruses. The viral supernatant was stored in aliquots at −70°C until use. The EGFP+ and GATA-3+ retroviruses were used in conjugation in the absence of bystander effects.

**Retroviral gene transfer**
Sorted CD3+CD4−CD8− thymocytes were cultured in complete IMDM, supplemented with human IL-7 (10 ng/ml) and SCF (10 ng/ml) for 1 day. The next day, the cells were transduced once for 24 h. For transduction, 5–15 × 106 cells per well were seeded on RetroNectin- (Takara Shuzo, Otsu, Japan) coated 96-well culture plates in a final volume of 150 μl, containing 75 μl of retroviral supernatant and 75 μl of complete IMDM supplemented with 20 ng/ml IL-7 and 20 ng/ml SCF to keep the final cytokine concentration at 10 ng/ml for both IL-7 and SCF. After transduction, cells were washed and harvested to wash the virus particles, transduction efficiency was determined by FACS analysis, and the numbers of transduced cells were determined by FACS analysis. Transduction efficiencies varied between 8.5 and 30.3% for EGFP+ transduced thymocytes and 1.3 and 6.2% for GATA-3+ EGFP− transduced thymocytes.

**FTOC**
Thymic lobes were isolated from fetal day 14–15 NOD-SCID mice. Hanging drops were prepared in Terasaki plates by adding 25 μl of complete medium per well containing the progeny of 5 × 103 CD3+CD4−CD8+ thymocytes transduced as described above. To each of these wells, 1 fetal thymic lobe was added, and the plates were inverted that the clone used contained the published coding region of the human GATA-3 gene (37) (GenBank accession no. X55122).

**Calculation of absolute cell numbers**
The absolute number of transduced EGFP+ human cells at the different time points of analysis was calculated by multiplying the total number of cells transduced under the microscope by the percentage of mouse CD45 EGFP+ human cells as determined by FACS analysis. Due to the difference in virus concentration between the EGFP+ and GATA-3+ EGFP− retroviral batches used and the difference in transduction efficiency between different experiments, the number of transduced cells at the beginning of FTOC is different between both cultures and for each experiment. To compare the yields of human thymocytes obtained from EGFP+ and EGFP−GATA-3+ transduced CD3+CD4+CD8− thymocytes, we therefore multiplied the absolute number of EGFP+ cells at the different timepoints, obtained as described above, by a factor so that the number of
EGFP+ cells at the time of hanging drop was $5 \times 10^4$ for both EGFP+ and EGFP+/GATA-3+-transduced CD34+CD3 CD4 CD8 thymocytes.

**Immunoblotting**

Cell lysates were run on 10% Tris-glycine polyacrylamide gels (NuPAGE; NOVEX, San Diego, CA) in Tris-glycine SDS running buffer (NOVEX) in reducing conditions, and proteins were blotted on polyvinylidene fluoride membranes (NOVEX). Blots were stained with the GATA-3 mAb (H925-31; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse IgG alkaline phosphatase conjugate (Santa Cruz Biotechnology).

**Flow cytometry**

Before labeling, cells were suspended in PBS/1% BSA/0.1% NaN₃. In all cases in which human cells were stained in the presence of mouse cells, the mixture of cells was preincubated for 15 min with saturating amounts of anti-mouse-FcRII/III mAb (clone 2.4G2; a kind gift of Dr. J. Unkeless, Mount Sinai School of Medicine, New York, NY) to avoid nonspecific binding of mAbs by the murine cells. Subsequently, the cells were stained with a panel of mAbs, as indicated. Murine and dead cells were gated out by mouse CD45 CyChrome and propidium iodide, respectively. Negative controls included isotype mAbs conjugated with the corresponding fluorochrome. For intracellular TCR-β chain staining, cells were fixed and permeabilized using Fix & Perm (CalTag Laboratories, San Francisco, CA), according to the guidelines of the manufacturer. Briefly, cells were washed with PBS, resuspended in 100 μl of solution A, and washed with PBS. Subsequently, cells were resuspended in solution B and incubated with the β-F1 mAb for 1 h. Cells were then washed twice with PBS, resuspended in solution B, and incubated with rat anti-mouse-PE for 30 min. Finally, the cells were washed with PBS/1% BSA/0.1% NaN₃, and analyzed. For annexin V staining, cells were washed three times with annexin V binding buffer (10 mM HEPES, 5 mM KCl, 150 NaCl, 1 mM MgCl₂, and 1.8 mM CaCl₂), labeled in 100 μl of this buffer with the addition of annexin V-biotin and streptavidin-APC, and analyzed without washing. The cells were analyzed on a FACSCalibur (BD Biosciences) with an argon-ion laser tuned at 488 nm and a red-diode laser at 635 nm. Forward light scattering, orthogonal scattering, and four fluorescence signals were determined and stored in list mode data files. Data acquisition and analysis was done using CellQuest software (BD Biosciences).

