Role of GATA Transcription Factors in the T Cell Lineage

Rol van GATA transcriptie factoren in T-cellen en hun ontwikkeling

ISBN: 978-90-73436-82-4

Cover:
Scanning electron microscopic image showing a detailed view of the characteristic shape and cellular surface of T lymphocytes. This picture was kindly provided by Prof. Dr. W. van Ewijk, University of Leiden, The Netherlands.

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
prof. dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 1 oktober 2008 om 13.45 uur

door

Jan Pieter van Hamburg

geboren te Oud-Beijerland

PROMOTIECOMMISSIE

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IMMUNOLOGY
ROTTERDAM

The studies described in this thesis were performed at the Department of Immunology, Erasmus MC, Rotterdam, The Netherlands.

The studies were financially supported by the Dutch Cancer Society (KWF: grant 2002-2699) and partly by the Association for International Cancer Research (AICR: grant 02-243).

The printing of the thesis was supported by the Dutch Cancer Society, Erasmus University Rotterdam and the Department of Immunology, Erasmus MC, Rotterdam.

Illustrations: T. van Os, R.W. Hendriks, K.W. Ling, J.P. van Hamburg
Printing: Ridderprint B.V., Ridderkerk
Cover: W. van Ewijk, T. van Os
Lay-out: M. IJdo-Reintjes, A.D. Korpershoek, W. Netten

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GATA transcription factors and T cell development and T cell function

Parts of this chapter have been published as a review in Stem Cell Research Developments

INTRODUCTION

T lymphocytes play a central role in the mammalian immune response against potentially hazardous pathogens, such as parasites, bacteria, viruses and fungi. These cells have the remarkable capacity to specifically recognize foreign substances, termed antigens, to which they respond by clonal amplification and cellular differentiation, conferring lifelong protective immunity to reinfection with the same pathogen. T lymphocytes express an antigen-specific receptor, called the T cell receptor (TCR), which recognizes peptide fragments derived from foreign proteins or pathogens that have entered into host cells. Defective T cell development and function can result in increased susceptibility to infections or even development of leukemias, allergies and autoimmune diseases. On the other hand, T lymphocytes can be manipulated to eradicate tumor and control graft rejection after organ transplantation. Therefore, in addition to biological interest, knowledge on T cell biology is important for understanding the etiology of a wide variety of diseases and potentially improves current therapies.

Virtually all T lymphocytes are produced in the thymus, a bi-lobed greyish organ in the upper chest above the heart. The thymus is seeded by precursor cells in the adult bone marrow, but the precise identity of these thymus-seeding cells is currently under debate [1-5]. It is clear that adult T cells are derived from hematopoietic stem cells (HSCs), which can differentiate into progenitors with a more restricted lineage potential and generate all the lympho/hematopoietic lineages via a cascade of commitment events [6]. During embryonic development, when the immune system is first established, progenitors with lymphoid potential are detectable before the first HSCs [7, 8]. Therefore, T cells may arise independent of HSC. Moreover, several embryonic sites harbor HSC activity and could thus be the origin of the first cells that seed the thymus [9-11]. Taken together, it is presently unclear how and where the first embryonic thymus-seeding cell is produced.

During early stages of thymocyte development, the TCR gene segments, which are inherited as large arrays of variable (V), diversity (D) and joining (J) gene segments, are irreversibly joined by DNA recombination to form a stretch of DNA, encoding a complete variable region. Developing thymocytes first rearrange the TCRβ and then the TCRα locus. After each TCR gene rearrangement, there is a stringent selection process to ensure that only cells expressing functionally relevant TCRs can survive and receive signals for further development. Although various molecules have been shown to be crucial for thymic T cell development [3], the molecular mechanisms guiding these selection processes are still quite unclear. In the peripheral lymphoid organs, early immune responses instruct naive T cells to differentiate into distinctive effector T cell subsets with specialized function. In mature T cell differentiation, cytokines provide crucial instructive signals leading to the generation of various T cell subsets, whereby the intracellular molecular pathways mediating the differentiation into one subtype and suppressing the others are currently being unraveled [12-14].

The differentiation of HSCs to T cells in the thymus, as well as the generation of effector T cells in the periphery, are tightly regulated processes involving interaction of

several signaling molecules, which result in expression of lineage-specific transcription factors. Such transcription factors define and restrict the developmental potential and function of the cells in which they are expressed. Interestingly, most of these transcription factors are critical at multiple stages during T cell development and their function can be different depending on the developmental stage. The family of GATA binding transcription factors is one out of many multigene families that are essential for the development of the lympho/hematopoietic system at multiple stages.

While the function of GATA1 in the development of the hematopoietic system has been reviewed by Ferreira *et al*., 15, the focus in this introduction will be on GATA2 and GATA3. GATA2 is essential for the production of embryonic hematopoietic stem and progenitor cells in a cell-autonomous fashion [16, 17]. In contrast, the role of GATA3 during in the lympho/hematopoietic system in the embryo is less clear. Expression of GATA3 is associated with HSC, but this factor may regulate the microenvironment of the developing embryonic lympho/hematopoietic system [18]. In the adult, GATA2 activity is required for HSC function [19]. Functional data on the role of GATA3 in bone marrow HSC and progenitors with T lineage potential are still lacking. Analyses in *Gata3*-LacZ knock-in mice [20], show that GATA3 expression marks a previously undescribed population that is present both in the bone marrow and the thymus, which may contain the potential thymus seeding cells. Within the thymus, GATA2 and GATA3 are required for the inhibition of the progenitor cells into the myeloid lineage, while GATA3 is necessary for the generation of all thymic T cells [20-23]. GATA3 is also required for the two developmental checkpoints after TCRβ and TCRα chain gene rearrangement [24]. Interestingly, there is a specific requirement of GATA3 for CD4 T cell development within the thymus and the subsequent differentiation into Th2 cells in the peripheral lymphoid organs [24, 25]. Recently identified upstream and downstream pathways linking these two GATA proteins to the other signal molecules and their targets will be described in this chapter.

The GATA protein family

All GATA family transcription regulators contain C4 zinc finger motifs with the characteristic Cys-X_2-Cys-X_{17-18}-Cys-X_2-Cys sequence, which bind to a six-nucleotide consensus sequence (A/T)GATA(A/G) [26, 27]. GATA proteins are present in a wide range of organisms from slime molds to vertebrates [28]. Although, the overall homologies for individual members are high between species and between different members of the same species [29, 30], sequence homologies outside the zinc finger regions are low among species. Therefore, unlike other evolutionarily conserved protein families, GATA transcription factors represent a family of proteins that are solely related to each other by their homologous zinc finger DNA binding domains. The founding member of the GATA family, GATA1 (also known as Ery-1, NF-E1, NF-1, and GF-1) was originally cloned as an erythroid nuclear protein [31]. GATA2 and GATA3 were subsequently discovered through cross-hybridization in a chicken reticulocyte cDNA library [29]. In total, six mammalian GATA transcription factors have been isolated, all containing two zinc fingers. The GATA factors show overlapping, but distinctive expression patterns. GATA1, -2 and -3 are predominantly expressed in

the hematopoietic system and are often collectively referred to as the hematopoietic subfamily. In contrast, GATA4, 5 and 6 have been implicated in the gene expression and cellular differentiation in a variety of embryonic tissues, including the heart, lung, gastro-intestinal epithelium, testis and ovaries [32, 33].

GATA1 and GATA2 expression patterns overlap substantially, because they are both expressed in erythroid cells, megakaryocytes and mast cells [34-38]. In addition, GATA1 is expressed in eosinophils [34], while GATA2 is expressed in immature hematopoietic multipotent cells [38] (Figure 1). GATA2 is also expressed in embryonic brain, inner ear, endothelial cells, urogenital organs, liver, adipose tissue and cardiac muscle.

Alternative promoters regulate transcription of the GATA2 gene: transcription initiates from two distinct first exons, both of which encode entirely untranslated regions, while the remaining five exons are shared by each of the two divergent mRNAs. The proximal first exon is utilized in general tissues, while in hematopoietic progenitor cells transcription is initiated at the distal first exon [39, 40]. Differential use of two exons, which was first described for the GATA1 gene, is also found for the GATA3 locus, which was shown to contain a brain-specific and a thymus-specific promoter in mouse and human [41]. The GATA3 expression pattern is quite unique: it is abundantly expressed in the developing nervous system, including the inner ear and different parts of the auditory nervous system, in hair follicles, in the adrenal gland and in the kidney [42-48], but within the hematopoietic system expression appears to be confined to the T, NK and invariant NKT cell lineages [20, 29, 48-52]. GATA factors are essential for the early development of the lympho/hematopoietic system at the embryonic stage. All *GATA* null mutations cause embryonic lethality [17, 35, 43].

The biochemical properties of GATA2 to GATA6 are less well characterized than those of GATA1. GATA1 protein contains at least 3 functional domains: the N-terminal activation domain, the N-terminal zinc finger and the C-terminal zinc finger (Figure 2). Initial studies showed that the C-terminal zinc finger is essential for GATA1 function, because it recognizes the GATA consensus sequence and therefore is responsible for DNA binding. Nevertheless, also the N-terminal zinc finger contributes to stable and specific DNA binding [27, 53-56]. Later *in vivo* transgenic rescue experiments confirmed that all these 3 domains can function collaboratively as well as independently of each other [57]. While similar *in vivo* analyses with respect to the functional domains of GATA2 and GATA3 are lacking, *in vitro* studies have revealed a comparable activation domain in the GATA3 N-terminus [58]. Intriguingly, the N-terminal domain of GATA2 was shown to be inhibitory [59]. The N-terminal zinc fingers of GATA2 and GATA3 are capable of strong independent binding with a preference for the GATC motif [60]. Even expression of GATA4 in the absence of GATA3 is competent to support development of CD4 single positive (SP) T cells. However, GATA4 cannot fully compensate for GATA3 in the induction of Th2 cytokines. More specifically, converting a proline residue in a partially conserved region C-terminal to the second zinc-finger of GATA4, to a methionine residue, which was present in GATA3, was sufficient to induce the expression of IL-13 by GATA4 [61]. This indicates that both a specific cell type dependent expression pattern and protein structure are required for GATA3 to exerts its function.

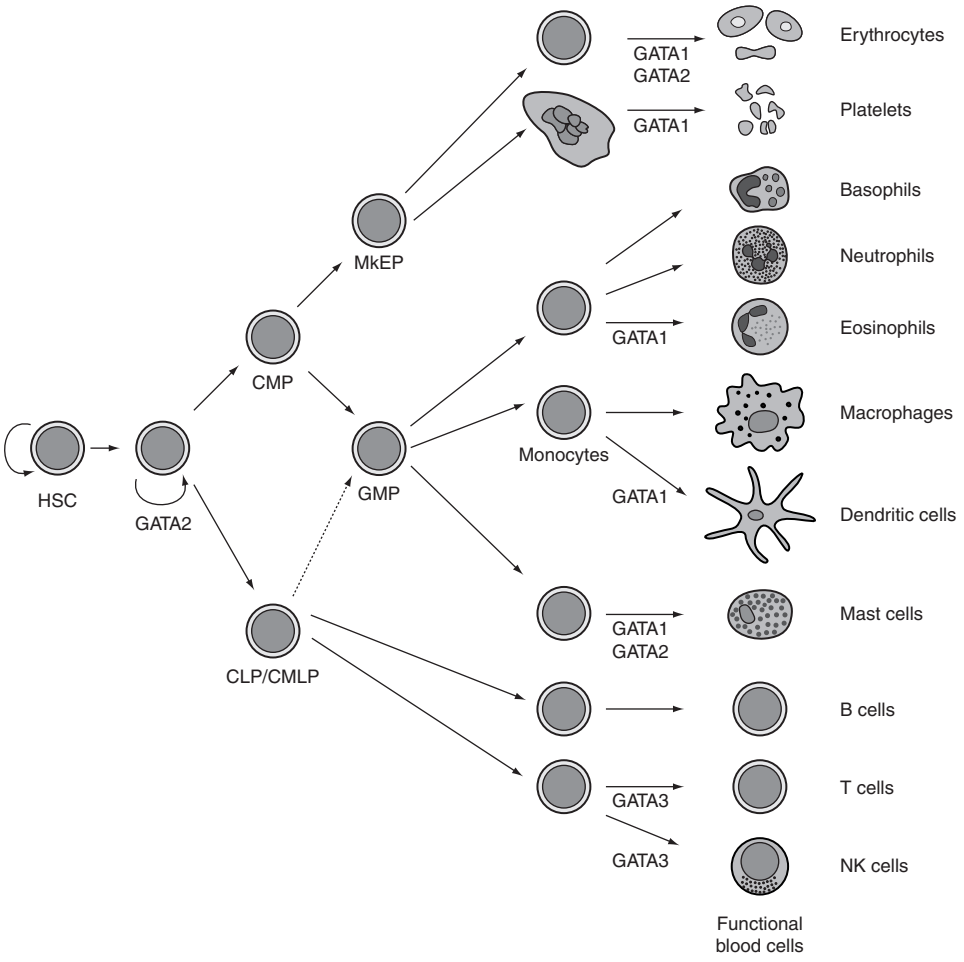

Figure 1. Schematic overview indicating the requirement of GATA transcription factors during hematopoiesis. This overview shows the main lineage commitment steps from HSC to functional blood cells during hematopoiesis. GATA transcription factors relevant for the development of specific commitment steps during hematopoiesis are indicated. MLP = multilineage progenitor, CLP = common lymphoid progenitor, CMP = common myeloid progenitor, CMLP, common myeloid lymphoid progenitor, MkEP = megakaryocytic/erythroid progenitor and GMP = granulocyte/monocyte progenitor. Adapted from Ferreira *et al*., 15.

This further implies that minor differences within the three functional domains of the GATA proteins collectively distinguish their specific roles in the tissues in which they are expressed.

While it is clear that GATA1 activity is subject to various forms of regulation on the protein level by post-translational modifications and protein degradation (reviewed

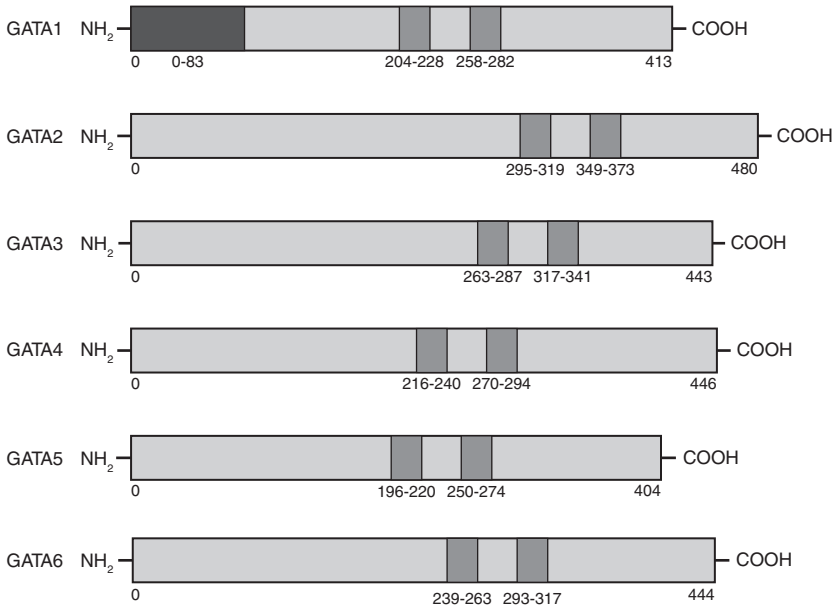

Figure 2. Schematic representation of the murine family of GATA transcription factors. The N-terminal activation domain (black) of GATA1 and the N-terminal and C-terminal zinc finger domains (dark grey) of the individual GATA transcription factors are indicated. Numbers indicate the corresponding amino acids of the various GATA protein domains (source: www.uniprot.org).

by Ferreira *et al.,* 15), knowledge on regulation of GATA2 and GATA3 activity are relatively limited. As in GATA1, acetylation of GATA2 and GATA3 affect both their DNA binding activities and physiological functions [15, 62, 63]. The stability of the GATA1 and GATA2 proteins is affected by caspase-mediated and ubiquitin-proteasome degradation pathways [64, 65]. Likewise, it was recently shown that stabilization of GATA3 is regulated by the polycomb group protein Bmi1. Biochemical studies indicated that in effector T cells Bmi1 binds to GATA3, whereby this interaction is dependent on the Ring finger of Bmi1 [66]. Overexpression of Bmi1 resulted in decreased ubiquitination and increased GATA3 protein stability. In *Bmi1*-deficient helper T cells, the levels of Th2 cell differentiation decreased as the degradation and ubiquitination of GATA3 increased. Yet, there is so far no report on the regulation of GATA3 turnover in developing thymocytes via similar mechanisms. Nevertheless, the identified co-expression of GATA2, GATA3 and Bmi1 in HSCs [67] would allow for a similar regulatory pathway in HSCs. In addition, the Fog1 (friend of GATA1) multitype zinc finger protein, which was originally identified as a cofactor for transcription factor GATA1 in erythroid and megakaryocytic differentiation [68], can repress GATA3-dependent activation of the IL-4 and IL-5 promoters in activated T cells [69, 70]. Another GATA3 interacting protein, termed repressor of GATA (ROG) represses GATA3-induced transactivation [71].

Recently, GATA1-containing complexes were identified by an *in vivo* biotinylation tagging, purification by streptavidin beads and subsequent mass spectometry

approach [72]. In addition to known GATA1 interacting factors, such as Fog1, T cell acute leukemia 1 (Tal1) and LIM domain binding 1 (Ldb1), new partners were identified including the essential hematopoietic protein growth factor independent 1b (Gfi-1b) and the chromatin remodeling and modification complexes MeCP1 and ACF/WCRF. It was concluded that GATA1 forms several distinct complexes, whereby Fog1 serves as a bridging factor between GATA1 and the methyl-DNA binding protein MeCP1 complex. Evidence was presented for the *in vivo* binding of the repressive GATA1/Fog1/MeCP1 complex to silenced hematopoietic genes in erythroid cells and of the activating GATA1/Tal1 complex to erythroid-specific genes [72]. It is expected that similar approaches will be applied to identify GATA2 and GATA3 protein complexes.

