Gene expression profiling in mice with enforced Gata3 expression reveals putative targets of Gata3 in double positive thymocytes

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1. Introduction

Gata3 is a member of a family of transcription factors that bind to a consensus GATA site through a highly conserved zinc-finger domain (Evans et al., 1988; Patient and McGhee, 2002). Within the hematopoietic system Gata3 is essential for T cell development from the earliest stages and is involved in NK and invariant NKT cell differentiation (Kim et al., 2006; Samson et al., 2003). In addition, Gata3 acts as master regulator of Th2 differentiation in mature CD4⁺ T cells, where it is crucial for transcription of the T helper 2 cytokines IL-4, IL-5 and IL-13, as well as for the inhibition of Th1 and regulatory T cells (Mantel et al., 2007; Ouyang et al., 1998; Zheng and Flavell, 1997). Gata3 is also expressed in non-hematopoietic tissues, including kidney, central nervous system, skin and mammary gland (Ho et al., 1989). Gata3-deficient mice die at day 11 of gestation due to noradrenaline deficiency of the sympathetic nervous system (Lim et al., 2000; Pandolfi et al., 1995).

The essential function of Gata3 in T cell development was demonstrated by the inability of Gata3-deficient ES cells to give rise to early CD4⁺CD8⁻ double negative (DN) T cell progenitors in the thymus (Hendriks et al., 1999; Ting et al., 1996). In this context, Gata3 function for T cell specification is dependent on Notch signaling (Hozumi et al., 2008). In DN thymocytes gene segments encoding the T cell receptor β (TCRβ) chain undergo V(D)J recombination and only cells with a functional TCRβ rearrangement are selected for further maturation. This process, termed β-selection, is associated with an increase in cell size and induction of proliferation (Ciofani and Zuniga-Pflucker, 2005). Analysis of Gata3-lacZ reporter mice showed that Gata3 expression is upregulated in cells that have undergone β-selection (Hendriks et al., 1999).
Furthermore, conditional deletion experiments demonstrated that Gata3-deficient DN cells failed to undergo β-selection, indicating the essential role for Gata3 in this process (Pai et al., 2003). After β-selection, cells upregulate expression of CD4 and CD8 and TCRα gene segments are recombined, resulting in surface expression of TCRβ at the CD4+CD8+ double positive (DP) stage. Upon engagement by self-major histocompatibility complex (MHC) peptide complexes, low- to intermediate-avidity interactions rescue DP thymocytes from death by neglect, resulting in positive selection to either CD8 single positive (SP) cells in the context of MHC class I or CD4 SP cells in the context of MHC class II (Bosselut, 2004; Kappes and He, 2005). In DP thymocytes TCR stimulation in the context of MHC class II results in the induction of Gata3 expression, which is maintained in CD4 SP thymocytes and downregulated in CD8 SP cells (Hendriks et al., 1999; Hernandez-Hoyos et al., 2003; Ling et al., 2007). An absolute requirement for Gata3 for CD4 positive selection became evident from the finding that conditional deletion of the Gata3 gene at the DP stage resulted in a failure of CD4 SP generation (Pai et al., 2003). We recently found that during CD4 positive selection Gata3 has the capacity to downregulate expression of CD5, which is a negative regulator of TCR signaling, and to upregulate TCR expression (Ling et al., 2007). Based on the observed induction of Gata3 by TCR stimulation in the context of MHC class II (Hernandez-Hoyos et al., 2003), we concluded that Gata3 probably acts in a positive feedback loop to upregulate TCR expression in developing CD4 SP thymocytes (Ling et al., 2007).

Accurate regulation of Gata3 expression levels is essential for correct T cell development. Early overexpression of Gata3 rapidly induced respecification of early T cell precursors to the mast cell lineage (Taghon et al., 2007). CD2-GATA3 transgenic mice, with enforced expression of Gata3 driven by the CD2 promoter, display impaired CD8 T cell maturation and develop thymic lymphoma (Nawijn et al., 2001b). These lymphomas appear to arise at the DP cell stage, where Gata3 overexpression induces a dramatic cell size increase and increased expression of the c-Myc oncogene (van Hamburg et al., 2008a).