**Results**

**Cloning and retroviral-mediated gene transfer of GATA-3**

To investigate the effect of enforced expression of GATA-3 on human T cell development, we cloned the coding region of the human GATA-3 gene into the LZIP retroviral vector containing the marker gene EGFP (35, 36). In this way we obtained two vectors, one expressing only EGFP (EGFP+) and one expressing both GATA-3 and EGFP (GATA-3+EGFP+). In the second vector, the two genes were separated by an IRES so that both genes were expressed from one bicistronic messenger RNA.

We transduced HL-60 cells, which do not express endogenous GATA-3 as determined by RT-PCR (data not shown), with both retroviruses to show that the encoded proteins are expressed after transduction. EGFP expression was detected by FACS analysis in both EGFP+ and GATA-3+EGFP+-transduced HL-60 cells, and after cell sorting of EGFP+ cells (purity >95% for both EGFP+ and GATA-3+EGFP+ HL-60 cells), immunoblotting revealed that as expected, only HL-60 cells transduced with GATA-3+EGFP+ retroviral supernatant expressed GATA-3 with the predicted molecular mass of ±50 kDa (Fig.1).

**Overexpression of GATA-3 severely affects thymic cellularity and induces apoptosis**

The most immature thymocytes that can be found in the human thymus express CD34 and are negative for the T cell markers CD3, CD4, and CD8. These cells are the precursors of ISP4+, DP immature thymocytes, SP mature thymocytes, and T cells. To study the effect of GATA-3 overexpression on human T cell development, CD34+CD3+CD4+CD8+ thymocytes were isolated by cell sorting and were transduced with EGFP+ or GATA-3+EGFP+ retroviral supernatant. Human T cell development from transduced progenitor cells was assayed in vitro in FTOC, and we performed a kinetic analysis to monitor the cells during the culture period. The percentage of EGFP+ cells in cultures started with EGFP+-transduced progenitor cells sustained during the culture period in accordance with previous data (35, 38), and most of these cells expressed high levels of EGFP. In contrast, the percentage of GATA-3+EGFP+ thymocytes severely declined from day 10 of FTOC onward, with an especially significant reduction in EGFP+, compared with EGFP+transduced cells (Fig. 2). This also reflected on absolute cell numbers of EGFP+ cells, calculated as described in Materials and Methods. Whereas the number of transduced EGFP+ and GATA-3+EGFP+ cells was similar during the first time points of analysis, a 2.5-fold decrease in transduced cells was observed in the GATA-3+EGFP+ cultures as compared with the EGFP+ cultures at day 10 of FTOC, and this difference increased to a factor of 4.5 at day 17 of FTOC (Fig. 3). The non-parametric paired Wilcoxon test showed that these differences were statistically significant (p = 0.028 for day 10, p = 0.018 for day 14, and p = 0.028 for day 17). There was no significant difference in the absolute numbers of untransduced cells in both EGFP+ and GATA-3+EGFP+ cultures at all time points analyzed (data not shown).