The GATA target motif appears in a variety of regulatory contexts throughout the genome. GATA3 for example, binds to the promoter regions immediately upstream of the transcription start-point, as well as in enhancers of the Th2 cytokine locus (reviewed by Lee *et al*., 13). Therefore, GATA proteins can function as classical transcription factors, or by modifying chromatin structure and thereby facilitating interactions among enhancers, promoters and factors associated with the basal transcription machinery. The result can be activation or inhibition of the expression of target genes. Different GATA proteins can compete for their target sites, leading to a switching of associated DNA binding proteins and therefore different effects on their target genes. There is also accumulating evidence showing that GATA proteins have the capacity to regulate their own expression and cross-regulate the expression of each other [73-76]. The presence of autoregulation and network regulation between the individual GATA family members appears to be essential. The outcome of GATA transcription factor regulation is highly complex, unpredictable and often cell-context and expression-level dependent.

Expression of GATA factors during embryonic lympho/hematopoiesis

During embryonic development the lympho/hematopoietic system is generated from HSCs in the fetal liver. Shortly after birth, this function is replaced by the bone marrow. It is unclear how the initial immune system is established before lympho/hematopoiesis begins in the fetal liver. Owing to the differences in functional requirements, the embryonic lympho/hematopoietic system is different from the adult. For example, HSCs in fetal liver have greater repopulating capacity than HSCs derived from adult bone marrow in irradiated recipients [77]. Primitive embryonic erythrocytes and macrophages are different from the definitive types found in the adult. Embryonic immunoglobulin and TCR repertoires are unique: only fetal liver HSCs can generate the innate-like $V\gamma5^+$ $\gamma\delta$ T cells (originally termed $V\gamma3^+$) in fetal thymuses, whereas adult bone marrow HSCs do not have this capacity. Likewise, $CD5^+$ B-1a B cells, which are predominantly localized in the peritoneum and pleural cavities, are readily generated from fetal/neonatal precursors, but inefficiently from precursors in the adult [78]. Furthermore, it has been shown that, whereas IL-7 is required for adult B cell development, it is dispensable during fetal hematopoiesis [79, 80].

Also in the embryo the thymus is the main site of T cell production, but the first lympho/hematopoietic progenitors seeding the thymus may arise from various

hematopoietic sites, including the extra-embryonic yolk sac (YS) and the intra-embryonic splanchnopleura (Sp), para-aortic splanopleura (P-Sp) or aorta-gonads-mesonephros (AGM) region (Figure 3). Influx of lympho/hematopoietic progenitors to the thymus begins at about embryonic day 10.5 (E10.5) [81, 82]. As early as E7, primitive erythroid cells can be observed in the YS blood islands [83]. Until recently, it was believed that the blood islands of the YS produce the cells that are required to establish the adult lympho/hematopoietic system that seeds the liver and the thymus. However, it has now become clear that lymphoid potential in the YS is limited and is not detectable beyond E8 [84]. Rather, the intraembryonic Sp tissue has both erythro/myeloid and lymphoid potential before E8 [7]. Although cells with long-term multi-lineage potential can be detected in both YS and P-Sp when transplanted into conditioned newborn recipients [85], adult HSCs that are able to provide long-term multi-lineage reconstitution in adult recipients are only detectable in the E10.5 P-Sp /AGM region and only later in the YS at E11 [86]. Progenitors with lymphoid potential detected before

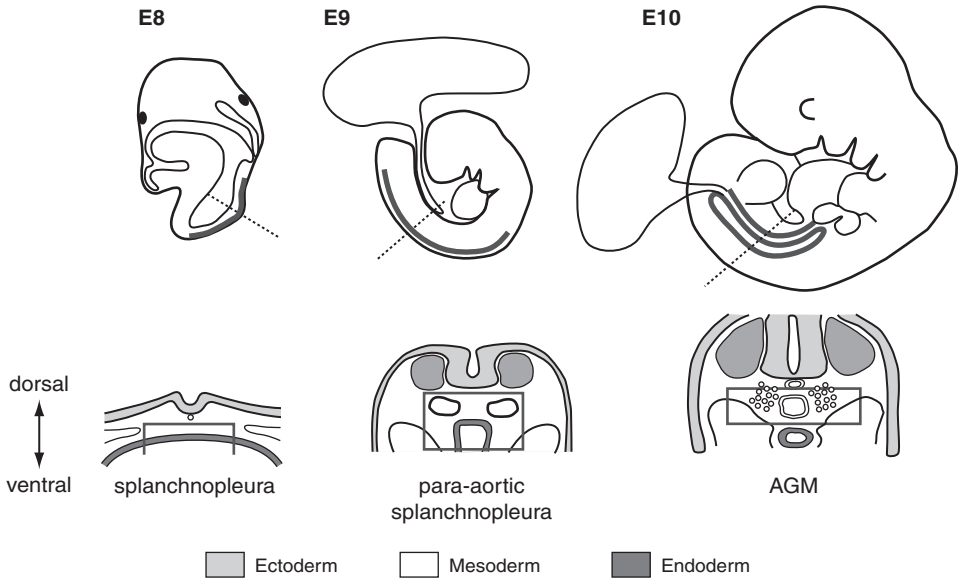

Figure 3. Location and development of the intra-embryonic sites with lympho/hematopoietic potential in the mouse embryo. The top panel shows a schematic representation of a whole mouse embryo at E8 (left), E9 (middle) and E10 (right). Cross sections marked by dotted lines of the corresponding stages are shown in the lower penal. Red boxes in the lower panel are the embryonic sites in which lympho/hematopoietic activity and progentors can be detected. At E8, splanchnopleura (Sp) is defined as the mesoderm that is associated with the endoderm. The dorsal aortae will develop from this region. At E9, when the paired aortae have developed, this embryonic region is defined as the para-aortic splanchnopleura (P-Sp). At E10, the paired aortae fuse, forming a single aorta and the development of the gonads and mesonephros become apparent. Collectively, this region is referred to as the aorta, gonads and mesonephros (AGM) region. (See Appendix page 215 for a full-color representation of this figure).

E10.5 in the AGM region and E11 in the YS could arise independently of HSC [8]. Thus, the developmental hierarchy of the embryonic lympho/hematopoietic system appears different from that of the adult. The molecular pathways required for the maintenance of the embryonic lympho/hematopoietic system may therefore also differ from that of the adult. This would imply that the lympho/hematopoietic progenitors detected at E10.5 in the thymus could be either derived from the YS or from the AGM region. At the moment, the relative contribution of these two embryonic sites is still unknown.

The expression patterns of GATA2 and GATA3 in the P-Sp/AGM region suggest that these factors have distinctive roles in the control of the generation of the lympho/hematopoietic system in the developing embryo. GATA2 is expressed in E7.5 lateral plate mesoderm, in E8.5 dorsal aorta and the surrounding mesenchyme, and in the YS blood islands [39, 59, 73, 87]. In a GATA2-GFP transgenic mouse line, in which the GFP expression pattern recapitulates the early embryonic GATA2 expression pattern, the GATA2-GFP expressing cell population in E9 YS and P-Sp contains a high proportion of c-kit$^+$CD34$^+$ cells [73]. The c-kit$^+$CD34$^+$ cells in these embryonic regions are enriched in neonatal repopulating HSCs [88]. Therefore, GATA2 possibly regulates the production of the cells that are required to generate the embryonic lympho/hematopoietic system. In contrast, GATA3 expression is undetectable in the YS (analyzed between E8 and E10) [18], but is present in the P-Sp/AGM region from E8 through E11. Although GATA3-positive cells are closely associated with hematopoietic molecular marker expressing cells, such as AA4.1, Lmo2 and CD45, they do not overlap [18]. GATA3 may therefore play a role in the determination of the embryonic stromal microenvironment for generation of the embryonic lympho/hematopoietic system. Interestingly, cell clusters within the E10.5 splenic mesoderm, closely associated with the aorta epithelium in the AGM region, express both GATA2 and GATA3 [89]. These sub-aortic clusters have been described in all vertebrates and are potentially involved in HSC generation. The overlapping expression patterns in these clusters suggest that GATA2 and GATA3 may cross-regulate or work coordinately with each other for the generation of HSCs in the E10.5 AGM region.

GATA3 and embryonic HSC

GATA3$^{-/-}$ embryos die between E11 and E12 and display massive internal bleeding, marked growth retardation, severe malformation of the brain and spinal cord, and gross aberrations in fetal liver hematopoiesis. YS hematopoiesis appears to be normal, but fetal liver shows a mild reduction in hematopoietic progenitors obtained *in vitro*, suggesting that definitive hematopoiesis is GATA3-dependent [43]. Subsequent *Rag2*$^{-/-}$ complementation analyses showed the intrinsic requirement of GATA3 for T cell production [20, 21], but the effects of GATA3 deficiency on the microenvironment of the embryonic hematopoietic system has remained unexplored. Also HSC production and lymphoid potential of the *GATA3*$^{-/-}$ AGM and P-Sp have not been analyzed to date.

GATA3$^{-/-}$ embryonic lethality is primarily due to noradrenalin deficiency of the sympathetic nervous system and secondarily due to heart failure [90]. This discovery

was based on a previous finding that a 625-kb GATA3 YAC transgene, mimicking endogenous GATA3 expression except in thymus and the sympatho-adrenal system, failed to overcome embryonic lethality. The hypothesis that a neuro-endocrine deficiency in the sympathetic nervous system might cause the embryonic lethality of GATA3 deficient mice was tested by feeding catechol intermediates to pregnant mice, which partially averted GATA3 mutation-induced lethality [90]. This pharmacological rescue raised the possibility to further study the development of the lympho/hematopoietic system using rescued *GATA3*$^{-/-}$ embryos at a later developmental stage. The rescued *GATA3*$^{-/-}$ thymuses were small, which could be either due to an intrinsic role of GATA3 directly on the development of the thymic epithelium or to the lack of lympho/hematopoietic progenitors seeding the thymus. It is clear that more analyses on the *GATA3*$^{-/-}$ mutants have to be done to get a better understanding of the role of GATA3 in the establishment of the embryonic lympho/hematopoietic system.

Control of GATA2 expression in embryonic HSCs

Given the network regulation between GATA factors, one possible downstream target of GATA3 could be the GATA2 gene. Six GATA factor binding sites are present upstream of the GATA2 locus, and both GATA1, -2 and -3 are able to bind to these GATA sites [73]. Only five out of these six GATA binding sites are necessary for the expression of GATA2 in the YS and the major arteries, while only one is essential for the expression in the dorsal aorta. Thus, GATA2 expression in the embryonic hematopoietic sites is GATA factor-dependent and the expression in different sites is differentially regulated. However, GATA2-GFP expression is maintained in both GATA2 and GATA3 deletion mutants. Collectively, these data suggest that GATA2 expression in the P-Sp is GATA factor dependent and GATA2 and GATA3 may compensate each other, at least for the expression of GATA2 [73].

GATA2 expression in the P-Sp/AGM region is also regulated by the Notch (neurogenic locus notch homolog) signaling pathway [91]. Molecules necessary to transduce the Notch signal (the receptors Notch-1 and -4, the ligands Delta-like-1 (Dll-1, -3 and -4) and Jagged-1 and -2) as well as Notch target genes, such as the Hes related proteins Hrt-1 and -2) and bone morphogenetic protein 4 (Bmp4) are expressed in the endothelium of the P-Sp/AGM region. Expression of these genes was found in particular in the hematopoietic clusters on the ventral side of the dorsal aorta [91, 92]. This is the region where emerging HSCs are labeled in a recently developed transgenic mouse model [93] and where the HSC-associated transcription factors Aml1/Runx1, GATA2, Scl/Tal1 are expressed [16, 17, 94-96].

Notch1 null mutants have reduced c-kit^{+}CD34^{+} cell numbers and neonatal repopulating HSC activity [92]. In the *Notch1*$^{-/-}$ endothelium in the AGM region GATA2 and Bmp4 expression are reduced. As GATA2 is under the control of Bmp4 [97], Notch1 could regulate GATA2 indirectly through Bmp4 [92]. In addition, Rbp-jκ, a mediator of feedback regulation of Notch signals, binds to the promoter of GATA2. Moreover, GATA2 mRNA levels in the *Rbp-jκ*$^{-/-}$ P-Sp region are reduced [91]. Robert-Moreno *et al*., 91, have also shown that (i) in wild type (WT) embryos, Notch1 and GATA2 are co-expressed in cells lining the aorta endothelium at E9.5, (ii) Notch1 specifically

associates with the GATA2 promoter in E9.5 WT embryos and 32D myeloid cells by chromatin immunoprecipitation, and (iii) the Notch1/GATA2 interaction is lost in *Rbp-jκ*$^{-/-}$ mutants. Taken together, these data strongly suggest that activation of GATA2 expression by Notch1/Rbp-jκ is a crucial event in the generation of HSC in the developing embryo.

GATA2 and adult HSC function

Long-term (LT) repopulation efficiency of adult HSC is directly related to their cell cycle status and is highest in the G0 phase [98]. It is estimated that about 75% of LT-HSCs are normally in G0 phase, but these quiescent HSCs do regularly enter the cell cycle. It has been estimated that almost all LT-HSCs are recruited into the cell cycle on average every 57 days [98]. It is clear that the regulation of the cell cycle of HSCs plays a central role in HSC function, but the molecular mechanisms regulating HSC cell cycle entry are not defined.

Adult HSCs express high levels of GATA2 and when they are induced to proliferate *in vitro*, the GATA2 expression levels are down-regulated [99]. These observations suggest that the GATA2 level plays a role in the maintenance of HSC quiescence, as high GATA2 levels may block HSC cell cycle entry. The early embryonic lethality of *GATA2*$^{-/-}$ mutants precludes the direct analysis of the role of this gene in adult HSC function. Early *in vitro* studies involving overexpression of ligand-inducible GATA2 chimeric proteins in hematopoietic cell lines were contradictory [99-101]. Conditional activation of a GATA/estrogen receptor chimera produced essentially opposite effects to those observed with conditional, drug-inducible, GATA2 expression. GATA2 and GATA2/ER differ in their binding activities and transcriptional interactions [102]. Recently, it has been shown that this discrepancy is due to the interaction between GATA2 and PU.1. While transcription of the PU.1 gene is regulated by GATA2, the function of GATA2 is modified in a context-dependent manner by expression of PU.1 [103]. Nonetheless, overexpressing GATA2 in bone marrow cells blocks their engraftment to lethally irradiated recipients [104]. Engrafted cells did not die, differentiate nor expand, thus GATA2 expression appears crucial for the function of HSC. Because enforced expression of GATA2 in pluripotent hematopoietic cells blocked both their amplification and differentiation, Persons *et al*.,104, concluded that there is a critical dose-dependent effect of GATA2 on blood cell differentiation in that down-regulation of GATA2 expression is necessary for stem cells to contribute to hematopoiesis *in vivo* [104]. On the other hand, it was demonstrated, using mouse ES cells co-cultured on the stromal cell line OP9, that GATA2 increased the proliferation of immature hematopoietic cells [102]. Recently, GATA2 function was analyzed by combining *in vitro* ES cell differentiation with tetracycline-based conditional gene expression [103]. In this system, GATA2 expression inhibited macrophage differentiation and redirected the fate of hematopoietic differentiation to other lineages, including the megakaryocytic and erythroid lineages. In agreement with the previous finding that generation of mast cells requires co-operative functions of GATA2 and the transcription factor PU.1, also in these experiments, interaction between GATA2 and PU.1 appeared to play a critical role. The authors concluded that GATA2 function is modified in a context-dependent manner by expression of PU.1, which is in turn regulated by GATA2 [103].

Early thymic progenitors and lymphoid lineage commitment

The thymus is seeded via the blood, but the identity of thymus-seeding cells in the circulation of adult mice is unknown [1-4]. Likewise, there is no consensus on where and how lymphoid lineage commitment of these early thymic progenitors occurs. Because HSCs can be isolated from the circulation, they would have the capacity to seed the thymus directly [105]. In this case, T cell specification would be initiated shortly after HSCs enter the thymus. However, oligopotent progenitors with lymphoid developmental potential can be isolated from the bone marrow, suggesting lymphoid lineage specification can occur before they leave the bone marrow [106-109]. Recent data show the presence of bone marrow progenitors with robust lymphoid and limited myeloid potential, but lacking erythroid potential [109], suggesting that lymphoid commitment is not a simple single event but a gradual loss of developmental potential to other non-lymphoid lineages. Nonetheless, bone marrow HSC and various progenitors with different lineage developmental potential could seed the thymus and the significance of each individual population may depend on different physiological requirements.

HSC activities in the mouse bone marrow are restricted to the Lin$^-$Sca-1^{hi}c-kit^{hi} population [110], which is commonly referred to as the LSK population, whereby Lin$^-$ refers to the negative expression of several hematopoietic lineage markers. In particular, a single CD34$^{-/lo}$ cell (and in some mouse strains Thy-1.1^{lo}) within the LSK population can engraft a lethally irradiated recipient and regenerate the whole lympho/hematopoietic system [111, 112]. LSK CD34^{lo}Thy-1.1^{lo} cells are LT-HSCs with extensive self-renewal capacity, which can sustain lympho- and hematopoiesis for at least the lifespan of an animal. Upon commitment to differentiation, LT-HSCs progressively lose their self-renewal capacity and become short-term (ST)-HSCs that are still multipotent while their self-renewal capacity is transient [113]. The expression of the fms-like receptor kinase Flt3 (also known as Flk2), divides LSK cells into LT-HSC and ST-HSC with increasing expression of this receptor kinase, and so they are designated the Flt3- and Flt3$^{lo/-}$ subset of LSK cells, respectively (Figure 4) [114]. The first step of HSCs differentiation is therefore the progressive loss of self-renewal capacity, probably by changes in the responsiveness to the environment.

The first lineage-committed progenitors isolated from the bone marrow are the common lymphoid progenitors (CLPs), which were defined as Lin$^-$ IL-7Rα^+ Sca-1^{lo} c-kit^{lo} cells [108,115]. These cells have the potential to differentiate into B and dendritic cells *in vitro*, and also clonally into T and NK, cells but lack myeloid potential. Subsequently, an equivalent myeloid-lineage counterpart, the common myeloid progenitors (CMPs) were defined as Lin$^-$ IL7Rα^- Sca-1$^-$ c-kit^{hi}. These cells can give rise to granulocyte/macrophage/dendritic cell (G/M/DC) progenitors and megakaryocyte/erythroid (Meg/Ery) progenitors with exclusive G/M and Meg/Ery potential, respectively [116] (Figure 4). These early data suggest that the main branching of hematopoietic cell differentiation is the separation of lymphoid and myeloid lineages. Lacking all myeloid potential, CLP can possibly seed the thymus to produce all thymic T cells. Recently, an additional progenitor subset, defined as Flt3^{lo}VCAM1$^+$ LSK cells, which lacks erythroid but has potent lymphoid and G/M developmental potential was isolated in the bone marrow.

