Enforced Expression of GATA-3 Severely Reduces Human Thymic Cellularity

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Enforced Expression of GATA-3 Severely Reduces Human Thymic Cellularity

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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.
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; 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 CD8β (2ST5.8H7 PE) and TCR panαβ (BMA031 PE) mouse anti-human mAbs were from Coulter (Miami, FL). The mAb used for intracellular TCR-α chain (β-F1; Endogen, Woburn, MA) staining was revealed with rat anti-mouse-PE (BD Biosciences). For annexin V staining, cells were stained with annexin V-biotin (Boehringer Mannheim, Mannheim, Germany) plus streptavidin-APC (BD Biosciences). Sorted cells were checked for purity, which was always at least 99.5%.

Data acquisition and analysis was done using CellQuest software (BD Biosciences) equipped with an argon-ion laser tuned at 488 nm.

Purification of human CD34+/H9262

Therefore, the search for regulated expression systems for human CD34+ cells at the different stages of T cell development can have opposite results. The search for regulated expression systems for human therapeutic use remains essential.

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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 (H9–31; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-mouse IgG alcaline phosphatase conjugate (Santa Cruz Biotechnology).

**Flow cytometry**

Before labeling, cells were suspended in PBS/1% BSA/0.1% NaN₃, 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-Ig-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 LZRS retroviral vector containing the marker gene EGFP (35, 36). In this way we obtained two different thymocyte subsets. Whereas the differentiation of untransduced thymocytes present in 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
percentage of DP cells gradually increased in both cultures and became equal after 17 days of FTOC. Taking into account the absolute cell numbers of EGFP<sup>+</sup> cells (Fig. 3), the number of DP cells in cultures initiated with GATA-3<sup>+</sup> EGFP<sup>+</sup>-transduced progenitors was significantly higher, on average an 8-fold difference, at day 4 of FTOC compared with cultures with EGFP<sup>+</sup>-transduced 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<sup>+</sup> EGFP<sup>+</sup>-transduced progenitors was always significantly lower compared with the number generated from EGFP<sup>+</sup>-transduced cells (p = 0.028 at days 14 and 17 in a nonparametric paired Wilcoxon test). The drastic decrease in the percentage of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes, and the increase in the percentage of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes, which were CD3<sup>-</sup> (data not shown) at day 4 of FTOC starting with GATA-3<sup>+</sup>EGFP<sup>+</sup>-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.

Although GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced thymocytes showed an enhanced differentiation toward the CD4<sup>-</sup>CD8<sup>+</sup> stage of T cell development, these cells did not acquire cell surface expression of CD3 and TCR-αβ earlier than EGFP<sup>+</sup>-transduced cells, which was between days 7 and 10 of FTOC in both cultures (Fig. 6). Despite the fact that the percentage of CD3<sup>+</sup> TCR-αβ<sup>+</sup> cells was slightly higher in GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced thymocytes compared with EGFP<sup>+</sup>-transduced cells at day 17 of FTOC (Fig. 6), the absolute number of CD3<sup>+</sup> TCR-αβ<sup>+</sup> thymocytes was severely reduced in the former cultures as a result of the drastic reduction in total cell number (Fig. 3).

We also analyzed the development of TCR-γδ T cells. The percentage of CD3<sup>+</sup> TCR-γδ<sup>+</sup> T cells was at all time points lower than 3% in both EGFP<sup>+</sup>- and GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced cells, with no significant difference between them (data not shown).

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Kinetic analysis of the percentage of EGFP-expressing cells in FTOC initiated with EGFP<sup>+</sup>- and GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced CD34<sup>+</sup> thymocytes. Dot plots, gated on human cells, show forward scatter (FSC) vs EGFP for EGFP<sup>+</sup> (top) and GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced CD34<sup>+</sup> thymocytes (bottom) at the indicated time points of FTOC. Figures at the bottom of the corresponding dot plots show the total percentage of EGFP<sup>+</sup> and EGFP<sup>+</sup> cells. Data shown are representative of seven independent experiments with seven different donors.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Reduction of thymic cellularity in FTOC initiated with GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced CD34<sup>+</sup> thymocytes. Absolute number of EGFP<sup>+</sup> thymocytes per lobe, calculated as described in Materials and Methods, generated in FTOC from 5000 EGFP<sup>+</sup>- (■) or GATA-3<sup>+</sup>EGFP<sup>+</sup>- (□) transduced CD34<sup>+</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> thymocytes at the indicated time points of FTOC. Differences in cell number between EGFP<sup>+</sup>- and GATA-3<sup>+</sup>EGFP<sup>+</sup>-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.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Increased apoptosis during intrathymic development in FTOC initiated with GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced CD34<sup>+</sup> thymocytes. Histograms show annexin V staining of EGFP<sup>+</sup>-gated cells of EGFP<sup>+</sup>- (top) and GATA-3<sup>+</sup>EGFP<sup>+</sup>-transduced CD34<sup>+</sup>CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> 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.
OVEREXPRESSION OF GATA-3 DISTURBS T CELL DEVELOPMENT

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-\(\alpha\)\(\beta\) thymocytes is severely reduced. There was no skewing toward the \(\gamma\delta\) T cell lineage.

GATA-3 transduced thymocytes fail to express a functional TCR-\(\beta\) 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\(^+\) transduced thymocytes, we analyzed the intracellular expression of the TCR-\(\beta\) chain. As immature CD4\(^+\)CD8\(^+\)CD3\(^-\)CD8\(\beta\) thymocytes undergo a process called \(\beta\) selection before they acquire CD8\(\beta\) expression, all CD4\(^+\)CD8\(^+\)CD3\(^-\)CD8\(\beta\) DP thymocytes normally express a functional TCR-\(\beta\) chain intracellularly. Despite the fact that the percentage of CD4\(^+\)CD8\(^+\)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-\(\beta\) 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-\(\beta\) 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-\(\beta\) chain intracellularly, suggesting that GATA-3 overexpression interferes with TCR-\(\beta\) 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 CD3\(^+\)CD4\(^-\)CD8\(\alpha\)\(\beta\) progenitor cells. However, the observation that in FTOC, the frequency of CD3\(^+\)CD4\(^-\)CD8\(\beta\) cells, which characterizes the preceding differentiation stage, was also higher, whereas the frequency of the most immature CD3\(^+\)CD4\(^-\)CD8\(\beta\) precursor cells was severely reduced, strongly argues against this. Moreover, sorted GATA-3-transduced CD34\(^+\)CD3\(^+\)CD4\(^-\)CD8\(\alpha\)\(\beta\) thymocytes were unable to express CD8\(\beta\) 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-\(\beta\) chain revealed that the percentage of

<table>
<thead>
<tr>
<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-CD3ε treatment in FTOC initiated with thymic lobes derived from SCID-, 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 promotor 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
OVEREXPRESSION OF GATA-3 DISTURBS T CELL DEVELOPMENT

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 expression in mice suppresses adipocyte differentiation and traps cells at the preadipocyte stage, showing that GATA-2 and GATA-3 down-regulation is necessary for final adipocyte maturation. Also, overexpression of GATA-1 in the erythro-myeloid lineage leads to inhibition of differentiation and to lethal anemia in mouse (32), despite the fact that GATA-1 is essential 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 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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References