The CD4/CD8-cell fate choice of double positive (DP) thymocytes is mainly determined through differences in T cell receptor (TCR) signal strength and duration (Singer et al., 2008). Both Gata3 and the zinc-finger transcription factor ThPOK have been implicated in positive selection of DP thymocytes towards the CD4 lineage. The finding that forced expression of ThPOK in thymocytes can redirect cells that should become CD8 cells into the CD4 lineage, indicated that ThPOK is a master regulator that is necessary and sufficient for the CD4 T cell fate (He et al., 2005; Sun et al., 2005). Because ThPOK is not expressed in the absence of Gata3 and Gata3-binding sites are present in the ThPOK locus, it has been hypothesized that Gata3 directly regulates ThPOK expression (Wang et al., 2008). However, transgenic expression of ThPOK failed to promote CD4 lineage differentiation of Gata3-deficient cells (Wang et al., 2008). Therefore, Gata3 acts via additional targets to initiate the CD4 lineage program and thus Gata3 expression may be required to reach a developmental stage at which ThPOK expression is induced.

In this report, we aimed to identify putative Gata3 targets in DP thymocytes. We compared gene expression profiles of sorted DP thymocytes from wild-type mice and from CD2-GATA3 transgenic mice. To specifically identify Gata3 target genes in the context of positive selection towards the CD4 lineage, we also investigated wild-type and CD2-GATA3 transgenic DP cells harboring the DO11.10 transgenic MHC class II-restricted TCRαβ specific for ovalbumin (Murphy et al., 1990). We found that Gata3 overexpression did not affect ThPOK expression levels. Instead, our findings implicate Gata3 in the termination of TCRα gene rearrangement and in the regulation of TCR signal strength.

2. Materials and methods

2.1. Mice

The generation and genotyping of CD2-GATA3 (FVB) (Nawijn et al., 2001b), CD2-GATA3:DO11.10 (BALB/c) (Ling et al., 2007) and Lck-Cre Tg Gata3f/f (Ling et al., 2007) have been described. Mice were kept under pathogen-free conditions in the Erasmus MC animal care facility. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

2.2. Flow cytometry, antibodies and cell sorting

The generation of single-cell suspensions and cell labeling for flow cytometry have been described previously (Nawijn et al., 2001b). Antibodies were purchased from BD Bioscience (San Diego, CA). FACS sorting of CD4+CD8+ T cells was performed with a FACSVantage VE, equipped with Diva Option and BD FACSdiva software (BD, San Diego, CA). Purity of fractions was >98%.

2.3. Chromatin immunoprecipitation (ChIP)

ChIP analysis of GATA3 binding to the TCRα enhancer (Ho et al., 1991) was performed as described previously (Ribeiro de Almeida et al., 2009) using anti-GATA3 antibodies (HG3–31 AC). Quantitative real-time PCR on immunoprecipitated DNA was performed using SYBR Green qPCR Master Mix (Fermentas). Enrichment was calculated relative to Necdin (Necdin-F: GGTCCTGCTCTGATCCGAAG and Necdin-R: GGGTCCCTACGTCTCTTACTT) and values were normalized to input measurements.

2.4. Preparation of probes, microarray hybridization and data analysis

Oligonucleotide arrays printed with the Operon Mouse Genome Oligo Set version 3.0 (32K mouse) were obtained from The Netherlands Cancer Institute central microarray facility (NKI-CMF, Amsterdam, The Netherlands). Protocols for sample preparation and array hybridization were supplied by NKI-CMF (http://microarrays.nki.nl). In brief, total RNA was isolated using the GeneElute mammalian total RNA miniprep system (Sigma–Aldrich, St. Louis, MO). The quantity and quality of RNA were determined using a NanoDrop spectrometer (Nanodrop Technologies, Wilmington, DE). Samples with a 260/280 nm optical density ratio >1.8 were used. Two micrograms of total RNA was used for amplification using T7 MEGAscript Kit (Ambion, Austin, TX), whereby aminoallyl-UTP (Ambion) was incorporated into amplified RNA (arRNA). Subsequently, Cy5- or Cy3-dye (Amersham, GE Healthcare, Piscataway, NJ) was coupled to the aminoallyl-modified arRNA. Labeled arRNA was purified and concentrated using Microcon YM30 columns (Millipore, Billerica, MA). Oligonucleotide arrays were co-hybridized with purified probes derived from DP thymocytes from individual mice and control probes derived from pooled wild-type DP thymocytes (n=6) and scanned with a Scanarray Express HT scanner (PerkinElmer, Boston, MA). Data were extracted using Imagene software 6.0 (Biodiscovery, CA, USA). Each experiment consisted of 2 oligonucleotide arrays, whereby dyes were reversed between arRNA from sorted DP cells of CD2-GATA3, CD2-GATA3:DO11.10 or DO11.10 transgenic mice and the control wild-type DP T cells pool.

Obtained array data were uploaded in the NKI-CMF database, analyzed using NKI-CMF software (http://dexter.nki.nl) and normalized per subarray using the Lowess normalization method. Normalized data from dye-reversed hybridizations were combined by means of a weighted average. Experiments were combined and normalized.