The drastic decrease in the number of GATA-3+EGFP+ thymocytes from day 10 of FTOC onward can be either due to a lower proliferation rate of these cells as compared with EGFP+ transduced cells, to induction of apoptosis in part of the GATA-3+EGFP+ cells, or to a combination of both. To determine whether GATA-3+EGFP+ transduced thymocytes were undergoing apoptosis at later stages of differentiation, we determined the profile of annexin V staining on EGFP+- and GATA-3+EGFP+-transduced thymocytes during culture. Fig. 4 shows that at days 14 and 17 of FTOC there was a much higher percentage of apoptotic cells in the GATA-3+EGFP+-transduced thymocytes compared with the EGFP+ control-transduced cells. Thus, GATA-3 overexpression ultimately leads to a drastic reduction of thymic cellularity and induces apoptosis.

**Enforced expression of GATA-3 induces enhanced differentiation toward the CD3+CD4+CD8β+ DP stage of T cell development**

In addition to the effect on thymic cellularity, we also analyzed the effect of GATA-3 overexpression on the development of the different thymocyte subsets. Whereas the differentiation of untransduced cells was similar in both EGFP+ and GATA-3+EGFP+ cultures (data not shown), we noticed that after 4 days of FTOC there was a much higher percentage of CD4+CD8β+ DP immature thymocytes present in GATA-3+EGFP+-transduced thymocytes compared with control EGFP+-transduced cells (Fig. 5). The
Methods at day 4 of FTOC compared with cultures with EGFP genitors was signifi-
cantly higher, on average an 8-fold difference, cells in cultures initiated with GATA-3 and 17 in a nonparametric paired Wilcoxon test). The drastic de-

...generate from GATA-3 coxon test). However, from day 14 onward, the number of DP cells (Table I; \( p = 0.018 \) at day 4 in a nonparametric paired Wilcoxon test). However, from day 14 onward, the number of DP thymocytes generated from GATA-3 EGFP \(^{-}\)-transduced progenitors was always significantly lower compared with the number generated from EGFP \(^{-}\)-transduced cells (\( p = 0.028 \) at days 14 and 17 in a nonparametric paired Wilcoxon test). The drastic decrease in the percentage of CD4 \(^{+}\)CD8\(\beta\) \(^{-}\) thymocytes, and the in-
crease in the percentage of CD4 \(^{+}\)CD8\(\beta\) \(^{-}\) thymocytes, which were CD3\(^{-}\) (data not shown) at day 4 of FTOC starting with GATA-3 EGFP \(^{-}\)-transduced precursor cells (Fig. 5), strongly suggests that the higher percentage of DP thymocytes in these cultures is caused by an enhanced differentiation of the precursor cells.

...also analyzed the development of TCR-\(\gamma\delta\) T cells. The per-
centage of CD3\(^{+}\) TCR-\(\gamma\delta\) T cells was at all time points lower than 3\% in both EGFP \(^{-}\)- and GATA-3 EGFP \(^{-}\)-transduced cells, with no significant difference between them (data not shown).

![FIGURE 2. Kinetic analysis of the percentage of EGFP-expressing cells in FTOC initiated with EGFP \(^{-}\)- and GATA-3 EGFP \(^{-}\)-transduced CD34 \(^{-}\) thymocytes. Dot plots, gated on human cells, show forward scatter (FSC) vs EGFP for EGFP \(^{-}\) (top) and GATA-3 EGFP \(^{-}\)-transduced CD34 \(^{-}\) thymocytes (bottom) at the indicated time points of FTOC. Figures at the bottom of the corresponding dot plots show the total percentage of EGFP \(^{\text{low}}\) and EGFP \(^{\text{high}}\) cells. Data shown are representative of seven independent experiments with seven different donors.](image)