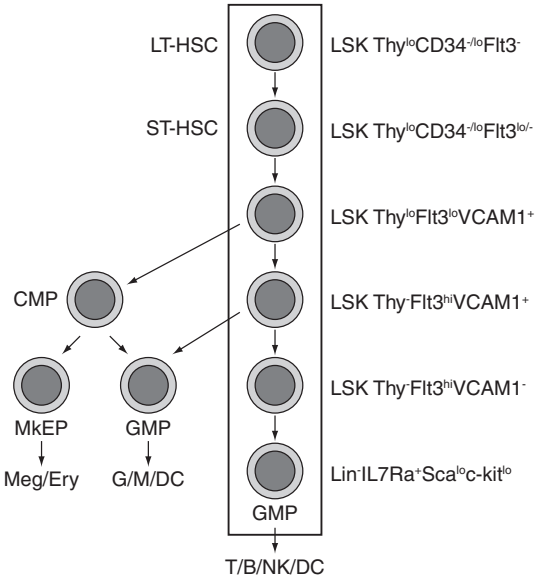

Figure 4. Proposed model of hierarchy in progenitor subset within the bone marrow. In this model, when HSCs differentiate into the lymphoid lineage, they progressively lose their self-renewal capacity and myeloid developmental potential. HSCs and progenitor cells that could possibly seed the thymus are marked in the white box. Only phenotypic markers of HSC and progenitor cells with lymphoid potential, which are the potential source of T cells in the thymus are noted. Adapted from Lai *et al.*,109.

These cells can give rise to Flt3^{hi}VCAM1^{+} LSK and subsequently Flt3^{hi}VCAM1^{-} LSK cells, with increased lymphoid potential, and reduced G/M/DC potential, but lacking Meg/Ery potential [109] (Figure 4). Therefore, bone marrow HSC may differentiate into the lymphoid lineage by sequentially and gradually losing the Meg/Ery and G/M/DC differentiation potential, before commitment to the lymphoid lineage. Any of the intermediate progenitors with limited and biased developmental potential could also be the source for thymic T cell development.

While direct lineage relationships between these various lymphoid progenitors and CLPs are lacking, thymopoiesis can carry on without detectable CLPs in the bone marrow of Ikaros-deficient mice [117]. In the same study performed by Allman *et al*., 117, the authors found that instead of the CLPs, c-Kit^{hi}Sca-1^{+}IL-7Rα$^{neg/lo}$ cells could give rise to the T cell lineage and therefore this subpopulation was named early thymic T lineage progenitors (ETPs). From this it was concluded that TPs were not derived from CLPs an that ETPs arise from a earlier BM progenitor pool, more closely related to HSC [117]. Most of the ETPs, in the thymus lacked B cell potential, but possessed myeloid potential [118]. This finding indicates that myeloid potential is not lost in ETPs, but that structural and molecular mechanisms taking place within the thymus are critical for T cell and myeloid development [118]. The presence of CLPs in the circulation remains controversial and physiological T lineage precursors may

not be present in circulation [119]. On the other hand, it is possible to isolate LSK cells from peripheral blood with efficient T lineage potential [105]. This would indicate that peripheral LSK cells, more closely related to HCSs are the principal progenitors that could seed the thymus even before lymphoid commitment is initiated.

GATA3 and T cell commitment

Flow cytometric and immunocytochemical analyses of fetal thymus cells in the mouse showed that GATA3 is already present in the most immature population of fetal thymus cells, which have the DN1 phenotype ($CD44^+CD25^-$ $CD4^-CD8^-$ double negative (DN) cells [119]. Antisense GATA3 oligonucleotides inhibited T cell development from fetal liver precursor cells in fetal thymic organ cultures, indicating the critical importance of GATA3 for T cell development [120]. Moreover, *Rag2*$^{-/-}$ complementation experiments *in vivo* demonstrated that the development of *GATA3*$^{-/-}$ embryonic stem cell-derived T cell precursors is arrested at or before the DN stage [21]. Yet, in such *GATA3*$^{-/-}$ *Rag2*$^{-/-}$ chimeric mice, the *GATA3*$^{-/-}$ cells did significantly contribute to non-hematopoietic tissues and to various hematopoietic lineages, including erythroid, myeloid en B cell lineages [21]. In chimeric mice generated by injections of GATA3 deficient ES cells (harboring an allele in which the GATA3 gene was targeted by insertion of a *lacZ* reporter) in WT blastocysts, we showed that *GATA3*$^{-/-}$ ES cells did not contribute to the T cell lineage, not even to the earliest subset of DN1 cells [20].

As (i) GATA3 is expressed within the hematopoietic system in a T cell specific fashion [20, 43, 49], and (ii) GATA3 is essential for proper development of early thymocytes, it is attractive to propose that GATA3 acts as the decisive T cell commitment factor, equivalent to Pax-5 in the B cell lineage [121, 122]. However, forced expression of GATA3 in hematopoietic precursors does not appear to enhance T specification. On the contrary, it was found that overexpression of GATA3 in HSCs resulted in cessation of cell expansion, followed by selective induction of megakaryocytic and erythroid differentiation and inhibition of myeloid and lymphoid precursor development [3, 123]. These findings indicate that in these experiments, GATA3 may mimic GATA1 function, suggesting functional redundancy among GATA proteins. Thus, induction of cell fate decisions by GATA proteins may well be regulated by their restricted expression patterns and not so much by the features of the individual GATA factors. Moreover, overexpression of GATA3 in developing thymocytes clearly has adverse effects and is associated with reduced cell survival [124-126] (Ling *et al*., unpublished).

Overexpression of GATA3 in early T lineage progenitors, depending on Notch signaling and developmental stage, can block the survival of these cells. Interestingly, when GATA3 is overexpressed at the DN1 or DN2 stage, but not the DN3 stage, mast cell differentiation is induced. This indicates that the timing and dosage of GATA3 expression is essential in early T cell development [127].

In support of a role for GATA3 as transcription factor important for T lineage identity, it was recently shown that GATA3 has the capacity to counteract C/EBPα-induced macrophage reprogramming of DN thymocytes cultured on OP9-Dll-1 stromal cells [23]. The restriction of multipotent precursors in the thymus is normally accompanied by a downregulation of the transcription factors C/EBPα and PU.1.

Retroviral transduction of C/EBPα in DN cells induced the formation of functional macrophages, but this reprogramming could be inhibited by expression of intracellular Notch or GATA3 [23]. A similar antagonism for C/EBPα and GATA3 has been described in adipocyte differentiation, whereby both GATA2 and GATA3 form protein complexes with C/EBPα and C/EBPβ [128]. In this context, it is intriguing that GATA3 was also shown to regulate the balance between hair follicle and epidermal cell fates by integrating various signaling networks including the Wnt, Notch and Bmp pathways [48]. Additional links with GATA3 and Notch include the finding that activation of the Notch1 pathway controls lineage commitment of early thymic precursors by altering the levels between GATA3 and Spi-B, which is an Ets family member transcription factor controlling plasmacytoid dendritic cell development [129]. Moreover, impairment of T lymphopoiesis in *GATA3*$^{-/-}$ hematopoietic progenitor cells was rescued only by introduction of both GATA3 and the intracellular region of Notch1 but not by either alone. This indicates that Notch signaling is necessary for the function of GATA3 and that Notch signaling alone is not sufficient for T cell fate specification [130]. Taken together, these findings indicate that GATA3 function is closely associated with T cell specification and development.

T cell differentiation in the thymus

Early T lineage restricted precursors express neither CD4 nor CD8 co-receptors and are therefore referred to as DN cells (Figure 5). Within the DN population, the CD44^{+}CD25^{-} DN1 subset contains the most immature progenitors originating from the bone marrow [117, 131]. DN1 cells develop into CD44^{+}CD25^{+} DN2 thymocytes, in which specification is initiated and commitment is completed [132]. When DN2 cells have differentiated into CD44^{-}CD25^{+} (DN3), the pro-T cells begin controlled locus-specific recombination of their TCRγ, -δ or -β genes, initiated by the Rag1 and Rag2 proteins [3]. Pro-T cells that successfully rearrange TCRγ and TCRδ will express a γδ TCR and are eligible to develop further as γδ T cells [133]. By contrast, cells that produce a functional TCR β chain, which associates with an invariant pTα chain to form a pre-TCR, are selected for further development, enter the cell cycle and differentiate into DN4 cells, a process referred to as β-selection [134-136]. Subsequently, they acquire both CD4 and CD8 co-receptors. In most mouse strains CD8 is expressed first, resulting in the generation of large cycling CD8 immature single positive (ISP) cells. In double positive (DP) thymocytes productive TCRα locus recombination results in the expression of the αβ TCR complex on the cell surface. After engagement of the αβ TCR by self-MHC peptide complexes, low- to intermediate-avidity interactions rescue DP thymocytes from death by neglect through a process termed positive selection [137-139]. By contrast, strong TCR signals resulting from interactions with endogenous peptides trigger apoptosis, which eliminates auto-reactive thymocytes, a process termed negative selection. Positive selection results in the differentiation of CD4 and CD8 SP cells, which express an αβ TCR that recognizes peptide antigens presented by MHC class II and class I molecules, respectively. Mature SP cells exit the thymus to circulate to the periphery as naive CD4 SP T helper (Th) cells and CD8 SP cytotoxic T cells.

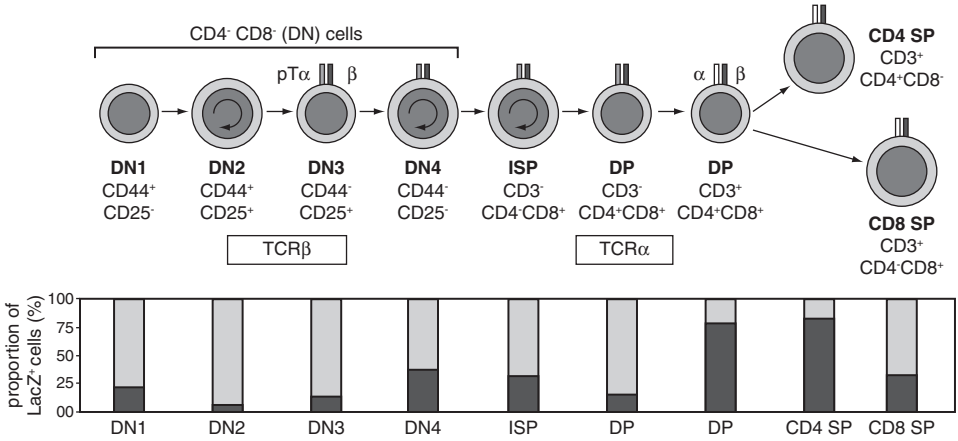

Figure 5. Schematic overview of T cell development and GATA3 expression. Developmental markers used to separate different developmental T cell subsets are shown in the upper panel. Proportion of *LacZ*+ cells (analyzed by flow cytometry using GATA3-*LacZ* knock-in mice and FDG substrate [20, 124]) is shown in the lower panel. DN = double negative; DP = double positive; ISP = immature single positive; SP = single positive.

GATA3 and its role in TCRß-selection

Using mice with the GATA3 *LacZ* reporter on one allele, the proportion of *LacZ*+ cells were examined as a function of T cell development (Figure 5). Although GATA3 is expressed throughout T cell development, the fraction of *LacZ*+ cells was highly variable between different stages, indicating that expression levels of GATA3 may be crucial for regulation of T cell development [20, 125]. High proportions of *LacZ*+ cells in the DN4, ISP and CD3+ DP cell populations suggested a role for GATA3 in β-selection and in positive selection of CD4 SP T cells [20, 125]. In particular, the finding of low GATA3 expression during the two waves of TCR gene recombination, separated by a stage of high GATA3 expression, suggested that GATA3 may function as a regulator of proliferation events associated with the essential coupling of V(D)J recombination activity to the cell cycle [140]. In agreement with this, transgenic overexpression of GATA3 was found to be associated with thymic lymphoma [125].

Conditional deletion of the *GATA3* gene at the DN stage using the Cre-loxP system, whereby the Cre transgene was driven by the proximal Lck promoter, resulted in a developmental arrest at the DN3 stage, implicating GATA3 in β-selection [24]. *GATA3*-/- DN3 cells can rearrange and express intracellular TCRβ chain, but the DN3 TCRβ+ cells fail to increase their cell size (Figure 6) and do not downregulate CD25 surface expression. Interestingly, there is no evidence suggesting a survival defect in these arrested *GATA3*-/- DN3 cells. These findings indicate that β-selection is partially induced, because cells are rescued from apoptosis, but fail to expand or differentiate. Introduction of a pre-rearranged TCR transgene into these *GATA3*-/- thymocytes did not overcome the developmental block [24]. On the other hand, expression of GATA3

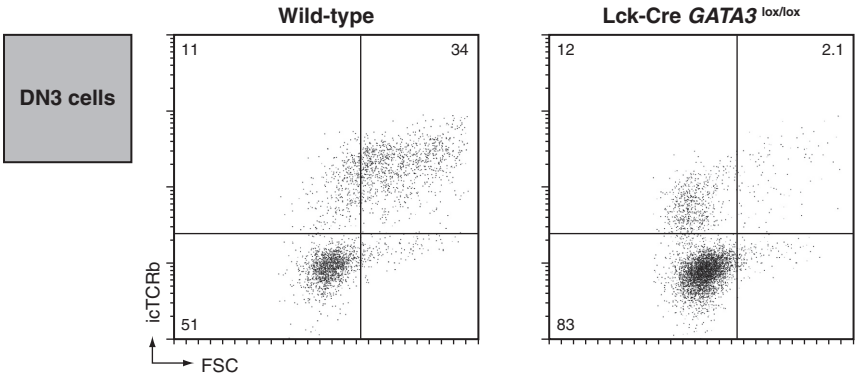

Figure 6. GATA3 deficient DN3 cells fail to increase cell size after β-selection. Flow cytometric analysis of wild-type and Lck-Cre mediated GATA3 deficient DN3 cells for intracellular TCRβ expression and cell size, based on FSC. CD4 and CD8 expressing thymocytes were excluded and DN cells expressing CD25, but not CD44, were gated as DN3 cells (Ling *et al*., unpublished and Pai *et al*., 24).

alone is not sufficient to initiate β-selection: the developmental arrest at the DN3 stage in *Rag2*$^{-/-}$ mice [141, 142] could not be corrected by enforced transgenic expression of GATA3. In these GATA3 transgenic *Rag2*$^{-/-}$ mice, cell size increased and CD25 downregulation in DN3 cells was negligible and no DN4 cells were detected. Taken together, these findings show that GATA3 functions as a key mediator for β-selection, but expression of GATA3 alone, in the absence of a functionally rearranged TCRβ chain, is not sufficient to initiate β-selection.

Role of GATA3 during positive selection and CD4 T cell development

Conditional deletion of the *GATA3* gene after β-selection, using CD4-Cre transgenic mice, resulted in a profound specific deficiency of CD4 SP cells. This finding demonstrated the absolute requirement of GATA3 for survival or development of CD4 committed thymocytes *in vivo*. Together with the unique expression pattern of GATA3 low in early immature DP cells and specifically up-regulated when DP cells develop towards the CD4 lineage (Figure 5). This suggests that GATA3 has a role in CD4 versus CD8 lineage commitment. Interestingly, TCR signal strength at the DP stage has been shown to affect CD4 versus CD8 lineage decision, i.e. strong TCR signals direct commitment towards the CD4 lineage [137-139]. GATA3 is upregulated by DP thymocytes in response to TCR stimulation, whereby its expression levels correlate with the strength of the TCR signal [143]. To assess the stages at which the GATA3 expression levels diverge between cells taking the CD4 or CD8 pathway, quantitative real-time (RT) PCR was used to analyze GATA3 transcripts from sorted thymocyte subpopulations from either MHC class I- or MHC class II-deficient mice to guarantee the CD4 or CD8 lineage of the sorted populations, respectively [143]. In the MHC class II selected cells, GATA3 mRNA levels increased to

a maximum in CD69$^+$ CD4 lineage intermediates and then gradually decreased as the cells develop into CD4 SP mature cells, while in the MHC class I selected cells GATA3 was not induced [143]. Taken together, these observations indicate that GATA3 could function to direct CD4 lineage commitment by translating the quantitative difference between TCR signals for CD4 versus CD8 lineage commitment. However, in the absence of GATA3, while MHC class I-restricted T cell commitment was not affected, MHC class II-restricted T cells were not diverted into the CD8 lineage [24]. Conversely, sustained over-expression of GATA3 in fetal thymic organ cultures favored selection of CD4 over CD8 SP cells, but did not divert MHC class I-restricted precursors into the CD4 lineage [143]. On the basis of these findings it has been concluded that GATA3 is necessary for post commitment CD4 generation, rather than for commitment to the CD4 lineage [24, 143]. Nevertheless, the possibility that GATA3 might promote CD4 lineage choice cannot be excluded, as it is still conceivable that MHC class II-restricted CD8 SP T cells or MHC class I-restricted CD4 SP T cells die as they fail to undergo MHC-TCR and CD4/CD8 co-engagement required for their survival [139].

c-Myb is a transcription factor that is a critical downstream regulator of positive selection, promoting the development of Th cells and blocking the development of cytotoxic T cells. Inducing c-Myb expression in mice, inhibits the development of CD8 SP cells. In contrast, in thymocytes deficient for *c-Myb*, Th differentiation is impaired and GATA3 is not up-regulated in response to TCR signaling. Furthermore, GATA3 appeared to be a direct target of c-Myb and this indicates an important regulatory role for c-Myb, via GATA3, in the regulation of Th differentiation [144].

GATA3 as a master regulator of Th2 differentiation

Upon antigenic stimulation naive CD4 SP T cells differentiate into effector T cells, which are classically divided into two functionally distinct subsets, termed Th1 and Th2 [145-148]. Th1 cells, which produce interferon-γ (IFNγ) and lymphotoxin-α are associated with the elimination of intracellular pathogens. Th2 cells, which produce IL-4, IL-5 and IL-13, are critically important for the eradication of parasitic worms, but are also implicated in allergic responses [149, 150].