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data were filtered for genes that were differentially expressed in experiments. Hierarchical clustering of genes and experiments was performed using Genesis 1.5.0 (Sturn et al., 2002).

2.5. Semi-quantitative and quantitative PCR analysis

Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma). One microgram of total RNA was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers.

Semi-quantitative PCR analysis was performed on samples serially diluted 3-fold before amplification. Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers and probes are listed in Suppl. Table S1. To confirm the specificity of amplified products, samples were analyzed by standard agarose gel electrophoresis. Threshold levels were set and analysis was performed using the SDS v1.9 software (Applied Biosystems). Obtained ΔCt values of the genes of interest were normalized to the G3 value of glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

3. Results and discussion

3.1. Enforced Gata3 expression results in increased occupancy of Gata3-binding sites in the TCRx enhancer region

To be able to identify Gata3 target genes in DP thymocytes in the context of positive selection towards the CD4 lineage, we took advantage of our CD2-GATA3 transgenic mouse model, which we crossed with D011.10 transgenic mice carrying a transgene encoding an ovalbumin peptide-specific TCRβ that is MHC class II restricted (Murphy et al., 1990). As previously shown (Hendriks et al., 1999; Hernandez-Hoyos et al., 2003; Ling et al., 2007), the presence of the CD2-GATA3 transgene did not affect D011.10 CD4 lineage restriction (Fig. 1A). The presence of the D011.10 transgene is associated with an upregulation of TCR expression levels, which was clearly more pronounced in CD2-GATA3 D011.10 double transgenic mice (Fig. 1B). Gata3 has been shown to bind to GATA sites present in the TCRx transcriptional enhancer located just 3’ to the TCR Cc gene segment (Ho et al., 1991). ChIP experiments on total thymocyte fractions from wild-type and CD2-GATA3 transgenic mice showed that transgenic overexpression of Gata3 resulted in increased binding of Gata3 protein to its recognition sites in the TCRx enhancer region (Fig. 1C).

Thus, overexpression of Gata3 leads to enhanced TCR expression and increased occupancy of Gata3-binding sites in the TCRx enhancer region. In this context, Gata3 may indirectly specify the developmental stage at which ThPOK expression is induced. Therefore, Gata3 expression in DP cells is not sufficient to induce the expression of ThPOK.

Taken together, these findings show that Gata3 overexpression did not affect ThPOK expression levels in DP, CD4 SP or CD8 SP cells. Therefore, Gata3 does not directly act as a CD4 specification factor by promoting the expression of ThPOK. Instead we conclude that, in the absence of Gata3, DP thymocytes do not reach the developmental stage at which ThPOK expression is induced.

3.2. Enforced Gata3 expression does not enhance ThPOK expression in DP or CD4 SP thymocytes

To investigate whether enforced expression of Gata3 was associated with increased ThPOK expression, we performed quantitative RT-PCR analyses. Whereas in DP cells the presence of the CD2-GATA3 transgene, either on the wild-type or the DO11.10 background, resulted in a ~4-fold increase in Gata3 transcription, we did not detect any effect on ThPOK transcription (Fig. 2A). We found that in CD4 SP thymocytes enforced Gata3 expression was even associated with reduced ThPOK transcription (Fig. 2B). Finally, we analyzed ThPOK transcription in our previously reported complex crosses of CD2-GATA3 transgenic mice with conditionally deleted CD4-Cre Gata3f/f mice. In these mice, the endogenous floxed Gata3 gene is deleted and developing CD4 SP and CD8 SP thymocytes express the same level of Gata3 transcription, contributed exclusively by the CD2-GATA3 transgene, irrespective of their developmental choice (Hendriks et al., 1999; Hernandez-Hoyos et al., 2003; Ling et al., 2007). Quantitative RT-PCR experiments showed that in the CD2-GATA3 transgenic CD4-Cre Gata3f/f mice ThPOK was exclusively expressed in CD4 cells and not in CD8 cells (Fig. 2C). Therefore, Gata3 expression in CD8 SP cells is not sufficient to induce the expression of ThPOK.

The DP compartment in the thymus is heterogeneous and contains (i) cells that are positively selected towards the CD4 or (ii) the CD8 lineage, (iii) cells that are negatively selected, and (iv) a major proportion of cells that are not selected and die by neglect. Because GATA3 expression is specifically induced in DP cells by TCR signaling in the context of MHC class II and maintained during positive selection towards the CD4 SP cell lineage, we considered two different expression profile comparisons. First, we compared wild-type and CD2-GATA3 transgenic DP thymocytes, allowing the identification of genes induced or repressed by GATA3 in a heterogeneous DP cell population containing many cells in which GATA3 is normally not induced. We also compared DP thymocytes from DO11.10 single transgenic and DO11.10:CD2-GATA3 double transgenic mice. Hereby, we investigated the effects of premature and enhanced GATA3 expression in DP cells in which Gata3 would normally be induced because DO11.10 transgenic DP cells are efficiently positively selected towards the CD4 lineage.