![FIGURE 3. Reduction of thymic cellularity in FTOC initiated with GATA-3 EGFP \(^{-}\)-transduced CD34 \(^{-}\) thymocytes. Absolute number of EGFP \(^{-}\) thymocytes per lobe, calculated as described in Materials and Methods, generated in FTOC from 5000 EGFP \(^{-}\)- (■) or GATA-3 EGFP \(^{-}\)- (□) transduced CD34 \(^{-}\)CD3 \(^{-}\}CD4 \(^{-}\}CD8 \(^{-}\) thymocytes at the indicated time points of FTOC. Differences in cell number between EGFP \(^{-}\)- and GATA-3 EGFP \(^{-}\)-transduced thymocytes were statistically significant at days 10, 14, and 17, with \( p < 0.05 \) as determined by a nonparametric paired Wilcoxon test. The lines indicate the SD on the averages of seven independent experiments.](image)

![FIGURE 4. Increased apoptosis during intrathymic development in FTOC initiated with GATA-3 EGFP \(^{-}\)-transduced CD34 \(^{-}\) thymocytes. Histograms show annexin V staining of EGFP \(^{-}\}-gated cells of EGFP \(^{-}\)- (top) and GATA-3 EGFP \(^{-}\)-transduced CD34 \(^{-}\}CD3 \(^{-}\}CD4 \(^{-}\}CD8 \(^{-}\) cells (bottom) on days 14 and 17 of FTOC as indicated. The percentage of annexin V-positive cells is shown in each histogram. Data shown are representative of three independent experiments with three different donors.](image)
Overall, these data show that overexpression of GATA-3 favors differentiation of thymic progenitor cells toward the DP stage of T cell differentiation. However, further differentiation is hampered, as the absolute number of CD3+ TCR-αβ+ thymocytes is severely reduced. There was no skewing toward the γδ T cell lineage.

GATA-3 transduced thymocytes fail to express a functional TCR-β chain

To investigate what caused the reduced T cell differentiation at the DP stage of T cell development and the higher level of apoptosis in GATA-3 EGFP+-transduced thymocytes, we analyzed the intracellular expression of the TCR-β chain. As immature CD4+CD8α+CD8β+ thymocytes undergo a process called β selection before they acquire CD8β expression, all CD4+CD8α+CD8β+ DP thymocytes normally express a functional TCR-β chain intracellularly. Despite the fact that the percentage of CD4+CD8β+ DP immature thymocytes was much higher during early time points in cultures initiated with GATA-3 EGFP+-transduced progenitor cells compared with cultures initiated with EGFP+-transduced cells (Fig. 5), the percentage of thymocytes expressing a TCR-β chain intracellularly was drastically decreased in GATA-3 EGFP+-transduced cells at days 4 and 7 of FTOC (Fig. 7). Therefore, the observation that T cell development from GATA-3-transduced human T cell progenitors is severely hampered is presumably associated with a defect in TCR-β chain expression.

Discussion

In this report we have shown that overexpression of GATA-3 in CD34+CD3+CD4+CD8+ thymic progenitor cells enhances the early differentiation of thymocytes toward the DP stage of T cell development in FTOC, but at later time points, it severely reduces thymic cellularity and results in an increased incidence of apoptosis. We further demonstrate that the defective T cell development probably correlates with failure of GATA-3-transduced thymocytes to express a functional TCR-β chain intracellularly, suggesting that GATA-3 overexpression interferes with TCR-β chain rearrangement or expression.

Early differentiation toward the immature DP stage of T cell development was strongly enhanced in thymic progenitor cells that overexpressed GATA-3, as the frequency and the absolute cell number of DP thymocytes was 8-fold higher after 4 days of FTOC. As GATA-3 binding sites are located in the enhancers of various T cell-specific genes, including CD4 and CD8 (39–41), it could be argued that the higher frequency of DP thymocytes was caused by a direct effect of GATA-3 on CD4 and CD8 gene expression, rather than due to thymic-dependent differentiation of the CD34+CD3+CD8α+ progenitor cells. However, the observation that in FTOC, the frequency of CD3+CD4+CD8β- cells, which characterizes the preceding differentiation stage, was also higher, whereas the frequency of the most immature CD3+CD4+CD8α+ precursor cells was severely reduced, strongly argues against this. Moreover, sorted GATA-3-transduced CD34+CD3+CD4+CD8- thymocytes were unable to express CD8β in cell suspension cultures supplemented with IL-7 and SCF (data not shown).