In response to chronic antigenic stimulation *in vivo*, progressive polarisation of the cytokine responses ultimately leads to the commitment of naive CD4 SP precursors into mutually exclusive Th effector phenotypes, which are thought to be maintained independent of extrinsic factors. Th1 development is facilitated by two major signaling pathways, one involving IL-12/signal transducer and activator of transcription 4 (Stat4) and the other involving IFNγ/Stat1/T-box transcription factor (T-bet). Th2 differentiation is dependent on IL-4-induced activation of Stat6, leading to expression of GATA3 [151, 152]. On its turn GATA3 auto-activates its own expression and increases the accessibility of the Th2 cytokine cluster containing the genes coding for IL-4, IL-5 and IL-13 [153-155]. Furthermore, GATA3 suppresses Th1 development by downregulating the expression of Stat4 and the IL-12 receptor β2 chain [156-158]. Only if T-bet is induced in naive cells at sufficient levels, such GATA3 suppression is counteracted, permitting Th1 differentiation to occur. This ability of T-bet to oppose the

action of GATA3 now appears its most essential function in Th1 differentiation rather than any ability of T-bet to directly affect IFNγ gene transcription, as has been argued previously [159]. T-bet may act on GATA3 through multiple mechanisms, as it (i) inhibits GATA3 transcription and (ii) physically interacts with GATA3 and thereby prevents its binding to the IL-5 promoter [160]. Commitment of CD4 SP T cells to a particular Th phenotype is associated with the induction of epigenetic changes in the loci of effector cytokine genes, the Th2 cytokine locus (IL-4 / IL-13 / Rad50 / IL-5 locus) and the IFNγ locus [153, 155]. The mechanisms through which regulatory elements in these loci regulate cytokine expression by intra- and even inter-chromosomal interactions [161-163], has recently been by Ferreira *et al*., 15.

GATA3 or chromatin remodeling using pharmacological histone deacetylase- and cytosine methylation-inhibitors can replace the essential role of STAT6 in Th2 differentiation [154, 164, 165]. In activated Th2 cells GATA3, STAT6, c-Maf and the chromatin remodeling enzyme Brg1 and RNA polymerase II are all bound across the Th2 locus, whereby densely looped, transcriptionally active chromatin is packaged by the special AT-rich sequence binding protein (SATB1) [166]. The introduction of GATA3 into *in vitro* cultured T cells was shown to generate Th2-specific DNAse I hypersensitive sites independently of Stat6, implicating GATA3 in the process of chromatin remodeling [154]. These experiments also indicated that there is a positive autoactivation pathway, whereby GATA3, either directly or indirectly, activates its own expression.

Interestingly, Th cell differentiation is controlled by the cell cycle. Instruction for the Th2 cytokines IL-4 and IL-10 requires progression into S phase [164, 167]. In conditional *GATA3*-deficient T cells, normal Th2 acetylation patterns in the IL-4, IL-5, and IL-13 genes are impaired [168]. The effect is most prominent in the IL-5 gene, where there is a severe loss of Th2-associated hyperacetylation. The effect in the IL-4 and IL-13 genes is much less pronounced. The effect of GATA3 loss on Th2 cytokine production is more apparent in developing responses than in established Th2 cells. In a developing Th2 response, loss of GATA3 results in the inability to effectively mount a Th2 response, with a substantial reduction in the generation of IL-4-, IL-5-, and IL-13-producing cells [25, 168, 169]. In contrast, the loss of GATA3 in established Th2 cells causes a dramatic reduction in IL-5 production, a smaller effect on IL-13, but little effect on IL-4 [25, 169]. GATA3 acts in part by maintaining hyperacetylation of promoter regions of the IL-5 gene. The maintenance of acetylation patterns and transcriptional activity of the IL-4 and IL-13 genes may be controlled by additional mechanisms.

Both Th1 and Th2 differentiation can be induced by Notch1. Recently, GATA3 was found as a direct target for Notch1 signaling, whereby the intra-cellular part of Notch1 and its signaling component Rbp-J bind to the upstream promoter of GATA3. In the absence of GATA3, Notch turned into a strong inducer of Th1 differentiation. On the other hand, lack of Notch signaling leads to diminished GATA3 expression. These findings imply a critical function for GATA3 in Th2 differentiation by limiting Notch1 induced Th1 differentiation [170-172].

To examine the functional capacities of GATA3 *in vivo*, Th cell differentiation was investigated in CD2-GATA3 transgenic mice with enforced expression of GATA3 in all T cells. These mice manifested increased serum levels of the IL-4/Th2-dependent

isotype IgG1, when compared with non-transgenic littermates [173]. Upon immunisation with the TNP-KLH protein, antigen-specific IgG2a, which is an INFγ/Th1-dependent isotype, was significantly decreased and IgE, which is an IL-4/Th2-dependent isotype, was significantly increased [173]. In addition, a severe reduction in the KLH-induced footpad swellings was found in CD2-GATA3 transgenic mice, indicating that enforced GATA3 expression suppresses the Th1-dependent delayed type hypersensitivity response to KLH. The total population of CD4 T cells in spleen or mesenteric lymph node manifested rapid secretion of the Th2 cytokines IL-4, IL-5, and IL-10, reminiscent of Th2 memory cells. At the same time, the ability to produce IL-2 and IFNγ was decreased [173]. It was concluded that the increased functional capacity to secrete Th2 cytokines, along with the increased expression of surface markers for antigen-experienced Th2-committed cells, including T1/ST2, would argue for a role of GATA3 in Th2 memory formation [173].

Effects of GATA3 on regulatory T and Th17 differentiation

Recently, new lineages of CD4 T cells have been identified, including $CD25^+$ regulatory T cells [174] and a Th cell subset producing IL-17 and for this reason termed Th17 (Figure 7).

Regulatory T (Treg) cells are involved in the suppression of effector T cell function and are essential for tolerance against self-antigens. In the thymus naturally occurring Treg cells are generated, but Treg cells can be induced in the periphery as well. Both naturally occurring Treg cells and inducible Treg cells are dependent on the expression of the transcription factor forkhead box P3 (FoxP3) (Figure 7). An important factor to induce Treg cells *in vivo* and *in vitro* is Transforming growth factor-β (TGFβ) [175, 176]. However the precise molecular mechanisms underlying this induction are unclear. Recently it was found that GATA3 can regulate the expression of FoxP3 during Th2 differentiation. To establish this, GATA3 binds directly to the promoter region of FoxP3, resulting in a suppression of FoxP3 gene expression [177].

Classically, it was believed that Th1 cells were the important mediators for autoimmune diseases. However, conflicting data about this concept appeared over time, when for example analyses in mice deficient for IFNγ or deficient for Th1 signaling molecules, such as Interleukin-12p35 (IL-12p35), STAT1 and IL-12Rβ2, were performed. When autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS), was induced in these mice, even enhanced disease activity was observed [178-182]. A major breakthrough was the discovery that both IL-12 and IL-23 consist of the subunit p40. The subunits, p35 and p19, are specific for IL-12 and IL-23 respectively [183]. Mice deficient for p40 were not susceptible for EAE and collagen induced arthritis (CIA), suggesting that IL-12 and Th1 cells were involved in the induction of arthritis. However, the fact that in mice deficient for p19, EAE and CIA could not be induced, indicated the critical importance for IL-23 in the induction of these autoimmune diseases [184, 185]. Further studies showed that IL-23 was associated with the expansion of IL-17 producing CD4 cells [186].

IL-17 is a pro-inflammatory cytokine, which has been linked to autoimmune diseases, such as systemic lupus erythematosus (SLE), MS, rheumatoid arthritis (RA) and psoriasis and to allograft rejection [187-194]. In CIA and EAE, IL-17 expression

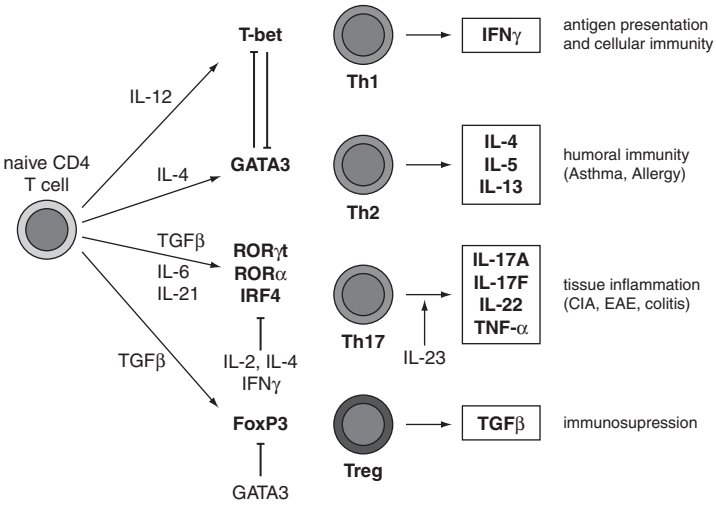

Figure 7. Overview of murine CD4 effector T cell differentiation in the mouse. When naive CD4 T cells are activated in the presence of specific cytokines, different developmental programs are initiated to restrict towards the Th1, Th2, Th17 or Treg lineages, which are characterized by their unique cytokine expression profile. The developmental programs are mainly based on the induction of transcription factors that serve as master regulators of specific lineages. Various ways of counter regulation exist, which occur both on the level of transcription and via protein-protein interactions.

has been implicated in inflammation [184, 186, 195-201]. Although IL-17 expression has been shown to be pathogenic in the induction of allergic asthma in mice, IL-17 expression is protective in the allergic response during the effector phase [202]. The implication of IL-17 in human diseases or in experimental mouse models for these diseases has been recently reviewed by Dong *et al.*, 12.

Differentiation towards individual T cell subsets and their maintenance is critically dependent on specific transcription factors, such as T-bet for Th1 [203], GATA3 for Th2 [151, 152] and FoxP3 for Treg cells [174, 204]. The retinoid related orphan receptor-γt (RORγt) was initially identified as the regulator of Th17 cells [205]. However, differentiation of Th17 cells appeared to be more complex, since the transcription factors IRF4 and RORα were found to be required for Th17 cells as well [206, 207] (Figure 7).

Induction of murine Th17 differentiation is dependent on TGFβ and IL-6 [190, 208, 209] and followed by the induction of IL-21 and IL-23R expression. On its turn IL-21 functions in a positive feedback loop to establish Th17 differentiation by inducing more IL-21 and IL-23R [210-212]. The functional role of IL-23 in establishing Th17 differentiation is still unclear. It is assumed that IL-23 has a role in expanding and stabilizing Th17 cells [213]. However, *in vitro* culture experiments indicate that the addition of IL-23 besides IL-6 and TGFβ does not result in enhanced Th17 differentiation. Another function for IL-23 might be the induction of a more pathogenic Th17 state, by inducing factors such as IL-22 [214]. Besides IL-17A and IL-22, Th17 cells produce IL-17F, and

tumor necrosis factor-α (TNFα) [186-188] (Figure 7).

Down-stream signaling via TGFβ and IL-6 is dependent on Janus kinase (JAK), resulting in STAT3 phosphorylation, which is required to induce IL-17 expression. STAT3 activation is on its turn negatively regulated by suppressor of cytokine signaling-3 (SOCS3) [215]. Another inhibitor of Th17 differentiation appeared to be IL-2 acting via STAT5 signaling [216].

While GATA3 has been implicated for the negative regulation of Th1 and Treg cell associated factors, so far no role for GATA3 has been found in the regulation of Th17 associated factors. It has been shown that both IL-4 and IFNγ inhibit Th17 differentiation [187, 188]. For this reason it could be that GATA3 inhibits Th17 differentiation via the induction of IL-4. On the other hand, it could well be that GATA3 supports Th17 differentiation via the inhibition of IFNγ production. Another possibility might be that GATA3 directly acts on the regulation of Th17 associated factors.

Implications of GATA factors in human disease

As GATA factors are crucially involved in the regulation of self-renewal, cellular proliferation and differentiation, it is not surprising that there is evidence for a role of GATA factors in the etiology of specific tumors.

Acquired missense mutations in the GATA1 gene have been identified in almost all cases of Down's syndrome-related acute megakaryoblastic leukemia (AMKL) and transient myeloproliferative disorder [217]. Mutations are clustered and each mutation results in the introduction of a premature stop codon in the N-terminal activation domain region, preventing the synthesis of full-length GATA1. The mutations do not block the synthesis of a shorter GATA1 variant that is initiated downstream and lacks the N-terminal activation domain but can still bind to Fog1. To date, the molecular mechanism by which this short GATA1 variant induces AMKL remains unclear. Furthermore, a high incidence of leukemia was found in female mice heterozygous for the knock-down mutation allele GATA1.05, the expression of which is reduced to 5% of the WT level. Hemizygous male mice ($X^{GATA1.05}$/Y) die *in utero* due to severe anemia, but heterozygous females ($X^{GATA1.05}$/X) survive, although they show various degrees of anemia and thrombocytopenia. These $X^{GATA1.05}$/X female mice may be a valuable animal model for myelodysplastic syndrome (MDS), as they suffer from multilineage cytopenia which progresses to acute leukemia in aging mice [218, 219].

Two new GATA2 gain-of function mutations were identified in chronic myeloid leukemia (CML) patients. L359V substitutions within zinc finger domain 2 of GATA2 were found in 8 cases, whereas an in-frame deletion of 6 amino acids, spanning the C-terminal border of zinc finger was found in a single case. The L359V mutation resulted in enhanced GATA2 transactivation activity and in an inhibition of PU.1 activity. Transduction of this mutation in the HL-60 cell line or in mice harboring the Bcr/Abl fusion protein, resulted in disturbed myelomonocytic differentiation and proliferation [220]. GATA2 was further found to be located near 3q21 breakpoints in acute myeloid leukemia (AML) or MDS. The majority of 3q21 breakpoints are located telomeric to the transcribed portion of the GATA2 gene in a region that is necessary for proper promoter function in mice. GATA2 was found to be reproducibly overexpressed in 7 out

of 9 patient samples with 3q21 abnormalities [221]. GATA2 can form a complex with the promyelocytic leukemia (PML) protein and can potentiate its transactivation capacity [222]. GATA2 can also physically interact with the PML-RARα (retinoic acid receptor α) oncogenic fusion protein generated by the t(15;17) translocation characteristic of acute PML leukemia. Functional experiments further showed that this interaction has the capacity to render a subset of GATA2 target genes subject to regulation by the retinoid acid signalling pathway [223]. Although GATA2 can interact with these leukemic chimera proteins, it has not been demonstrated to date that GATA2 indeed functions in the induction of leukemia.

It has been reported that GATA2 transcription is decreased in $CD34^+$ bone marrow-derived hematopoietic stem and progenitor cells in aplastic anemia [222, 223] Aplastic anemia is a bone marrow failure syndrome due to impaired stem cell function, characterized by hypocellular bone marrow, reduced hematopoiesis and peripheral pancytopenia [224]. It was postulated that the primary cause of aplastic anemia is not intrinsic to the HSC, but due to a cytotoxic T lymphocyte-mediated attack on bone marrow $CD34^+$ cells [225]. Recent DNA micro array analyses confirmed the reduced GATA2 expression in $CD34^+$ bone marrow cells from aplastic anemia patients. However, it also revealed an abnormal expression of genes involved in signal transduction pathways for apoptosis and terminal cytolytic enzyme generation and the reduced expression of anti-apoptotic genes in these cells. Furthermore, there is an increased expression level of immune response genes in aplastic anaemia patients [226]. Taken together, these data support the idea that the primary cause of aplastic anaemia is an immune attack on bone marrow $CD34^+$ cells. The observed reduced GATA2 expression level is therefore not likely the primary cause of aplastic anemia.

GATA3, which is highly expressed and required for the maintenance of cell fate of luminal epithelial cells in the breast, was found mutated in cases of human breast tumors [227, 228]. Recently, GATA3 has been identified as a prognostic marker in breast cancer [229]: patients whose tumors expressed low GATA3 had significantly shorter overall and disease-free survival when compared with those whose tumors had high GATA3 levels. In breast cancer cells GATA3 acts as a transcriptional regulator of the glycoprotein Mucin1 (Muc1). Abnormal Muc1 expression leads to a loss of cell-cell and cell-extracellular-matrix adhesion [230]. *In vitro* studies showed a function for GATA3 in the regulation of tumor differentiation and the suppression of tumor dissemination in breast cancer [231]. Also GATA4, -5 and -6 have been implicated in various human tumors, including gastric and colon cancer, esophageal cancer, ovarian carcinoma and adrenocortical tumors [232-235].

GATA3 haplo-insufficiency is associated with HDR syndrome (hypopara-thyroidism, sensorineural deafness and renal anomalies) [236, 237]. However, hematological disorders have not been reported so far in HDR syndrome patients. Consistently, as GATA3 haplo-insufficiency can cause HDR syndrome, GATA-L*acZ* knock-in heterozygous mice have a normal hematopoietic profile. In contrast to $GATA2^{+/-}$ mice, regeneration of the hematopoietic system, including the T cell lineage, after treatment with the cytotoxic drug 5-FU in the GATA3-*LacZ* heterozygous mice is comparable to that of the wild-type (Ling *et al*., unpublished observations).

T cell leukemia and implications of Notch1 and GATA3

T cell acute lymphoblastic leukemia (ALL) is a disease, associated with unfavorable clinical features, such as a high white blood cell count and enlarged or swollen lymph nodes (reviewed by Aifantis *et al*., 238). A large fraction of newly diagnosed patients are children and adolescents. The induction of T-ALL is thought to arise from malignant transformation of T cell progenitors. Certain genes, which are normally associated with the regulation of cell cycle control, differentiation and proliferation during specific stages of normal T cell development, are known to be involved in T-ALL. Aberrant expression of these genes or other oncogenes in T-ALL is often caused by chromosomal abnormalities, such as translocations. In many of those translocations the enhancer and promoter regions of TCR genes are involved. In other T-ALL cases, chromosomal abnormalities lead to fusion proteins with oncogenic properties. Factors involved in T-ALL are: basic-helix-loop-helix (bHLH) proteins such as, Tal1, Tal2, Lyl1 and E2A, homeobox genes HoxA-HoxD, Hox11 and Tlx3 and Lim-only domain binding proteins Lmo1 and Lmo2.

An important factor in T-ALL and normal T cell development is Notch1 (reviewed by Aifantis *et al*., 238 and Radtke *et al*., 239). In more than 50% of all human T-ALL cases, activated Notch1 mutations are found [240]. Members of the Notch family are critical regulators of differentiation and proliferation [241]. Membrane bound Notch protein consists of an extra-cellular and an intra-cellular subunit. Upon activation with Notch ligand, the intracellular part of Notch1 is cleaved off and translocates to the nucleus, where it associates with the transcriptional repressor Csl. This results in the recruitment of co-activator proteins, such as mastermind-like 1 and the histone acetylase p300, leading to transcription of Notch target genes, such as Deltex1, Hes1, preTα and CD25 [242]. Notch1 is essential for early T cell development as the deletion of *Notch1* in HSCs results in a complete inhibition of T development [243, 244]. Furthermore, T cell progenitors cannot develop in the absence of Notch1 signaling [245]. In addition Notch1 is involved in β-selection of DN thymocytes and progression towards the DP cells [246].