FACS-sorted DP thymocyte fractions were obtained from three individual 11-week-old mice from the following four groups: wild-type, CD2-GATA3 transgenic, DO11.10 transgenic and CD2-GATA3:DO11.10 double transgenic mice. Oligonucleotide arrays were co-hybridized with RNA from these DP cell fractions and RNA from a control pool of sorted DP T cells from 12 wild-type mice. After performing a non-supervised hierarchical clustering of individual mice and genes, we were able to identify differential gene expression profiles for the four different groups of mice. The gene expression profiles of the three individual mice present in each group clustered together, indicating that enforced expression of GATA3 and the presence of the DO11.10 TCR transgene resulted in unique gene expression signatures (Fig. 3A). The almost complete absence of differentially expressed genes in the individual wild-type mice, when compared to the control pool, provided a good quality control indicating that the observed differential gene
expression profiles represent specific effects of enforced GATA3 expression.

To be able to identify genes, which are affected by enforced GATA3 expression at the DP T cell stage, the expression profiles of the individual mice were grouped per genotype, and a non-supervised clustering of all four groups and genes was performed (Fig. 3B). In this analysis 3751 differently expressed genes were identified within the four groups of mice. To further identify the most relevant differentially expressed genes, the following selection criteria were used: (i) only genes which were >1.4-fold upregulated or <1.4-fold down-regulated with a p value <0.0001 were allowed, and (ii) genes which were differently expressed in wild-type DP T cells or genes with unknown function were excluded. This resulted in a total number of 1126 genes that were differentially expressed in CD2-GATA3 or DO11.10 single transgenic or CD2-GATA3:DO11.10 double transgenic DP thymocytes.

3.4. Clustering of differentially expressed genes into three groups of genes

Next, a Venn diagram was composed, allowing for the identification of differentially expressed genes that are common or unique for the four DP genotypes. Our expression profiling revealed 276 (85 + 114 + 26 + 51, clusters A–D, Fig. 3C) differentially expressed genes in CD2-GATA3 transgenic DP thymocytes, when compared with wild-type DP cells. The comparison of DO11.10 single transgenic and wild-type DP thymocytes revealed differential expression of many more genes: 711 genes (114 + 26 + 173 + 408, clusters B, C, F and G), suggesting that the introduction of the DO11.10 TCR transgene, which induces positive selection towards the CD4 lineage, has a larger impact on gene expression in DP cells than the presence of the CD2-GATA3 transgene.

276 genes of clusters A–D represent genes that were induced or repressed by Gata3 in total DP cells, apparently irrespective of their cell fate (positive selection, negative selection, or death by neglect). From these 276 genes 136 genes (clusters A and D) were not differentially expressed in DO11.10 transgenic DP cells. The A/D cluster therefore represents genes that were upregulated or downregulated by transgenic Gata3 expression but were not involved in positive selection towards the CD4 lineage. Likewise, the 140 genes in the B/C cluster represent genes that were not only upregulated or downregulated by transgenic Gata3 expression in total DP cells but also in DO11.10 transgenic DP cells undergoing positive selection towards the CD4 lineage.

Finally, we found that enforced Gata3 expression in DO11.10 transgenic DP cells undergoing positive selection towards the CD4 lineage was associated with differential expression of 336 genes (85 + 251, clusters A and E), when compared with DO11.10 single transgenic DP cells. Importantly, only cluster E contained genes that were upregulated or downregulated by transgenic Gata3 expression in DP cells exclusively in the context of positive selection towards the CD4 lineage, but not in the heterogeneous total DP.
Fig. 2. Enforced Gata3 expression does not enhance ThPOK expression in DP or CD4 SP thymocytes. (A and B) Quantitative RT-PCR analysis of Gata3 and ThPOK expression in sorted DP thymocytes (A) or CD4 SP thymocytes (B) from wild-type (wt, white bars), CD2-GATA3 transgenic (G3, black bars), DO11.10 TCR transgenic (DO, white bars) and CD2-GATA3:DO11.10 double transgenic (G3:DO, black bars) mice. Expression was normalized to Gapdh and expression levels of Gata3 or ThPOK in wild-type DP (A) or CD4 SP (B) thymocytes were set to one. Average values and SEM are given for 4–10 mice per group (*p < 0.05; **p < 0.01; ***p < 0.001; t-test). (C) Quantitative RT-PCR analysis of ThPOK expression in the indicated sorted CD4 SP or CD8 SP thymocyte subpopulations from CD2-GATA3 transgenic (G3, white bar) and CD2-GATA3 transgenic Lck-Cre Gata3<sup>−/−</sup> mice (G3: Gata3<sup>−/−</sup>). Expression was normalized to Gapdh and ThPOK expression levels in wild-type DP were set to one.