At later time points of FTOC, overexpression of GATA-3 caused a drastic reduction in thymic cellularity. This reduction was most pronounced in cells expressing high levels of EGFP and thus probably high levels of GATA-3. Analysis of the intracellular expression of the TCR-β chain revealed that the percentage of

<table>
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<th>Days of Culture</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP+</td>
<td>823 ± 1,193*</td>
<td>3,083 ± 3,192</td>
<td>35,148 ± 40,828</td>
<td>171,025 ± 150,747</td>
<td>274,202 ± 133,699</td>
</tr>
<tr>
<td>GATA-3' EGFP+</td>
<td>6,350 ± 5,964</td>
<td>8,479 ± 3,196</td>
<td>13,866 ± 9,477</td>
<td>39,248 ± 28,267</td>
<td>62,390 ± 33,400</td>
</tr>
</tbody>
</table>

* Figures indicate the average of seven independent experiments with seven different donors. Differences in cell number between EGFP+ and GATA-3' EGFP+-transduced thymocytes were statistically significant as determined by a nonparametric paired Wilcoxon test, with p < 0.05 for all days, except for day 10.
TCR-β⁺ cells was strongly reduced in GATA-3⁺EGFP⁺-transduced thymocytes at all time points analyzed. As thymic cellularity mainly depends on proliferation of double negative thymocytes that express a functional TCR-β chain, absence of this chain in GATA-3-transduced cells presumably inhibits expansion of these T cell progenitors and is therefore responsible for the reduced thymic cellularity. We could also show that there was a higher level of apoptosis in GATA-3⁺EGFP⁺-transduced thymocytes, which can be explained by the fact that most of the CD4⁺CD8β⁺ DP cells did not express a functional TCR-β chain and were therefore unable to undergo positive selection. These CD4⁺CD8β⁺ thymocytes presumably died through “death by neglect.” This supports the suggestion of Blom et al. (42) that TCR-β⁻ cells are dead-end cells. As a result, despite the early enhanced differentiation of GATA-3-transduced thymocytes, CD3⁺ TCR-αβ⁺ cells do not arise earlier than in the control-transduced cultures and are strongly reduced in absolute cell numbers.

During normal T cell development, TCR-β chain expression is required for differentiation into CD4⁺CD8β⁺ cells. In this report, it is shown that GATA-3-transduced human T cell progenitors are able to differentiate into DP thymocytes in the virtual absence of a TCR-β chain. However, it has been shown previously in TCR-β-deficient animals that specific treatments can result in the generation of DP thymocytes, e.g., sublethal irradiation of recombination-activating gene-deficient mice (43), low-dose γ radiation or bleomycin treatment of SCID mice (44), or anti-CD3e treatment in FTOC initiated with thymic lobes derived from SCID-CD1− recombination-activating gene-1−, and TCR-β-deficient mice (45). This clearly shows the existence of a TCR-β-independent pathway for DP thymocyte development.

At this point, however, it is unclear whether the inhibition of TCR-β chain expression in GATA-3-transduced thymocytes is due to inhibition of rearrangement or to inhibition of transcription and/or translation of a rearranged β-chain. We observed that enforced expression of GATA-3 did not have any effect on TCR expression in transduced Jurkat cells (data not shown), indicating that overexpressed GATA-3 does not inhibit transcription or translation of a rearranged β-chain. It is also interesting to note that Hendriks et al. (25) showed that GATA-3 expression is downregulated at the stages of both β- and α-chain rearrangement.