The role of Notch in human T-ALL has been reviewed by Aster *et al*. and Grabher *et al*. [247, 248]. In human T-ALL, activating mutations of Notch1 are found within specific subdomains. The intra-cellular part of Notch1 harbors a PEST (proline-, glutamic acid-, serine- and threonine-rich domain), which is important for the stabilization of Notch1. Furthermore, a heterodimerization domain (HD) is present in the intra-cellular part that is involved in the cleavage of intracellular Notch1. Activating mutations in the PEST domain affect ubiquitination, resulting in a more stable form of Notch1. Activating mutations within the HD domain probably result in a form of Notch1, that is more susceptible to cleavage of the intracellular part of Notch1 [249]. In some T-ALL cases, Notch1 activation is caused by a t(7:9) translocation of Notch1 to the TCR locus [244].

Important targets and signaling pathways, whereby Notch1 acts to induce T-ALL were recently identified. One of these is the oncogene and cell size regulator c-Myc. Notch1 is capable to directly bind to the c-Myc promoter and induces c-Myc gene expression [250-252]. Notch1 has been implicated in the regulation of the metabolic

Phosphoinositide 3-kinase-Akt (PI3K-Akt) pathway. Notch1 affects the phosphatase Pten (phosphatase and tensin homolog), which is a negative regulator of this pathway, via its targets Hes1 and c-Myc [253]. It has been shown that in mice deficient for *Pten* or transgenic for Akt lymphomas are induced [254, 255]. Finally, Notch1 affects the NFAT (nuclear factor of activated T cells) signaling pathway, via the activation of calcineurin [256].

While Notch1 is a major target in human T-ALL, the role of GATA3 in T-ALL is not that clear. GATA3 is expressed in human T-ALL [257] and GATA3 has the capacity to form a complex with Tal1 and Lmo in T-ALL, which is required to induce the transcription of retinaldehyde dehydrogenase 2 [258].

Furthermore, GATA3 was shown to be required for the survival of lymphoma cells [259]. In mice, enforced expression of GATA3 throughout T cell development results in the induction of DP lymphoma [125]. At 9 months of age, ~50% of the CD2-GATA3 transgenic mice developed thymic lymphomas, which were typically noticed as mice displaying respiratory distress. The thymic lymphomas often had a $CD4^+CD8^+$ phenotype and were generally positive for CD3ξ and TCRαβ. Tumor frequencies were similar in two independent CD2-GATA3 transgenic mouse lines and were not seen in non-transgenic littermates. Several animals with thymic lymphoma exhibited enlargement of spleen or lymph nodes. Lymphoma cells were found in the spleen, liver, lymph nodes, and kidney, indicating that the thymic lymphomas metastasized to the periphery. This was confirmed by the presence of identical clonal TCRβ rearrangement patterns in Southern blotting analyses using probes specific for Jβ1 or Jβ2 gene segments [125].

Although these findings suggest that GATA3 plays a role in T-ALL, so far no evidence exist for a direct involvement of GATA3 in the induction of human T-ALL.

AIMS OF THE THESIS

Defective T cell development or effector T cell function can result in decreased responsiveness against infections and even in development of allergies, autoimmune diseases and leukemias. Therefore, knowledge about the molecular pathways essential for the generation of T lymphocytes is essential for understanding the etiology of a wide variety of diseases and potentially improves current therapies.

Members of the GATA family of transcription factors play a crucial role in the regulation of T cell development from HSCs. The expression of GATA transcription factors is tightly regulated and aberrant expression has been implicated in human diseases. This thesis describes a detailed analysis of GATA gene targeting and transgenic approaches in mice to increase our understanding of the molecular mechanisms underlying the role of GATA transcription factors in the T cell lineage.

Deficiency of GATA2 in the mouse leads to severe anemia and embryonic lethality. The role of GATA2 and in HSC development within the embryo and adult mice *in vivo* are largely unexplored. For this reason the effects of GATA2 gene dosage on the generation and expansion of HSCs were examined in **Chapter II,** both in the developing embryo and in adult mice.

GATA3 is essential for early T cell development and differentiation of Th2 cells.

In DP cells GATA3 is specifically induced upon TCR stimulation in the context of MHC class II, where it is essential for developmental progression of DP cells undergoing positive selection towards the CD4 lineage. However, direct target genes for GATA3 in developing T cells in the thymus are currently unknown. In **Chapter III**, we aimed to identify new GATA3 targets in DP thymocytes. The induction of GATA3 within this cell population, together with previous findings that enforced GATA3 expression in CD2-GATA3 transgenic mice, affects DP cell size and induces DP T cell lymphoma, made DP cells the prime subset for the identification of putative GATA3 target genes. For the identification of these targets a comparison was made between gene expression profiles of DP cells from wild-type and CD2-GATA3 transgenic mice.

GATA transcription factors are critical regulators of proliferation and differentiation implicated in various human cancers, including leukemia and breast cancer. The direct oncogenic potential of GATA3 in T-ALL has been shown by the induction of thymic lymphoma in CD2-GATA3 transgenic mice, which appear to arise at the DP stage. However, molecular pathways and downstream targets of GATA3 that may explain the oncogenic potential of GATA3 have not been identified yet. Therefore, in **Chapter IV** we characterized non-malignant and transformed DP thymocytes and identified molecular mechanisms involved in malignant transformation of CD2-GATA3 transgenic thymocytes in detail.

Although GATA3 is essential for positive selection of DP cells towards the CD4 lineage, the exact role of GATA3 in the development of the CD4 cell lineage is largely unclear. In **Chapter V** the functional role of GATA3 during CD4 positive selection was studied and a novel mechanism in the regulation of the TCR expression during CD4 positive selection in the thymus is described.

GATA3 serves as a master regulator of Th2 differentiation by inducing expression of Th2 cytokines and by suppressing Th1 development. Yet, it is currently not known whether GATA3 activity inhibits or stimulates differentiation of Th17 cells. **Chapter VI** describes how enforced GATA3 expression affects Th17 differentiation in vitro. In addition, **Chapters VI and VII** describe the *in vivo* effects of enforced GATA3 expression on Th17 mediated pathology in EAE and AIA, mouse models for MS and RA.

Chapter VIII discusses the implications of the findings described in this thesis.

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II

GATA2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells

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J Exp Med 2004;200:871-82

ABSTRACT

GATA2 is an essential transcription factor in the hematopoietic system that is expressed in hematopoietic stem cells (HSC) and progenitors. Complete deficiency of GATA2 in the mouse leads to severe anemia and embryonic lethality. The role of GATA2 and dosage effects of this transcription factor in HSC development within the embryo and adult are largely unexplored. Here we examined the effects of *GATA2* gene dosage on the generation and expansion of HSCs in several hematopoietic sites throughout mouse development. We show that a haploid dose of *GATA2* severely reduces production and expansion of HSCs specifically in the aorta-gonad-mesonephros (AGM) region (which autonomously generates the first HSCs), while quantitative reduction of HSCs is minimal or unchanged in yolk sac, fetal liver and adult bone marrow. However, HSCs in all these ontogenically distinct anatomical sites are qualitatively defective in serial or competitive transplantation assays. Also, cytotoxic drug induced regeneration studies show a clear *GATA2* dose-related proliferation defect in adult bone marrow. Thus, GATA2 plays at least two functionally distinct roles during ontogeny of HSCs: the production and expansion of HSCs in the AGM and the proliferation of HSCs in the adult bone marrow.

INTRODUCTION

Hematopoietic stem cells (HSC) at the foundation of the adult hematopoietic differentiation hierarchy have the ability to self-renew and produce all the distinct blood cell lineages [1, 2]. HSCs capable of complete long-term hematopoietic repopulation of irradiated adult recipients are first generated in the aorta-gonads-mesonephros region (AGM) at mid-embryonic day 10.5 (E10.5) [3, 4] and localize to the dorsal aorta, vitelline and umbilical arteries [5]. Subsequently, at mid/late E11, HSC activity is also found in the yolk sac (YS) and fetal liver (FL). HSC activity increases significantly in E11 AGM and E12 YS when they are cultured as whole tissue explants for 3 days [3, 6]. While the spatial and temporal appearance of HSCs during development has been described and quantitated, the molecular mechanisms underlying HSC generation, expansion and maintenance are less well explored.

One molecule important during hematopoietic ontogeny is GATA2, a member of the GATA family of zinc finger transcription factors [7, 8]. RT-PCR analysis shows high expression of *GATA2* in adult hematopoietic progenitor cells and HSCs [9, 10]. Furthermore, immunohistochemistry, *in situ* hybridisation and transgenic analyses show *GATA2* expression as early as E8 in the para-aortic splanchnopleura (precursor tissue to AGM) and subsequently in the AGM [11, 12]. In the E11.5 AGM, GATA2 is expressed in the aortic endothelium and neighboring mesenchymal cells, which are both considered putative hemogenic cell populations. Embryos lacking GATA2 are anemic, have moderately reduced numbers of primitive erythroid cells as well as hematopoietic progenitor cells [13] and die at E10.5, the time of HSC induction and expansion. Due to this lethality, the role of GATA2 has been examined mainly by *in*

vitro colony forming assays of cells from early embryonic tissues and hematopoietic cultures of *GATA2*$^{-/-}$ ES cells. In both cases, hematopoietic progenitor numbers are severely reduced. Further studies in chimeric mice produced with *GATA2*$^{-/-}$ ES cells, show no contribution of the mutant cells to any hematopoietic tissues. Taken together, these data indicate that GATA2 is crucial for the maintenance, proliferation and/or survival of immature hematopoietic progenitors [13].

The function of GATA2 had also been studied through enforced over-expression achieved by retroviral transduction or transfection of genes encoding either a wild type GATA2 or an inducible GATA2/estrogen receptor (ER) fusion protein [12, 14-19]. Irrespective of these attempts, a simple conclusion on the function of GATA2 in the HSC or progenitor cells is difficult. On one hand, expression of the inducible form of GATA2/ER fusion protein in the multipotent hematopoietic progenitor cell line FDCP promotes differentiation [17]. On the other hand, constitutive expression of GATA2 in murine bone marrow (BM) cells blocked progenitor-derived colony formation [14]. The opposing results may be due to the cell types chosen for these experiments. However, it is also suggested that the biochemical behavior of the artificially generated GATA2/ER fusion protein may not be the same as the wild type unmodified GATA2 protein [18]. Thus, the most relevant data on *GATA2* dose effects on hematopoiesis may be best obtained within the physiological context of the whole organism wherein *GATA2* is expressed under the endogenous regulatory machinery in the appropriate cell types.

The study of transcription factor dose and function at the earliest stages of hematopoietic development is of particular interest for an understanding of HSC generation. Studies on the *runx1* transcription factor have shown that a haploid dose results in changes in HSC induction, expansion and distribution in the midgestation mouse embryo [20]. Moreover, haploinsufficiency of human Runx1 (AML-1) results in thromobocytopenia and a propensity to develop myeloid leukaemia [21]. Recently, a correlation between a reduction in *GATA2* expression and aplastic anemia [22] has been demonstrated. Hence, to further understand the role of GATA2 in the ontogeny of HSCs, we examined the effects of *GATA2* haploinsufficiency on induction and expansion of HSCs during development by *in vivo* hematopoietic transplantation assays and phenotypic analysis of compound transgenic embryos (*GATA2*$^{+/-}$*:Ly-6A GFP*) [23].

Here we present data showing that the numbers of hematopoietic progenitors in *GATA2*$^{+/-}$ embryos are reduced. More importantly, we observe a dramatic quantitative reduction in HSC activity specifically in *GATA2*$^{+/-}$ AGMs and a further reduction in the serial repopulating ability of these HSCs. In contrast, *GATA2*$^{+/-}$ HSC numbers appear quantitatively normal in the adult BM but are qualitatively defective in the setting of competitive transplantation. In addition, *GATA2*$^{+/-}$ HSCs exhibit a delay in regeneration of the hematopoietic system following cytotoxic drug challenge, suggesting that GATA2 levels play a role in HSC proliferation. Thus, GATA2 plays functionally distinct roles in the production of HSCs in the AGM region and the proliferation of HSCs throughout ontogeny.

MATERIALS AND METHODS

GATA2 mutant mice and embryos

GATA2 mutant mice [13] were backcrossed onto the C57BL/6 background for over 10 generations and were housed in the Erasmus Medical Center Animal unit according to the institution guidelines with food and water provided *ad libitum*. The day of vaginal plug discovery from overnight matings (*GATA2*$^{+/-}$ male x C57BL/6 *GATA2*$^{+/+}$ or *GATA2*$^{+/-}$ female) was counted as day 0. Pregnant dams were sacrificed and embryos isolated from the uterus as described previously [24]. Embryos (E10 to E11) were staged by counting somite pairs [25]. Genotyping was performed by PCR as described previously [13]. Compound transgenic embryos were obtained by mating *Ly-6A GFP* hemizygous [23] and *GATA2*$^{+/-}$ mice.

Dissection, explant culture, cell preparation and *in vivo* transplantation

Dissections, tissue explants and cell preparation were performed as described previously [24]. Recipient mice (C57BL/6 or (129Sv x C57BL/6) F1 females, 8 - 16 weeks old) received a split dose of 1000 rad (for CFU-S_{11}), 900 rad (for HSC) or 640 rad (for competitive repopulation assay) at a 3 hour interval from a ^{137}Cs source on the day of donor cell injection. Cells were injected intravenously into the tail veins. Except for CFU-S, serial and competitive transplantation assays, 2 x 10^5 female spleen cells from the recipient strain were co-injected to provide short-term survival. Secondary transplantations were performed with 3 x 10^6 BM cells from the primary recipients. Cell dose for competitive repopulation assays was 3 x 10^5 to 3 x 10^7. Injected animals were provided with 0.16 % Neomycin (Sigma, Zwijndrecht, The Netherlands) supplemented water. For CFU-S_{11}, recipients were sacrificed at 11 days post-transplantation by cervical dislocation, spleens isolated and microscopic colonies scored after fixing with Teleynesnicki's solution overnight.

Semi-quantitative PCR for donor contribution

Blood, tissue or specific cell lineage DNA (100ng) was used for semiquantitative PCR to detect the donor HSC contribution to the recipient. For male-derived donor cells, *YMT*-specific PCR (350 bp product) was used together with myogenin-specific PCR (250 bp) for DNA normalization. The detection of *GATA2* mutant-derived donor cells was performed with *GATA2/NEO* (950 bp) and GATA2 wild type- (600 bp) PCR. Primers and PCR conditions were as previously described [13].

Cell sorting and flow cytometry analysis

FACS was performed on a FACS Vantage SE [23] and flow cytometric analyses were performed on a FACSCalibur dual laser instrument with CellQuest software (BD Biosciences, Mountain View, CA). Staining of embryonic tissue cell suspensions was performed in PBS supplemented with 10% FCS, and 2µg/ml 7AAD (Molecular Probes, Leiden, The Netherlands) was added for dead cell exclusion. Staining of adult HSCs was performed in PBS supplemented with 0.5% BSA. Biotin-conjugated anti-Gr-1 (ER-MP20) was a kind gift of Dr. P.J.M. Leenen, Erasmus MC, Rotterdam,

The Netherlands. All other antibodies were obtained from BD Biosciences including APC conjugated anti-c-kit (clone 2B8), PE-conjugated anti-Sca-1 (clone D7), PerCP-Cy5.5 conjugated anti-CD8a (clone 53-6.7), anti-B220 (clone RA3-6B2), anti-CD19 (1D3), anti-CD11b (anti-Mac-1, clone M1/70) and biotin-conjugated anti-CD3 (145-2C11), anti-CD4 (H129.19), Ly-76 (TER-119) anti-IgM (II/41), and anti-NK1.1 (PK136). A secondary step was sometimes performed with streptavidin conjugated PerCP-Cy5.5.

Immunohistochemistry

Embryos were fixed for 30 min with 2% paraformaldehyde/PBS at 4°C and equilibrated in 20% sucrose/PBS overnight at 4°C. They were immersed in Tissue Tek, quick frozen on dry ice and stored at -80°C until ready for use. Serial cryosections (10 µm) were treated in 100% cold acetone for 10 min, washed 3 times with PBS (0.05 % Tween), blocked with 0.5% BSA and 50% v/v Avidin D block solution (Vector Laboratories Inc.) for 15 min, washed 3 times, blocked with Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA) for 15 min, and washed 3 times. Subsequently, sections were incubated with a biotin-conjugated ant-CD34 (clone RAM34; BD Biosciences) diluted 1:50 in 1% BSA/ 0.05% Tween/PBS at room temperature for 1 hr, washed 3 times, incubated with the detection reagent Streptavidin-Cy5 (Rockland, Gilbertsville, PS) diluted 1:500 in 1% BSA/ 0.05% Tween/PBS at room temperature for 30 min, washed 3 times, dehydrated in ethanol (from 70% to 100%), and mounted with vectashield (Vector Laboratories Inc.).

Confocal images were taken of every tenth section starting caudally at the point where the urogenital ridges first appeared up to the rostral bifurcation of the dorsal aorta.

5-Fluorouracil treatment

GATA2$^{+/+}$ and $^{+/-}$ mice (9-10 weeks old) were intravenously injected with 150mg 5-fluorouracil (5-FU; Sigma, Zwijndrecht, The Netherlands) per 10g body weight. Treated mice were then sacrificed at 4, 8, 12 and 16 days post-treatment and BM cells were isolated and analysed by flow cytometry and *in vitro* culture assay.

Progenitor Colony Assay

BM cells were plated in triplicates from $2x10^4$ to $5x10^5$ cells per plate in methylcellulose medium (Methocult GF M3434, StemCell Technologies Inc., Vancouver, BC) supplemented with SCF, IL-3, IL-6 and Epo. All cultures were incubated at 37°C in a humidified chamber under 5% CO_2. CFU-GM (colony forming unit -granulocyte-macrophage) were scored with an inverted microscope at day 7 of the culture.