Fig. 3. Identification of specific gene expression signatures in DP cells as a result of enforced GATA3 expression. Gene expression profiles were generated from FACS-sorted DP cells from wild-type (wt), CD2-GATA3 transgenic (G3), DO11.10 TCR transgenic (DO) and CD2-GATA3:DO11.10 double transgenic (G3:DO) mice. (A) Non-supervised hierarchical clustering of both genes (rows) and individual mice (columns). Gene expression profiles were analyzed for 3 individual mice per group. (B) Non-supervised hierarchical clustering of both genes (rows) and grouped (n = 3) mice of the indicated genotype. Dendograms above and on the left side of the matrices indicate the average linkage clustering of respectively individual mice and genes. Green and red colors indicate the level of downregulation or upregulation of genes, when compared with a pool of DP cells obtained from wild-type mice (n = 6). The color scale above the matrix correlates with gene expression and the given value numbers represent -log values. (C) Differentially expressed genes in DP thymocytes from CD2-GATA3 and DO11.10 single transgenic and CD2-GATA3:DO11.10 double transgenic mice, displayed in a Venn diagram. Genes are either up- or downregulated 1.4-fold (p < 0.0001) and are not differentially expressed in wild-type mice. Numbers of differentially expressed genes per cluster are given. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).
cell fractions. Therefore, cluster E is expected to contain putative GATA3 target genes associated with the crucial function of Gata3 in CD4 SP cell development.

Taken together, we have now identified three gene clusters of putative GATA3 targets: target genes in total DP cells that are (cluster B/C) or are not implicated in positive selection (cluster A/D), and target genes that are differentially expressed exclusively in the presence of a MHC class II-restricted positively selecting TCR signal (cluster E).

### 3.5. Putative GATA3 target genes that are not specifically implicated in positive selection

First, we focused on cluster A/D, which contains 136 genes that were induced or repressed by Gata3 in DP cells, irrespective of their cell fate, but were not implicated in positive selection (Table 1 and Suppl. Table S2). We found a striking ~18.5-fold upregulation of very low density lipoprotein receptor (Vldl-R), which is like apolipoprotein E (~2.7-fold upregulated) involved in lipoprotein metabolism (Takahashi et al., 2004). Together with the upregulation of multiple genes associated with structural cellular organization, including α-actinin 2, collagen alpha 2 (I) chain and myosin IF, this might be linked to the observed increased cell size of CD2-GATA3 DP thymocytes. We also found increased expression of c-Myc and its target cyclin D2, which we recently described as a putative Gata3 target in lymphomagenesis and cell size regulation (van Hamburg et al., 2008). In addition, we found induction of Ctla-4, which is a negative regulator of TCR-proximal signaling proteins, and the IL-18 receptor. Gata3 also induced significant expression of thrombospondin-2 (Thbs2), a member of the thrombospondin family of extracellular matrix proteins that regulate cell adhesion, growth, survival, differentiation and motility. Although Thbs receptors can elicit co-stimulating signals, Thbs1 has been shown to inhibit TCR signaling and CD69 induction (Li et al., 2001). Thbs receptor ligation on human T cells induced the expression of Ctla-4, OX40, GITR, and Foxp3, associated with regulatory T cell function.

### Table 1

Selection of putative GATA3 target genes.

<table>
<thead>
<tr>
<th>Upregulated genes</th>
<th>G3</th>
<th>DO</th>
<th>G3:DO</th>
<th>Down regulated genes</th>
<th>G3</th>
<th>DO</th>
<th>G3:DO</th>
</tr>
</thead>
<tbody>
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<td></td>
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<tr>
<td>Very low-density lipoprotein receptor (VldlR)</td>
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<td>Matrix metalloproteinase 14</td>
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<tr>
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<td>8.6</td>
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<td>9.2</td>
<td>T-cell surface glycoprotein CD5</td>
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<td>T cell transduction molecule SAP</td>
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<td>1.1</td>
<td>3.9</td>
<td>C-X-C chemokine receptor type 4 (Cxc4)</td>
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<td>C-ETS-2 protein</td>
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<td>Interleukin-12 receptor beta-1 chain</td>
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<tr>
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<td>1.3</td>
<td>2.9</td>
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<td>1.4</td>
<td>3.2</td>
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<td>1.7</td>
<td>2.1</td>
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<td>1.4</td>
<td>3.1</td>
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<td>2.3</td>
<td>2.8</td>
<td>V(DJ) recombination activating protein 2 (Rag2)</td>
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<td>2.7</td>
<td>2.5</td>
</tr>
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<td>2.9</td>
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<td>2.2</td>
<td>3.7</td>
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<td>1.3</td>
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<td>C-ETS-2 protein</td>
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<td>3.2</td>
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</tbody>
</table>