Combined with our results, this would suggest that GATA-3 down-regulation during TCR rearrangement is a prerequisite for rearrangement to occur. The mechanism by which GATA-3 could interfere with TCR-β chain rearrangement is unclear. As GATA-3 binding sites are present in the enhancer of the TCR-β gene promoter (46), it might be that activation of this promoter before initiation of rearrangement results in an irreversible inhibition of β rearrangement. Interestingly, several GATA-3 binding sites are present in the TCR-β enhancer, but apparently these have a different function in the enhancer activity (46). Although it seems unlikely that GATA-3 inhibits TCR-β gene rearrangement as GATA-3 is already expressed at the earliest stages of T cell development, it might be that a cofactor of GATA-3, such as the recently identified repressor of GATA (47), is titrated out by overexpression of GATA-3, where normally this cofactor inhibits the early binding of GATA-3 to the TCR-β gene enhancer. Further investigation is necessary to determine this.

A small fraction of the GATA-3⁺EGFP⁺-transduced DP thymocytes did express a functional TCR-β chain intracellularly, and these must be the few remaining cells that could acquire surface expression of CD3 and TCR-αβ. The fact that this small fraction apparently escaped from the effect of enforced GATA-3 expression can be explained by differences in GATA-3 expression levels in GATA-3⁺EGFP⁺ transduced cells as both EGFPlow and EGFPhigh populations were included in our analysis. The expression level of GATA-3 in EGFPlow-transduced thymocytes might be too low to interfere with β rearrangement. Indeed, by analyzing the intensity of EGFP fluorescence, we noticed that the reduction of GATA-3⁺EGFP⁺-transduced cells during the culture period was more severe in the EGFPhigh cells, suggesting that the frequency of apoptosis, and thus presumably of TCR-β⁻ cells, was higher in cells expressing high levels of GATA-3. If overexpression of GATA-3 indeed inhibits rearrangement, another possible explanation might be that some of the CD34⁺ progenitors cells had already rearranged the TCR-β chain before transduction, as β rearrangement is initiated in the CD34⁺CD1⁺ population (42), which is included in our starting population, and that once rearrangement is started, overexpression of GATA-3 can no longer interfere with this process. This is also supported by the fact that when CD34⁺CD1⁻ thymocytes were transduced with GATA-3⁺EGFP⁺ retroviral supernatant, the
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reduction in EGFP+ thymocytes was even more pronounced, whereas in FTOCs initiated with GATA-3−EGFP−-transduced ISP4+ thymocytes, the reduction was less dramatic (data not shown). The development of γδ T cells was similar in both EGFP− and GATA-3−EGFP−-transduced cells. There was no obvious skewing toward this lineage as a compensation for the hampered αβ T cell differentiation. Due to the low frequency of γδ T cells in FTOC initiated with CD34+CD3−CD4−CD8− thymocytes, it is unclear whether enforced GATA-3 expression also inhibits the development of this lineage.

Due to the fact that GATA-3 is a transcription factor, there is also a possibility that enforced expression of this gene would induce the expression of a soluble or membrane-bound protein that influences the development of the surrounding untransduced cells. As the differentiation and cell numbers of untransduced cells were similar in both EGFP+ and GATA-3−EGFP+ cultures, there is no evidence for this.

In this report, we have shown that enforced expression of GATA-3, although shown by others to be essential in the earliest stages of thymopoiesis (23–25), has severe effects on normal T cell development. These results parallel recent papers in which several GATA genes were continuously expressed in various differentiation processes. Tong et al. (33) have shown that constitutive GATA-2 and GATA-3 down-regulation is necessary for normal red blood cell development (20). Finally, it has been described that continuous GATA-2 expression in pluripotent hematopoietic progenitor cells inhibits both their proliferation and differentiation (34). Combined with our data, these papers show the importance of regulated expression of GATA genes in normal differentiation processes.

In conclusion, we have shown that overexpression of GATA-3 seriously affects human T cell development, that this is probably correlated with an inability to express a functional TCR-β chain, and that this results in a drastic reduction of thymic cellularity. These results highlight that the role of GATA-3 during T cell development needs to be clarified more precisely, and also put severe limitations on the therapeutic potential of continuously expressed GATA-3 to enhance thymic-dependent T cell development following BMT and show that a critical balance of gene expression must be maintained for normal differentiation processes.

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