RESULTS

GATA2$^{+/-}$ AGM and YS explants contain fewer CFU-S$_{11}$

To investigate if *GATA2* gene dosage affects the production of hematopoietic progenitors during development, colony forming unit-spleen activity was assayed at 11 days post-injection (CFU-S$_{11}$) so as to measure the more immature erythro-myeloid progenitors. AGM, YS and FL were isolated from *GATA2*$^{+/+}$ and $^{+/-}$ embryos at E10.5 to E12, explant cultured and cells transplanted into irradiated adult recipients (Figure 1). At all time points tested, CFU-S$_{11}$ activity was detected both in *GATA2*$^{+/+}$ and $^{+/-}$ AGM, YS and FL explants (Figure 2). As expected from previous data [3], high numbers of

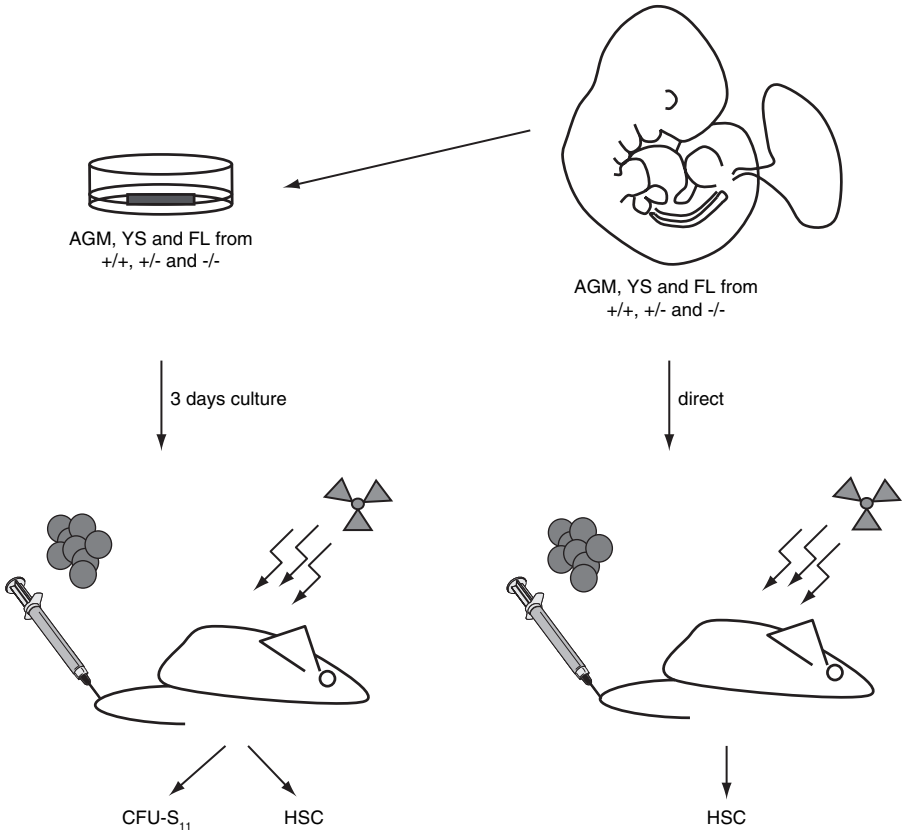

Figure 1. Strategy for studying HSC and hematopoietic progenitor cells in *GATA2* mutant embryonic tissues. *GATA2*$^{+/+}$, $^{+/-}$ and $^{-/-}$ aorta-gonads-mesonephros (AGM) regions, yolk sacs (YS) and fetal livers (FL) were harvested from midgestation mouse embryos. In some cases (left) they were then cultured for 3 days as whole tissue explants before preparation of single cell suspensions and injection into irradiated adult recipients to assay for CFU-S$_{11}$ (colony forming unit - spleen at day 11 post-transplantation) or hematopoietic stem cells (HSC). In some cases (right) single cell suspensions were prepared directly from freshly isolated tissues and injected into irradiated adult recipients for HSC activity.

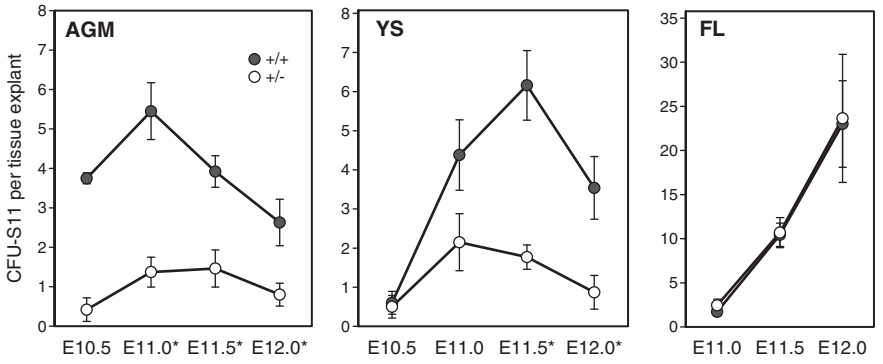

Figure 2. CFU-S_{11} activity in *GATA2* mutant tissue explants. CFU-S_{11} were assayed from AGM, YS and FL explants (E10.5 to E12.0) after 3 days of culture. Each point represents the average CFU-S_{11} number per embryo tissue equivalent ± SEM detected in the corresponding tissue and genotype. Three to 14 independent experiments were performed with 0.2 to 4.5 tissue equivalents injected per recipient. +/+ = *GATA2*$^{+/+}$ and +/- = *GATA2*$^{+/-}$. AGM = aorta-gonads-mesonephros; YS = yolk sac and FL = fetal liver. The total number of injected AGM explants: E10.5 +/+ = 6, E11.0 +/+= 21, E11.5 +/+ = 14, E12.0 +/+ = 12, E10.5 +/- = 7, E11 +/- = 22, E11.5 +/- = 12, E12.0 +/- = 12; YS explants E10.5 +/+ = 8, E11.0 +/+ = 8, E11.5 +/+ = 11, E12.0 +/+ = 10.75, E10.5 +/- = 8, E11.0 +/- = 11.5, E11.5 +/- = 15.5, E12.0 +/- = 9.5; FL explants E11.0 +/+ = 17, E11.5 +/+ = 18, E12.0 +/+ = 8.7, E11.0 +/- = 23, E11.5 +/- = 19.5, E12.0 +/- = 8. Embryonic tissues from E10.5 ranged from 36 to 40 somite pairs (sp), E11.0 ranged from 41-47 sp, E11.5 contain >48 sp and E12 contained >60 sp. * Denotes that there is a significant difference in the CFU-S_{11} number between *GATA2*$^{+/+}$ and *GATA2*$^{+/-}$ tissue explants: AGM E11.0, p<0.001; E11.5, p<0.01; E12.0, p<0.05; YS E11.5, p<0.001; E12, p<0.05. Note that fewer CFU-S_{11} are detected in both the *GATA2*$^{+/-}$ AGM and YS explants in comparison to the *GATA2*$^{+/+}$ explants, while the FL CFU-S^{11} numbers are unaffected.

E10.5 CFU-S_{11} are first detected in the *GATA2*$^{+/+}$ AGM explants, they increase at E11 and thereafter decline in number. In *GATA2*$^{+/-}$ AGM and YS explants, CFU-S_{11} were reduced by 3-9 fold and 1-4 fold respectively, as compared to wild type tissues. In contrast, CFU-S_{11} activity in *GATA2*$^{+/-}$ FL explants was normal. No reductions in FL CFU-S_{11} numbers were observed at any time. Therefore, *GATA2* gene dosage affects the generation and/or proliferation of immature hematopoietic progenitor cells in the YS and AGM of the midgestation embryo.

HSC activity is severely reduced in the *GATA2*$^{+/-}$ AGMs

To investigate if *GATA2* dose also affects midgestation HSC development, we performed the most stringent functional HSC test: the long-term, high level, multilineage repopulation of irradiated adult mouse recipients. E11 and E12 *GATA2*$^{+/+}$ and $^{+/-}$ AGM, YS and FL cells were transplanted directly into irradiated adult recipients (Figure 1). Engraftment by *GATA2*$^{+/+}$ and $^{+/-}$ cells was assayed by semiquantitative PCR of the male Y-chromosome specific marker *Ymt* and the *GATA2/NEO* mutant allele (respectively) in recipient peripheral blood DNA at 4 months post-transplantation. Only those recipients showing more than 10% engraftment with donor-marked cells were considered positive for high-level repopulation. PCR results of one

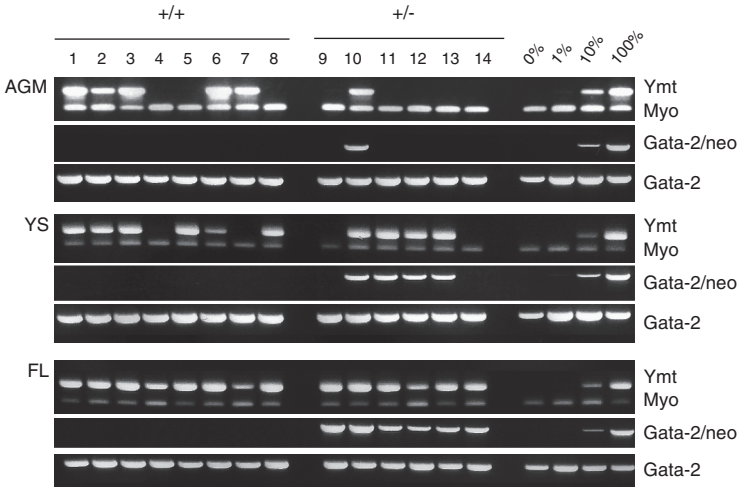

Figure 3. Detection of donor hematopoietic cell contribution in transplantation recipients by peripheral blood DNA PCR analysis. A representative PCR analysis for donor cell contribution to the peripheral blood of transplantation recipients. DNA was isolated from the corresponding recipients (at >4 months post-transplantation) of *GATA2*$^{+/+}$, $^{+/-}$, and $^{-/-}$ AGM (aorta-gonads-mesonephros), YS (yolk sac) and FL (fetal liver). Lanes 1- 8 and 9-14 are blood DNA samples isolated from recipients receiving cells from E12 *GATA2*$^{+/+}$ and $^{+/-}$ tissues, respectively. Each sample was analysed with primers specific for y-chromosome (*ymt*), and *GATA2* (*GATA2/NEO* for targeted allele). DNA samples were normalized by PCR with two endogenous gene controls (*myo* = *myogenin* and *GATA2* = wild type allele). Control DNA: 0%, 1%, 10% and 100% represents percentage of the male *GATA2*$^{+/-}$ DNA mixed with female DNA. Only when the donor marker specific PCR product was greater then 10%, as compared to controls, was the recipient considered to be positive.

representative experiment are shown in Figure 3. Briefly, each recipient received 1/3 of the cells obtained from an individually prepared E12 tissue (0.33 tissue equivalents). At 4 months post-injection, progeny of transplanted *GATA2*$^{+/+}$ AGM cells were found in the peripheral blood of 5 out of 8 recipients (*Ymt* PCR). In contrast, reduced HSC activity was found in E12 *GATA2*$^{+/-}$ AGMs. Only 1 out of 6 recipients was highly engrafted (*GATA2* mutant and *Ymt* PCR). Similarly, recipients transplanted with YS cells revealed some reduction of HSC activity in *GATA2*$^{+/-}$ embryos. However, no reduction in HSC activity was found in *GATA2*$^{+/-}$ FLs. Further analysis of recipients repopulated with *GATA2*$^{+/-}$ cells revealed high level multilineage engraftment within all hematopoietic tissues (blood, thymus, lymph nodes, BM and spleen) and subsets tested (splenic T and B lymphocytes, erythroid and myeloid cells) (not shown).

The cumulative results of all transplantation experiments are shown in Table 1 and reveal that at both E11 and E12, HSC activity is severely reduced in *GATA2*$^{+/-}$ AGMs. The percentage of recipients repopulated with E11 *GATA2*$^{+/-}$ AGM cells is only 6%, while 25% of recipients are repopulated with E11 *GATA2*$^{+/+}$ AGM cells. This represents a greater than 4-fold decrease in HSC activity in E11 *GATA2*$^{+/-}$ AGMs.

Table 1. HSC activity in *GATA2* mutant embryonic tissues.

	AGM		YS		FL	
Stage	+/+	+/-	+/+	+/-	+/+	+/-
E11.0	1[a]/4[b] (25[c])	1/16 (6)	2/7 (28)	4/17 (23)	0/6 (0)	1/19 (5)
E12.0	11/16 (69)	1/13 (8)	11/15 (73)	6/13 (46)	19/19 (100)	11/14 (78)
2[o]E12.0	6/6 (100)	0/3 (0)	6/6 (100)	4/9 (44)	6/6 (100)	4/9 (44)

E11 and E12 AGM, YS and FL tissues were made into a single cell suspension and injected into irradiated adult recipients. Each result represents [a]the number of recipient mice showing donor cells in peripheral blood (DNA) at >4 months post-transplantation, [b]the total number of mice transplanted and [c]the percentage of repopulated recipients. Only when the donor cells represented more than 10% was the recipient considered to be positive. Three and 2 independent experiments respectively were performed for E11 (41-47 somite pairs) and E12 tissues (>60 somite pairs). One and 0.33 tissue equivalents transplanted for E11.0 and E12.0 respectively. 2[o]E12 = secondary transplantation with 3 x 10^6 BM cells isolated from high level repopulated primary recipients that received cells from E12 tissues (two independent experiments). +/+ = *GATA2*$^{+/+}$ and +/- = *GATA2*$^{+/-}$. AGM = aorta-gonads-mesonephros, YS = yolk sac and FL = fetal liver.

Furthermore, at E12 *GATA2*$^{+/-}$ AGMs are 9-fold reduced in HSC activity as compared to *GATA2*$^{+/+}$ AGMs. Reductions in the HSC activity of *GATA2*$^{+/-}$ YS and FL tissues are less severe and stage dependent. The percentage of mice repopulated by E11 *GATA2*$^{+/-}$ YS (23%) is comparable to that of *GATA2*$^{+/+}$ YS (28%) and the FL at this stage contains only limited HSC activity. However, at E12 slight reductions in HSC activity are observed for both, *GATA2*$^{+/-}$ YS and FL (1.6-fold and 1.3-fold respectively) as compared to *GATA2*$^{+/+}$ tissues. Thus, two copies of the *GATA2* gene are required for the normal generation, expansion and/or survival of HSCs in the AGM region.

Ex vivo expansion and maintenance of AGM HSC activity is sensitive to *GATA2* dose

Since it was shown previously that HSC activity generated in the AGM can be amplified (either by induction or proliferation) when whole tissues are cultured for 3 days [3], we examined the effects of *GATA2* gene dosage on HSCs in such explant cultures of AGM, YS and FL from *GATA2*$^{+/+}$ and $^{+/-}$ embryos. Tissues (E10.5 to E12) were dissected, cultured as explants for 3 days, made into a single cell suspension and injected into irradiated adult recipients (Figure 1). Repopulation was measured at 4 months post-transplantation and only those recipients showing greater than 10% donor cell multilineage hematopoietic repopulation (measured in several hematopoietic tissues and lineages) were considered positive. The results are summarized in Table 2.

Compared to the results of the direct transplantation experiments (Table 1), *GATA2*$^{+/-}$ AGM explants were even more severely reduced in the HSC activity. At E10.5, only 8% of recipients receiving *GATA2*$^{+/-}$ AGM cells were repopulated, representing an 8-fold decrease in HSC activity from *GATA2*$^{+/+}$ AGM cells. The *GATA2*$^{+/-}$ cell contribution to the various hematopoietic organs (thymus, spleen, lymph node and BM) and purified cell lineages (B and T lymphocytes, myeloid

Table 2. HSC activity in GATA2 mutant tissues after explant culture.

Stage	AGM explants			YS explants			FL explants	
	+/+	+/-		+/+	+/-		+/+	+/-
E10.5	2[a]/3[b] (66)[c]	1/12 (8)		0/3 (0)	0/11		ND	0/2 (0)
E11.0	1/2n (50)	0/4 (0)		1/4n (25)	1/5 (20)		0/3 (0)	0/5 (0)
E11.5	11/12 (50)	0/27 (0)		7/25 (28)	7/29 (24)		6/25 (24)	3/28 (11)
E12.0	7/12 (58)	0/13 (0)		4/16 (25)	2/14 (14)		11/12 (92)	10/19 (53)
	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-
E10.0	0/7 (0)	0/8 (0)	0/3 (0)	0/7 (0)	0/8 (0)	0/3 (0)	ND	ND

E10 to E12 AGM, YS and FL explants were cultured for 3 days, made into a single cell suspension and injected into irradiated adult recipients. Each result represents [a] the number of recipient mice showing donor cells in the peripheral blood (DNA) isolated at >4 months post-transplantation, [b] the total number of mice transplanted and [c] the percentage of repopulated recipients. ND = transplantation not performed. Only when donor contribution was more than 10% was the recipient considered to be positive. Two to 4 independent experiments performed for each stage and tissue type. For E10, 1 to 5 tissue explant equivalents were transplanted per recipient. One tissue explant equivalent was transplanted per recipient for E10.5 and 0.33 tissue explant equivalents for E11.0, E11.5 and E12.0. +/+ = *GATA2*$^{+/+}$ and +/- = *GATA2*$^{+/-}$. AGM = aorta-gonads-mesonephros, YS = yolk sac and FL = fetal liver. E10 = 31-35 somite pairs (sp); E10.5 = 36-40 sp; E11.0 = 41-47 sp; E11.5 > 48 sp and E12.0 >60 sp.

and erythroid) was tested and found to be multipotent, thus demonstrating that *GATA2*$^{+/-}$ AGMs do generate functional HSCs, albeit at much reduced levels. At later developmental time points (E11, E11.5 and E12), HSC activity while increasing in *GATA2*$^{+/+}$ AGM explants, is completely absent from *GATA2*$^{+/-}$ AGM explants. As seen in the *GATA2*$^{+/+}$ AGM explants, HSC generation and expansion occurs from E10.5 to E11.5, with HSC numbers maintained thereafter (E12). Thus, the severely reduced HSC activity in *GATA2*$^{+/-}$ AGM explants can be attributed to reduced HSC expansion, survival and/or homing in the irradiated recipient.

The HSC activity of *GATA2*$^{+/-}$ YS and FL explants was also reduced in comparison to the *GATA2*$^{+/+}$ explants. However, this reduction was only slight as compared to the AGM. At the first appearance of HSCs in the YS on E11, *GATA2*$^{+/-}$ YS HSC activity begins to decrease and by E12 is decreased by 1.8-fold from *GATA2*$^{+/+}$ YS. The decrease in YS explant HSC activity at E12 corresponds with that seen in the directly transplanted YS, suggesting that the expansion but not the maintenance of YS HSC activity is sensitive to *GATA2* dose.