G3 = CD2-GATA3, DO = DO11.10, G3:DO = CD2-GATA3:DO11.10

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a Genes sorted by G3,
b Genes sorted by G3:DO.
Fig. 4. Expression analysis of genes upregulated as a result of enforced Gata3 expression in DP thymocytes. (A) Quantitative RT-PCR analysis of GATA3, c-Myc and Ctlα-4 expression in sorted DP cells from wild-type (wt), CD2-GATA3 (G3), DO11.10 (DO) and CD2-GATA3:DO11.10 double transgenic (G3:DO) mice. Expression was normalized to Gapdh and expression levels of c-Myc and Ctlα-4 in wild-type DP thymocytes were set to one. Data are from 4 to 10 mice per group. (B) Semi-quantitative PCR analysis for IL-18R, Granzyme A, Rorα, VldLR and β-actin on serial 3-fold cDNA dilutions prepared from sorted DP cells of the indicated mice. Data are representative for 4 mice analyzed per group. (C and D) Quantitative RT-PCR analysis of the indicated transcripts in sorted DP cells from DO11.10 (DO) and CD2-GATA3:DO11.10 (G3:DO) transgenic mice. Expression was normalized to Gapdh and expression levels in wild-type DP thymocytes were set to one. Average values ± SEM are displayed for 5–8 mice per group (*p < 0.05; **p < 0.01; ***p < 0.001; t-test).

(Grimbert et al., 2006). Thbs receptors include various integrins, CD36, CD47, low density lipoprotein receptor-related protein and heparan sulfate proteoglycans, some of which are also expressed on DP cells. Therefore, the induction of Ctlα-4 and TSP2 on DP thymocytes would allow the formation of cell-autonomous regulatory loops that dampen cellular activation of DP thymocytes by TCR-mediated signaling. As quantitative RT-PCR showed that enforced Gata3 expression is also associated with high Thbs2 transcript levels in DO11.10 Tg mice (Fig. 4D), we conclude that Thbs2 is also induced during CD4 lineage differentiation.

Interestingly, we noticed a ~2-fold down-regulation of the transcription factors Tcf1 and Lef1 (Table 1 and Suppl. Table 2), both of which are regulated by the Wnt/β-catenin signaling pathway and have been implicated in a subset of peripheral T cell lymphomas (Dorfman et al., 2003; Weerkamp et al., 2006). A ~2-fold down-regulation was also observed for CD5, a negative regulator of TCR signaling, which we recently reported as a putative direct or indirect Gata3 target gene (Ling et al., 2007). Furthermore, Gata3 expression downregulated ~1.8-fold the SLAM-associated protein SAP, which is involved in TCR signaling.

Taken together, this cluster of putative Gata3 targets mainly consists of genes implicated in lipoprotein metabolism, cell size regulation and lymphomagenesis, including VldLR, c-Myc and cyclin D2. In addition, we identified target genes that affect TCR-mediated signaling, including Ctlα-4, Thbs2, CD5 and SAP. RT-PCR analyses for Thbs2 and flow cytometric studies for CD5 (Ling et al., 2007) showed that Gata3 regulates expression of these genes also in thymocytes that are positively selected for CD4 lineage differentiation.
3.6. Putative GATA3 target genes that are implicated in positive selection

Cluster B/C contained 140 genes that were differentially expressed in DP cells in the presence of CD2-GATA3 transgene, irrespective of their cell fate, that were also induced or repressed in DO11.10 TCR transgenic DP cells (Table 1 and Suppl. Table S3). In this group, we surprisingly found upregulation of granzyme A, which is expressed in CD8+ T cells and NK cells and Rorγ, which was recently implicated in Th17 cell differentiation (Yang et al., 2008). Differential expression of granzyme A and Rorγ was confirmed by semi-quantitative PCR, by which also upregulation of the cluster A/D genes Vldl-R and IL-18R could be verified (Fig. 4A and B). The relevance of Rorγ upregulation is not clear, because lymphocyte-specific Rorα-deficient mice do not display defects in thymocyte development (Dzhagulov et al., 2004).

In addition, IL-12Rβ1 and STAT4 involved in cytokine signaling, and CD44, Lmo4, and Flt3-ligand, were induced (Table 1 and Suppl. Table S3). Using quantitative RT-PCR, we could confirm that Flt3-ligand expression is induced in DP cells overexpressing Gata3 (Fig. 4D). Transcriptional regulators, such as Sp3 and Id3 were ∼2-fold down-regulated (Table 1 and Suppl. Table S3). The transcription factor Id3 has been shown to be an important regulator for thymocyte selection, via the regulation of E2A (Rivera et al., 2000). This in combination with our finding in CD2-GATA3 transgenic DP cells of increased expression of E2A and reduced expression of CD5 (Hendriks et al., 1999; Hernandez-Hoyos et al., 2003; Ling et al., 2007), might indicate a role of Gata3 in downregulating CD5 via Id3 during thymocyte selection. Finally, we observed that Gata3 overexpression was associated with downregulation of Notch1 transcription. This would suggest a negative feedback mechanism regulating Notch1 expression, given the fact that Gata3 was recently shown to be a direct target of Notch1 during T helper cell differentiation (Amsen et al., 2007; Fang et al., 2007).

We conclude that the B/C cluster consists of a very diverse set of genes most of which cannot obviously be linked to induction of positive selection towards the CD4 lineage, except for Id3 or Notch1. These findings may be consistent with the notion that GATA3 expression is insufficient to induce positive selection towards the CD4 cell lineage (Nawijn et al., 2001b; Pai et al., 2003).

3.7. Putative GATA3 target genes in the presence of a MHC class II-restricted TCR signal

Cluster E contained genes that were upregulated or downregulated by transgenic Gata3 expression in DP cells exclusively in the context of positive selection towards the CD4 lineage, but not in total DP cell fractions. Importantly, transgenic Gata3 expression induces enhanced differentiation of DP cells, as evidenced by high surface TCR and CD69 expression levels of CD2-GATA3 transgenic DP cells, which were close to those normally reached only at the CD4 SP stage (Ling et al., 2007). Therefore, cluster E was expected to contain natural Gata3 targets associated with its crucial function in CD4 SP cell development.

Within cluster E, 118 genes were upregulated and 133 were downregulated as a result of enforced Gata3 expression (Table 1 and Suppl. Table S4). Among the upregulated genes, Mad4 a suppressor of c-Myc (Hurlin et al., 1996), G protein G-α13 and CD62L were present. Upregulation of CD62L transcription would be in agreement with the previously reported increased surface expression of CD62L on CD2-GATA3 transgenic CD4 SP cells (Nawijn et al., 2001a). Enforced Gata3 expression was associated with reduced activity of the V(D)J recombination machinery, as recombination activating gene-1 (Rag1), Rag2 and terminal deoxynucleotidyltransferase (TdT) were ∼2–3-fold downregulated (Table 1 and Suppl. Table S4). Reduced gene expression of Rag1, Rag2 and TdT was verified by quantitative RT-PCR analysis (Fig. 4C). In the promoter of the Rag2 gene a Gata3 binding site has been identified, mutation of which resulted in the reduction of promoter activity in T cells, indicating a direct regulation of V(D)J recombination by Gata3 (Kishi et al., 2000). Thus, our findings indicate that the induction of Gata3 upon TCR signaling in DP cells results in termination of V(D)J recombination activity, precluding further TCRα gene rearrangement. Interestingly, overexpression of Gata3 in early DN fetal thymocytes also caused downregulation of Rag1 and Rag2 (Anderson et al., 2002). Because Gata3 expression is upregulated in DN3 cells that have undergone β-selection (Hendriks et al., 1999), in the context of downregulation of the V(D)J recombination system Gata3 has parallel functions in DN3 cells where it is induced by pre-TCR signaling and in DP cells where it is induced by TCR signaling.

Enforced Gata3 expression resulted in reduced expression of B cell translocation gene 1 (Btg1) and CCR4-associated factor 1 (Caf1). Btg1 and Caf1 have been reported to act together in negative regulation of the gene for estrogen receptor α (ERα) (Prevot et al., 2001). Using quantitative RT-PCR, we could confirm that Btg1 expression is reduced in DP cells overexpressing Gata3 (Fig. 4D). This is particularly interesting, since Gata3 has been shown to positively regulate the expression of estrogen receptor alpha in breast cancer, by binding to the ERα gene itself (Eeckhoute et al., 2007). These data suggest that Gata3 can regulate ERs in a direct manner, but as well indirectly via Btg1.