Similarly, we observed slight reductions in the HSC activity of *GATA2*$^{+/-}$ FL explants. At the first appearance of HSCs in FL explants on E11.5, HSC activity is reduced from 24% of recipients repopulated with *GATA2*$^{+/+}$ cells to 11% repopulated with *GATA2*$^{+/-}$ cells, representing a 2.2-fold decrease in HSC activity. At E12, *GATA2*$^{+/-}$ FL explants show a 1.7-fold decrease in HSC activity. The changes in FL HSC activity with time are most likely related to the numbers of incoming HSCs from the AGM and YS.

Thus, these findings suggest *GATA2* dose affects the expansion but not the survival of HSCs in the FL.

The onset of HSC activity in *GATA2*$^{+/-}$ embryos is normal

Previously, we reported CFU-S_{11} and HSC deficiencies in embryos with a haploid dose of the *runx1* transcription factor [20]. The spatial distribution of HSC activity was altered and an unexpected early appearance of HSC activity was found in *runx1*$^{+/-}$ AGM and YS. To examine if there was also a premature appearance of HSC activity in *GATA2* mutant embryos, AGM and YS explants from early E10 (31-35 somite pairs) *GATA2*$^{+/+}$, $^{+/-}$ and $^{-/-}$ embryos were isolated, cultured for 3 days and cells transplanted into irradiated adult recipients. As shown in Table 2, although high tissue equivalents (up to 5) of cells from *GATA2* mutant (+/- and -/-) AGM and YS explants were injected, HSC activity was not detected in any of the recipients. Also, *GATA2*$^{+/-}$ E10.5 YS and E10.5 and E11 FL explants showed no HSC activity. However, HSC activity initiates normally in *GATA2*$^{+/-}$ AGM explants at E10.5 at the same stage as in the *GATA2*$^{+/+}$ AGM. HSCs also appear at normal time points in *GATA2*$^{+/-}$ YS and FL explants (E11 and E11.5 respectively). Therefore, we conclude that HSC induction initiates on schedule and that there is no early onset of HSC activity in *GATA2*$^{+/-}$ AGM, YS or FL.

Serial transplantation potential of midgestation HSCs is severely reduced

HSC self-renewal can be tested by serial transplantation of HSCs from primary to secondary recipients. Since we found that *GATA2*$^{+/-}$ AGM HSCs are severely reduced in their expansion, we examined whether *GATA2*$^{+/-}$ embryo-derived HSCs are as potent in their serial repopulation ability as wild type HSCs. Whole BM cells from primary recipient mice showing high donor contribution from transplanted E12 *GATA2*$^{+/+}$ or $^{+/-}$ AGM, YS and FL cells were injected into irradiated secondary adult recipients. Consistent with previous published results, *GATA2*$^{+/+}$ AGM, YS and FL derived HSCs can successfully reconstitute secondary recipients; 100% of secondary recipients were repopulated with HSCs from primary recipients of these midgestation tissues (Table 1). In contrast, HSCs from a primary *GATA2*$^{+/-}$ AGM recipient failed to repopulate any of the secondary recipients analysed; 0% (0 out of 3)s. Reduced HSC activity was also observed in the secondary recipients receiving BM cells from *GATA2*$^{+/-}$ YS and FL primary recipients (44% as compared to 100% recipient repopulation with *GATA2*$^{+/+}$ primary BM cells). These results demonstrate that *GATA2* dose affects HSC serial repopulation ability and suggests a defect in HSC self-renewal.

***GATA2*$^{+/-}$ BM HSCs are at a competitive disadvantage**

The decreased serial repopulation ability of embryo-derived HSCs prompted us to investigate if adult BM HSCs are also affected by a reduction in *GATA2* dose. Initially, we injected limiting doses of *GATA2*$^{+/+}$ and *GATA2*$^{+/-}$ BM cells into lethally irradiated adult recipients but found no quantitative differences in repopulation. Hence, we performed reciprocal competitive transplantations in which different concentrations of unmanipulated *GATA2*$^{+/+}$ and $^{+/-}$ BM cells were injected into sublethally irradiated *GATA2*$^{+/+}$ and $^{+/-}$ adult recipients.

When 3 x 10^5 whole *GATA2*$^{+/+}$ BM cells were transplanted into *GATA2*$^{+/+}$ adult recipients, long-term high-level donor contribution was found in 2 out of 5 (40%) recipients, while *GATA2*$^{+/-}$ cells at this dose provided no repopulation (0 out of 5 recipients; 0%) (Figure 4). Only at a 10-fold higher cell dose were the *GATA2*$^{+/-}$ cells able to repopulate 4 out of 7 (57%) recipients. A dose of 3 x 10^6 *GATA2*$^{+/+}$ cells repopulated almost all recipients (6 out of 7; 86%), while 3 x 10^7 *GATA2*$^{+/-}$ cells were required for repopulation of all recipients (5 out of 5; 100%). To further examine the competitive abilities of *GATA2*$^{+/-}$ cells, GATA2$^{+/+}$ cells were injected into sublethally irradiated *GATA2*$^{+/-}$ recipients. Only 3 x 10^5 (or fewer) wild type cells were required to fully out-compete all the *GATA2*$^{+/-}$ HSCs in the recipients. Thus, *GATA2*$^{+/+}$ HSCs more effectively compete against *GATA2*$^{+/+}$ HSCs than do *GATA2*$^{+/-}$ HSCs, demonstrating that *GATA2*$^{+/-}$ adult BM contains fewer HSCs or that these HSCs are qualitatively less potent.

GATA2 dose affects the number of phenotypically defined HSCs in the embryo but not the adult

Our *in vivo* transplantation results clearly show that *GATA2*$^{+/-}$ HSC activity is affected throughout development. To more specifically investigate the cell types that

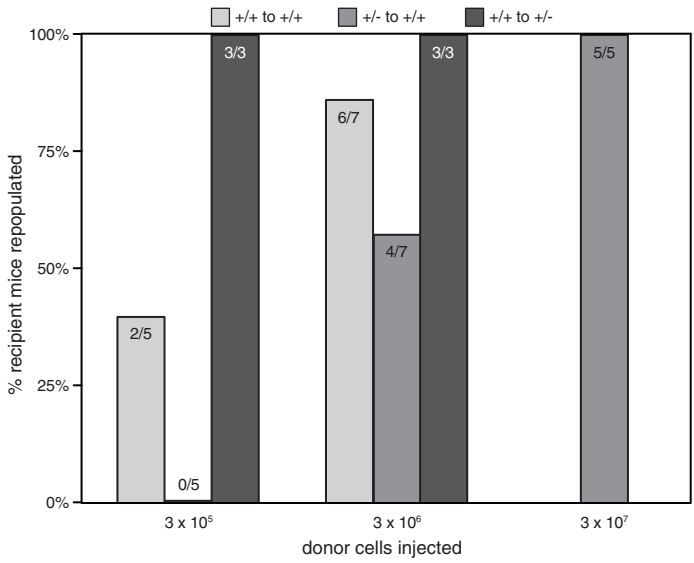

Figure 4. Competitive transplantation of *GATA2*$^{+/+}$ and $^{+/-}$ BM in sub-lethally irradiated adult recipients. Varying concentrations (3 x 10^5 to 3 x 10^7) of *GATA2*$^{+/+}$ or $^{+/-}$ BM cells were transplanted into sublethally irradiated *GATA2*$^{+/+}$ or $^{+/-}$ recipients to test for HSC competition in repopulation. The Y-axis shows the percentage of recipient animals engrafted with greater that 10% donor cells in hematopoietic tissues. Engraftment results are shown in grey striped bars for *GATA2*$^{+/+}$ donor cells transplanted into GATA2+/+ recipients, in grey bars for *GATA2*$^{+/-}$ donor cells transplanted into *GATA2*$^{+/+}$ recipients and in white bars for *GATA2*$^{+/+}$ donor cells transplanted into *GATA2*$^{+/-}$. The results show that *GATA2*$^{+/+}$ HSCs out-compete *GATA2*$^{+/-}$ HSCs.

are affected in the *GATA2*$^{+/-}$ mice, we crossed the *GATA2* mutant allele into *Ly-6A GFP* transgenic mice, in which HSCs can be detected by the expression of the *GFP* (green fluorescent protein) reporter under the transcriptional control of *Ly-6A* regulatory sequences 26. *Ly-6A* encodes the Sca-1 surface glycoprotein that is expressed on HSCs. Previously we have shown that all AGM, FL and adult BM HSCs express the *Ly-6A GFP* transgene and that GFP expression is highly restricted in the AGM region to a few aortic endothelial cells and hematopoietic clusters [23].

To determine whether *GATA2* dose affects these cells, we examined transverse sections through the E11 dorsal aorta from compound transgenics (*Ly-6A GFP: GATA2*$^{+/+}$ and *Ly-6A GFP:GATA2*$^{+/-}$). As shown in representative sections in Figure 5A, GFP positive cells are decreased in number in the *GATA2*$^{+/-}$ aorta as compared to the *GATA2*$^{+/+}$ aorta. Quantitations were performed by counting GFP positive and CD34 positive cells in 37 aorta sections from each genotype (CD34 immunostaining of endothelial cells provided a normalization control). GFP positive cells were present but decreased by a factor of 10 or more in the *GATA2*$^{+/-}$ aortas. Hence, *GATA2* haploinsufficiency leads to a significant decrease in HSCs and/or HSC precursors in the AGM.

Flow cytometric analyses were performed to verify these results and to examine if the phenotypic HSC content of the other hematopoietic tissues was also changed. As shown in Figure 5B, in such compound transgenic embryos a 6-fold decrease in GFP positive cells in the E11 *GATA2*$^{+/-}$ aorta was found, as compared to *GATA2*$^{+/+}$ aorta. E11 *GATA2*$^{+/-}$ YS showed a 1.9-fold decrease in GFP positive cells However, no decrease was found in E11 *GATA2*$^{+/-}$ FL. Similarly, no difference in the percentages of HSCs as defined by the Lin-Sca-1^{+}c-kit^{+} (LSK) phenotype was found when *GATA2*$^{+/+}$ and $^{+/-}$ adult BM was analysed (Figure 5C). Taken together, these phenotypic data support the transplantation data in showing that HSCs are quantitatively decreased in the AGM, but that HSCs increase to normal numbers in the FL and adult BM.

Cytotoxic drug treatment reveals a proliferation defect in *GATA2*$^{+/-}$ BM HSCs

To test if the qualitative defect in *GATA2*$^{+/-}$ HSCs is related to proliferation, *GATA2*$^{+/+}$ and $^{+/-}$ mice were treated with the cytotoxic drug 5-fluorouracil (5-FU) 27. At 0, 4, 8, 12 and 16 days after treatment, BM cells were tested in *in vitro* assays and analysed by flow cytometry for evidence of hematopoietic regeneration. As shown in figure 6A, both *GATA2*$^{+/+}$ and $^{+/-}$ mice showed similar reductions in total BM cell number at 4 days post 5-FU. Total BM cell numbers increased to starting numbers by 16 days post-5-FU. No significant differences were observed between the number of total *GATA2*$^{+/+}$ and $^{+/-}$ BM cells at any time point. In addition, no defect in hematopoietic differentiation was observed in *GATA2*$^{+/-}$ BM cells, as flow cytometric analysis showed the presence of all three lineages, lymphoid, myeloid and erythroid, at similar levels in *GATA2*$^{+/+}$ and *GATA2*$^{+/-}$ BM (data not shown).

To investigate whether specific immature hematopoietic progenitors were affected in 5-FU treated *GATA2*$^{+/-}$ mice, we performed *in vitro* colony assays for CFU-GM (colony forming unit-granulocyte-macrophage). As shown in figure 6B and C, the starting frequency and number of CFU-GM were the same in *GATA2*$^{+/+}$ and $^{+/-}$ BM.

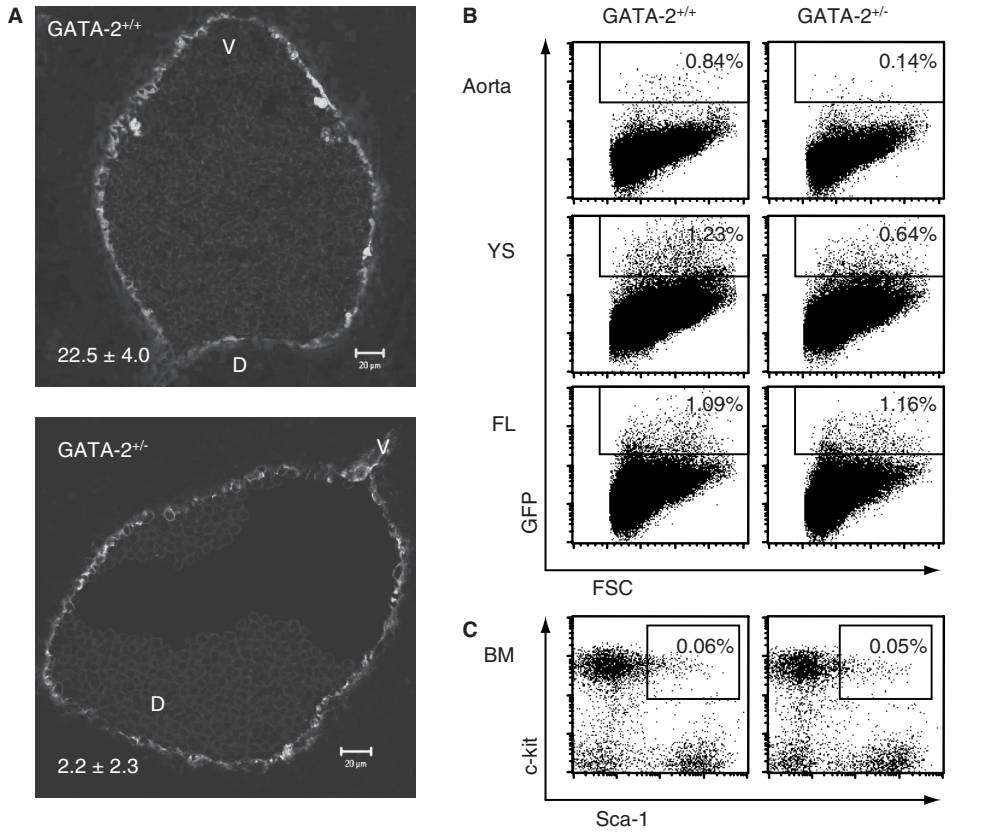

Figure 5. Phenotypic analysis of HSCs in *GATA2*$^{+/-}$ embryos and adults. *GATA2:Ly-6A GFP* compound transgenic embryos were generated by timed pluggings. (A) Representative transverse sections through the E11 dorsal aorta of a *GATA2*$^{+/+}$*:Ly-6A GFP* embryo (45 somite pairs; top panel) and a *GATA2*$^{+/-}$*:Ly-6A GFP* embryo (43 somite pairs; bottom panel). Sections were taken from the caudal end of the AGM, at the height of the hindgut and stained with anti-CD34 antibody. In total, 4 embryos were analysed (2 embryos and a total of 37 sections from each genotype) and cells counted in the aortic endothelium throughout the levels containing the gonads and mesonephroi. CD34$^+$ endothelial cells served as a control for section quality and normalization. Red fluorescence (CD34) and green fluorescence (GFP). The percentage of GFP$^+$/CD34$^+$ endothelial cells ± SEM is shown on the bottom left and is significantly reduced in the *GATA2*$^{+/-}$ embryos; $p < 0.05$. Flow cytometric analysis of phenotypically defined HSCs was performed on (B) embryonic hematopoietic tissues and (C) adult BM. Expression of the Ly-6A GFP HSC marker was analysed on E11 aorta, YS and FL cells. Adult BM cells were analysed for the percentage of cells in the Lin$^-$ fraction that are Sca-1$^+$c-kit$^+$. Percentages of GFP$^+$ cells in the embryonic tissue and Sca-1$^+$c-kit$^+$ cells enclosed in each gate are shown. (See Appendix page 216 for a full-color representation of this figure).

At 4 days post 5-FU treatment, frequency and number of CFU-GM reached a similar low point in both GATA2$^{+/+}$ and $^{+/-}$ BM. However, at 12 days following 5-FU treatment, CFU-GM frequency and number in *GATA2*$^{+/+}$ BM reached higher or the

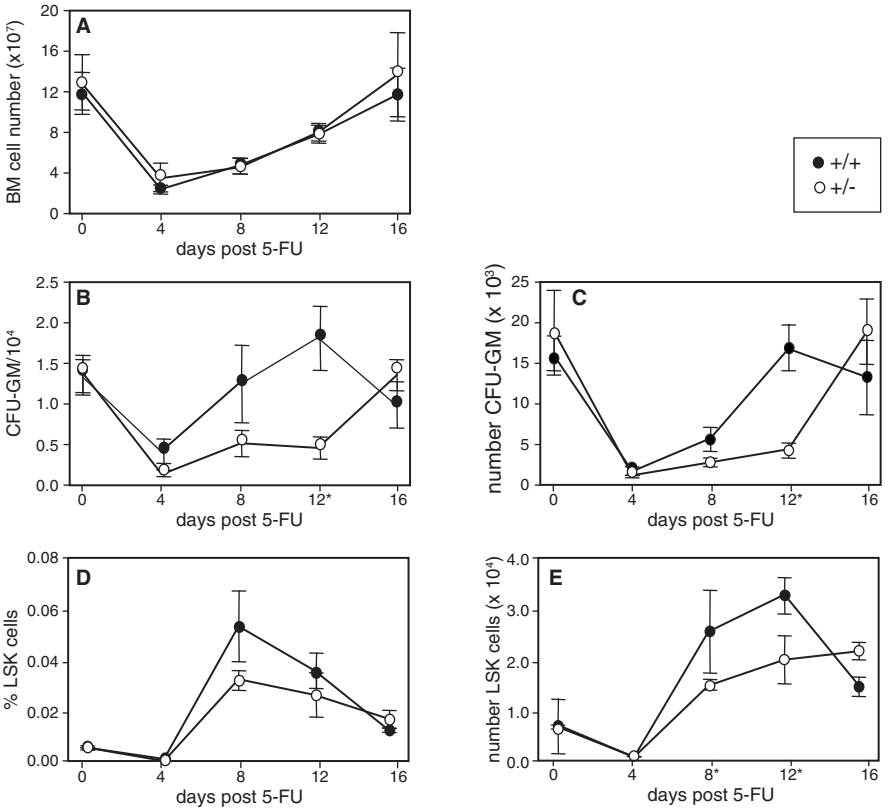

Figure 6. Hematopoietic regeneration following 5-FU treatment. The temporal regeneration of the hematopoietic system within the BM compartment of *GATA2*$^{+/+}$ and $^{+/-}$ mice was examined at days 0, 4, 8, 12 and 16 following 5-FU injection for (A) total BM cell numbers; (B) the frequency of CFU-GM in 10^4 BM cells; (C) the total number of CFU-GM in the BM (2 tibias plus 2 femurs). The regeneration of BM HSCs was examined by quantification of the Sca-1$^+$ and c-kit$^+$ cells within the lin$^-$ population by flow cytometry. (D) Percentage and (E) absolute number of lin$^-$Sca1$^+$c-kit$^+$ (LSK) BM cells was examined at days 0, 4, 8, 12 and 16 following 5-FU injection. *GATA2*$^{+/+}$ samples are represented by black circles and *GATA2*$^{+/-}$ samples are represented by white circles. Two complete time course experiments were performed. Each point represents an average of 2 to 3 animals with SEM. * Denotes that there is a significant difference between the *GATA2*$^{+/+}$ and $^{+/-}$: B) CFU-GM frequency day 12, $p<0.008$. (C) CFU-GM numbers day 12, $p<0.002$ and (E) LSK cell number day 8 and 12, $p<0.04$.

same levels as the untreated BM, while the *GATA2*$^{+/-}$ BM CFU-GM frequency and number remained low ($p<0.008$ and 0.002 respectively). Only at day 16 following 5-FU treatment did *GATA2*$^{+/-}$ CFU-GM frequency and number reach the same levels as in untreated BM. Thus, the 4 day delay in the regeneration of CFU-GM in *GATA2*$^{+/-}$ BM suggests a *GATA2* dose- related proliferation defect acting within these progenitors. Alternatively, a *GATA2* dose-related proliferation defect acts within HSCs and only secondarily influences CFU-GM.