Gata3 expression resulted in downregulation of genes involved in cellular signaling pathways such as, mitogen activated protein kinase4-2 (Mapk4-2), signal transducer and activator of transcription 5b (Stat5b), phosphatidylinositol-4-phosphate 5-kinase (Pip4PS5k1), and extracellular signal regulated kinase 3 (Erk3). In addition, the tyrosine protein kinases Tec and Itk, which are involved in proximal TCR signaling, were ∼2–3-fold downregulated. Also secreted modular calcium-binding protein-1, sarcomplasmic/endoplasmic reticulum calcium ATPase-3 and calpain L3, which are related to calcium signaling, were reduced. Secreted modular calcium-binding protein-1 was even reduced by a factor ∼9.

Enforced GATA3 expression also resulted in downregulated expression of genes encoding for cell surface molecules, such as MHC class I, IL-21R and CD4. Reduction of CD4 expression is of particular interest, because DP cells downregulate the expression of the CD4 and CD8 to become “double dull” thymocytes co-receptors when positive selection is initiated (Lucas and Germain, 1996). Although we did not detect a significant reduction in cell surface protein expression of CD4 by flow cytometric analysis of Gata3 transgenic DP cells, we did observe reduced CD4 expression in in vitro cultured CD2-GATA3 transgenic T helper-2 (Th2) cells, when compared to wild-type Th2 cells (data not shown). At the level of transcription, enforced Gata3 expression resulted in reduced expression of the nucleus class II transactivator (CIITA), p300/CBP and nuclear factor of activated T cells cytoplasmic 1 (Nfatc1).

3.8. Overlapping gene expression signature of CD2-GATA3 DP cells and lymphomas

When comparing the gene expression profiles of nontransformed CD2-GATA3 transgenic DP cells with those from thymic lymphoma samples obtained from CD2-GATA3 transgenic mice (van Hamburg et al., 2008b), we noticed striking parallels. Also in the DP T cell lymphomas we observed downregulation of Rag1 and Rag2, the cell surface molecules CD4, CD5 and IL-21R, TCR signaling molecules Itk, Tec and Sap, as well as the transcription regulators Stat5b and Nfat5. Moreover Ets2, Tcf1, Il-16, Cxcr4, were found to be downregulated in both non-malignant DP thymocytes as in Gata3-induced lymphoma cells. Among the upregulated
genes a similar expression profile in non-malignant DP cells and lymphoma cells was found of c-Myc, cyclin D2, transferrin, Apo-E, spermidine synthase and granzyme A.

4. Conclusions

In this report, we have shown that Gata3 overexpression does not affect Thpok expression levels in DP or CD4+ thymocytes, providing evidence that Gata3 does not directly regulate Thpok. Therefore, we propose that Gata3 is required during CD4+ lineage specification to reach the developmental stage at which expression of the CD4 commitment factor Thpok is induced. Our gene expression profiling studies allowed the identification of novel putative Gata3 target genes in DP thymocytes undergoing positive selection towards the CD4 lineage: Gata3 expression was associated with downregulation of the V(D)J-recombination machinery genes Rag1, Rag2 and Tdt, as well as various signaling molecules implicated in the transmission of TCR-mediated signals in DP thymocytes, including Tec, Itk, SAP, Pp450k-1, Mapk4-2, Erk3 and various molecules involved in calcium signaling. Gata3 overexpression was also associated with the induction of cIta-4 and thrombopon-2, which may act as modulators of TCR signaling in DP thymocytes. Together with our previous finding that Gata3 reduces expression of CD5, a negative regulator of TCR signaling, and upregulates TCR expression in a positive feedback loop (Ling et al., 2007), these findings indicate that Gata3 in DP cells mainly functions to (i) terminate TCR gene rearrangement and (ii) regulate TCR signal intensity or duration in cells undergoing positive selection towards the CD4 lineage. Validation of the identified targets in combination with functional experiments will provide improved insight into the essential physiological role of Gata3 during the developmental progression of DP cells that have received TCR-mediated positive selection signals into CD4 SP cells.

Acknowledgements

We would like to thank T. Schonewille, E. de Haas, K.W. Ling (Department of Immunology, Erasmus MC Rotterdam) and H. Diepstraten (Animal Care Facility, Erasmus MC Rotterdam) and Ron Kerkhoven and Mike Heimerink (Central Microarray Facility, The Netherlands Cancer Institute, Amsterdam) for assistance at various stages of the project. This work was partly supported by the Dutch Cancer Society, FCT Portugal and the International Association of Cancer Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.08.004.

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