To test this we analysed the regeneration of HSCs. We performed flow cytometric analysis for LSK BM cells at 0, 4, 8, 12 and 16 days post 5-FU treatment. In both *GATA2*$^{+/+}$ and $^{+/-}$ BM, HSC percentages and absolute numbers were similarly reduced at 4 days post 5-FU treatment (Figure 6 D and E). Both *GATA2*$^{+/+}$ and $^{+/-}$ BM HSC percentages and numbers began recovering at day 8 post 5-FU, when they surpassed the initial percentages and numbers. However, *GATA2*$^{+/-}$ BM HSC numbers remained significantly lower than in *GATA2*$^{+/+}$ BM at both day 8 and 12 post 5-FU treatment ($p < 0.04$). Furthermore, while *GATA2*$^{+/+}$ HSC numbers peaked at day 12 post-treatment and declined thereafter, *GATA2*$^{+/-}$ HSC numbers increased slowly up to day 16 post 5-FU treatment. The finding that HSC expansion in 5-FU treated *GATA2*$^{+/-}$ mice is delayed by at least 4 days strongly suggests a *GATA2* dose dependent proliferation defect in HSCs.

DISCUSSION

The data presented here show for the first time that a full dose of *GATA2* is required during embryonic and adult stages for quantitatively and qualitatively normal HSC activity *in vivo*. While HSCs are most likely not produced in *GATA2*$^{-/-}$ embryos, the effects of *GATA2* haploinsufficiency had been largely unexplored because such *GATA2*$^{+/-}$ animals grow normally and present an overtly normal adult hematologic profile. Here we have shown that with only half the dose of *GATA2*, HSC numbers are severely and specifically reduced in the AGM region, where the first induction and expansion of HSCs is occurring during midgestation. Moreover, AGM HSC quality is compromised. Thereafter, in the other tissues harboring HSCs, quantitative deficiencies in *GATA2*$^{-/-}$ HSCs appear to be compensated through normal (albeit delayed) expansion of HSCs, but qualitative deficiencies are retained through to adulthood. Therefore, given that only a few HSCs out of the whole cohort of HSCs are actively contributing to the hematopoietic system at any one time [28, 29] and that *GATA2*$^{+/-}$ cells are not defective in differentiation, no general hematologic defects would be expected in haploinsufficient adults. Only through stringent *in vivo* transplantations or cytotoxic stress are HSC functional defects observable. The results of these experiments strongly suggest an essential role for GATA2 in the induction and expansion of the first HSCs in the AGM and an additional, distinctive role for GATA2 in the proliferation of HSCs.

HSC quantitative processes are altered in *GATA2*$^{+/-}$ mice

In *in vivo* transplantation experiments we show quantitatively reduced HSC activity in *GATA2*$^{+/-}$ embryos. The 4- to 9-fold decrease in HSC activity in E11/E12 *GATA2*$^{+/-}$ AGMs as compared to *GATA2*$^{+/+}$ AGMs is the consequence of fewer HSCs, as aorta sections and flow cytometric analysis show a 6-fold decrease in phenotypically defined HSCs. Hence, *GATA2*$^{+/-}$ AGMs can neither expand nor maintain HSCs as compared to *GATA2*$^{+/+}$ AGMs. In contrast, HSCs are expanded and maintained in E11/E12 *GATA2*$^{+/-}$ YS (at a slightly decreased number), with the fold decrease in

phenotypically defined HSCs in the YS corresponding closely to the fold decrease in HSC activity. Considering the fact that HSCs are first detected in the AGM region and then in the YS and the FL, the reduced HSC content of the *GATA2*$^{+/-}$ YS and FL may well be a secondary effect of the reduction in the *GATA2*$^{+/-}$ AGM region.

Our *in vivo* analyses for hematopoietic progenitor cells in the AGM region and the YS show that CFU-S_{11} are also *GATA2* dose dependent. These data are consistent with previous *in vitro* studies on *GATA2*$^{-/-}$ YS and ES cells [13, 30] showing much reduced hematopoietic progenitor activity. The reduced CFU-S_{11} activity in *GATA2*$^{+/-}$ AGMs and YSs could further be a consequence of the reduced HSC activity we detected in these tissues. However, the source of cells providing the CFU-S_{11} activity in the embryo is not clear. While in the adult, hematopoietic progenitor cells are derived from HSCs, in the pre-E10.5 AGM region and the YS they may be derived from hemangioblasts and/or hemogenic endothelium rather than via a HSC ancestor. Hence, GATA2 may act directly on the *in vivo* generation, survival and/or expansion of the hematopoietic progenitor cells, HSCs and/or their direct precursors in the AGM and YS.

The YS as a compensatory generator of HSCs independent of *GATA2* dose

For over three decades, the origins of adult HSCs have been a focus of research. The view that the mammalian YS is able to provide hematopoietic cells that migrate and colonize the FL and then the BM during the neonatal/adult stages has been altered by the finding that the first fully functional adult HSCs are autonomously generated in the AGM region. Shortly thereafter, the YS contains HSCs, but due to the experimental constraints of mammalian embryos, it is difficult to definitively demonstrate whether these HSCs are AGM-derived or autonomously generated in the YS. Recent data suggest that indeed YS can autonomously generate and expand HSCs [6] and putative pre-HSCs [31]. Since we observe a dramatic reduction of HSCs in *GATA2*$^{+/-}$ AGMs but only a slight reduction of HSCs in *GATA2*$^{+/-}$ YSs, our transplantation data support the notion of YS HSC generation potential (albeit in a *GATA2*$^{+/-}$ embryo). However, since HSCs are still generated in the *GATA2*$^{+/-}$ AGM one day earlier than in the YS, it remains possible that YS HSCs are AGM-derived. Interestingly, the reduced HSC activity in the *GATA2*$^{+/-}$ YS can be expanded to a magnitude comparable to that of the *GATA2*$^{+/+}$ YS in explant cultures, suggesting that at least some of the reduced activity in the *GATA2*$^{+/-}$ YS is a secondary effect of the reduction of HSCs in the AGM. Furthermore, *GATA2*$^{+/-}$ HSC numbers are compensated to normal levels in the adult, possibly due to HSC generation and expansion in the YS and the further expansion in the FL and BM. Notwithstanding, these data imply that the underlying molecular mechanisms in which the AGM generates, maintains and expands HSCs are different from that of the YS; The AGM region is exquisitely sensitive to the level of the *GATA2* dose, while the YS is much less sensitive. Hence, the HSC defects in *GATA2*$^{+/-}$ AGMs do not result in severe anemia in adults since *GATA2*$^{+/-}$ YS can generate and/or expand sufficient HSCs irrespective of the haploinsufficiency.

HSC qualitative processes are altered in *GATA2*$^{+/-}$ mice

The results of adult BM competitive transplantation experiments clearly demonstrate a qualitative difference between the *GATA2*$^{+/+}$ and $^{+/-}$ HSCs. The high

percentage of *GATA2*$^{+/-}$ mice engrafted with *GATA2*$^{+/+}$ cells, even at low donor cell numbers, demonstrate that *GATA2*$^{+/+}$ BM HSCs have a proliferative advantage over the *GATA2*$^{+/-}$ BM HSCs. In the reciprocal transplantation, in which *GATA2*$^{+/-}$ BM cells were transplanted into *GATA2*$^{+/+}$ recipients, high numbers of cells were needed to obtain a high percentage of donor engrafted recipient mice and thus imply that: 1) the number of HSCs in *GATA2*$^{+/-}$ BM is quantitatively reduced; 2) the *GATA2*$^{+/-}$ HSCs have a lower proliferative advantage over the *GATA2*$^{+/+}$ HSCs; and/or 3) the homing efficiency is lower for *GATA2*$^{+/-}$ derived HSCs. The fact that no significant difference in the percentage or absolute number of LSK BM cells was found between *GATA2*$^{+/-}$ and wild type BM indicates that the decreased HSC activity is not due to a quantitative decrease in *GATA2*$^{+/-}$ BM HSCs. However, the delayed expansion of HSCs in the 5-FU recovery experiments does strongly suggest that the major GATA2 dose dependent defect is in HSC proliferation. Although homing of HSCs is not required in this experimental scenario, we cannot exclude an additional GATA2 dose dependent defect in homing.

How does *GATA2* dose affect the quantitative and qualitative development of HSCs?

We propose here that the *GATA2* dose effects we observe in the AGM act at the level of the hemogenic cells that differentiate into HSCs. Normally, a full dose of *GATA2* is required for the generation, maintenance and/or expansion of these precursor cells. In the haploinsufficient AGM region, these hemogenic cells fail to differentiate, survive and/or divide. However, owing to the stochastic nature of gene expression, some hemogenic cells still achieve a threshold level of GATA2 protein and therefore, the target genes (which are needed for the differentiation, survival and/or division of the precursor cells) can be activated at some low frequency in the E10.5 AGM. The outcome of *GATA2* haploinsufficiency is then a small production of HSCs, followed by an overall reduction in the absolute number of AGM HSCs that we can detect functionally in our transplantation assay and phenotypically in immunostained sections and flow cytometry analysis.

Recent GATA2 expression data in the AGM support the notion of a role for GATA2 in hemogenic precursors. Transgenic embryos with a GFP marker under the control of *GATA2* transcriptional regulatory sequences show high levels of GATA2 expression in CD45^{-} AGM cells with hemogenic potential and a significant decrease in the percentage of CD45^{+} cells in *GATA2*$^{+/-}$ E11.5 AGMs [12]. Moreover, during midgestation, at the time of the first induction of HSCs, GATA2 is expressed in the endothelial cells lining the dorsal aorta and some underlying mesenchymal cells. Hence, high GATA2 expression in hemogenic cells of the AGM suggests that GATA2 is acting on the cells just immediately preceding the induction of HSCs.

Since GATA2 is a transcription factor, its target genes within hemogenic AGM cells are of particular interest. Several markers of AGM HSCs and aortic hemogenic cells have been recently described; the Ly-6A (Sca-1) cell surface glycoprotein [23, 32] and Runx-1 transcription factor [33]. These molecules are overlapping with GATA2 in their expression patterns in hemogenic cells of the dorsal aorta. Targeted mutation of

these genes results in qualitative and/or quantitative defects in HSCs. While *Ly-6A*$^{-/-}$ embryos thrive into adulthood with no or little effect on HSC generation in the embryo, functional analyses of HSCs derived from *Ly-6A*$^{-/-}$ mutant BM show defects in their self-renewal ability [34], similar to our findings in *GATA2*$^{+/-}$ BM. In contrast, *runx-1*$^{-/-}$ embryos are completely devoid of HSCs and exhibit FL anemia leading to lethality at E12 [35-37]. Moreover, *runx-1* haploinsufficiency leads to a premature extinction of AGM HSCs [20]. Hence, the *Ly-6A* and *runx-1* genes could be targets of GATA2 or alternatively, contribute to the activation of the same pathways for HSC self-renewal and/or HSC generation. At present, although many GATA consensus-binding sites appear in the sequences surrounding these genes, there is no *in vivo* data showing that any of these sites are functional.

Nonetheless, two bona fide target genes of GATA2 have been proposed. These are SCL/tal-1, an essential early hematopoietic transcription factor and E4bp4, a transcription factor implicated in cell survival. *In vivo* studies show that GATA2 forms a multiprotein complex with Fli-1 and Elf-1 that binds the *SCL* enhancer and activates the expression in HSCs, endothelial cells and their bipotent progenitor, the hemangioblast [38]. However, *in vivo* mutation analysis on the HSC specific GATA sites within the *SCL* locus affects SCL expression not only in the AGM but also in YS and FL [38]. Therefore, it is unlikely that the selective defect in the *GATA2*$^{+/-}$ AGM HSCs can be attributed to defective SCL expression. Chromatin immunoprecipitation studies on BaF3 cell line stimulated with IL-3 show that GATA2 binds to a sequence downstream of the transcriptional start site of E4bp4 and is necessary for transcriptional activation of this gene [39]. Considering that IL-3 is a survival factor for HSCs, it is plausible that GATA2 is involved in the activation of this pathway.

In conclusion, GATA2 dosage is important in regulation of HSC production and expansion. Haploinsufficiency of *GATA2* results in quantitative decreases in HSCs in the AGM and qualitative defects in HSCs in both the embryonic derived and adult BM HSCs. The pivotal importance of GATA2 in these processes within HSCs now awaits the identification of the relevant target genes and the functional cascades that GATA2 activates, most likely in concert with other factors in multiprotein complexes.

ACKNOWLEDGMENTS

The authors thank all the members of the laboratory for assistance in embryo dissection and transplantation and for helpful discussions, especially Drs. Kirsty Harvey, Catherine Robin and Marian Peeters. We also thank the people from the EDC Animal Facility (Erasmus MC, Rotterdam), especially Yasmine Allen, for upkeep of transgenic mice and transplant recipients. This work was supported by NIH (RO1 DK51077), Dutch Cancer Society (2001-2442 and 2002-2699) and the Association of International Cancer Research (02-243). SHO is an Investigator of the Howard Hughes Medical Institute.

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III

Gene expression profiling in mice with enforced GATA3 expression reveals putative targets of GATA3 in double positive thymocytes

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ABSTRACT

The zinc-finger transcription factor GATA3 is essential for early thymocyte development and differentiation of T helper 2 cells (Th2). In DP thymocytes GATA3 is specifically induced upon TCR stimulation in the context of MHC class II where it is essential for developmental progression of DP cells undergoing positive selection towards the CD4 lineage. However, direct target genes for GATA3 in thymocytes are currently unknown. Using gene expression profiling in transgenic mice with enforced GATA3 expression we have now identified putative targets of GATA3 at the DP T cell stage. By comparing gene expression profiles of GATA3 transgenic DP cells in the presence or absence of the MHC class II-restricted DO11.10, we identified putative GATA3 targets implicated in positive selection towards the CD4 lineage. We found that GATA3 expression in DP cells, specifically in those undergoing positive selection, is associated with down-regulation of the V(D)J-recombination machinery genes Rag1, Rag2 and TdT and of various signal transduction proteins. Importantly, we found that in these cells GATA3 regulates the expression of key genes involved in cellular proliferation and survival, including Mad4, c-Myc, Caf1/Btg1 and Cop9. Moreover, GATA3 induces the intracellular Notch inhibitor Pin1 and downregulates Notch1 transcription. As both *GATA3*-deficiency and expression of activated Notch inhibit the DP to SP transition, we propose that GATA3 is essential for termination of Notch signaling in developing CD4 SP thymocytes.

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 [1, 2]. Within the hematopoetic system GATA3 is essential for T cell development from the earliest stages and is involved in NK and invariant NKT cell differentiation [3, 4]. GATA3 is also expressed in non-hematopoietic tissues, including kidney, central nervous system, skin and mammary gland [5]. *GATA3*-deficient mice die at day 11 of gestation due to noradrenaline deficiency of the sympathetic nervous system [6, 7].

The essential function of GATA3 in early 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 [8, 9]. In this context, GATA3 function for T cell specification is dependent on Notch signaling [10]. 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 [11]. Analysis of *GATA3*-lacZ reporter mice showed that GATA3 expression is up-regulated in cells that have undergone β-selection [8]. Furthermore, conditional deletion experiments demonstrated that *GATA3*-deficient DN cells failed to undergo β-selection, indicating the essential role for GATA3 in this process [12]. After β-selection, cells upregulate expression of CD4 and CD8 and TCRα gene

segments are recombined, resulting in surface expression of TCR$\alpha\beta$ 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 [13, 14]. However, strong TCR signals trigger apoptosis, leading to negative selection. 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 [8, 15, 16]. 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 [12]. 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 [16]. Based on the observed induction of GATA3 by TCR stimulation in the context of MHC class II [8, 15, 16], we concluded that GATA3 probably acts in a positive feedback loop to upregulate TCR expression in developing CD4 SP thymocytes [16]. In contrast, GATA3 expression is down-regulated in CD8 SP thymocytes. Finally, in mature $CD4^+$ T cells, GATA3 acts a master regulator of Th2 differentiation, whereby GATA3 is crucial for transcription of IL-4, IL-5 and IL-13 in the Th2 cytokine locus and for the inhibition of Th1 and regulatory T cells [17-19].

Accurate regulation of GATA3 expression levels is essential for correct T cell development. Early overexpression of GATA-3 rapidly induced respecification of early T cell precursors to the mast cell lineage [20] and mice with enforced expression driven by the CD2 promoter display impaired CD8 T cell maturation and develop thymic lymphoma [21]. 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 [22]. When CD2-GATA3 transgenic DP cells additionally express the DO11.10 transgenic MHC class II-restricted TCR $\alpha\beta$ specific for ovalbumine, and are therefore selected to the CD4 lineage, DP cell size increase and lymphoma induction is enhanced [23].

In contrast to Th2 cells where the Th2 cytokine genes have been defined as GATA3 transcriptional targets, in thymocytes direct target genes for GATA3 have not been identified. Some of the genes, previously regarded as GATA3 target genes, such as CD8α or various TCR genes are expressed in the absence of GATA3 in $CD8^+$ T cells or in conditionally deleted thymocytes [12, 24, 25]. In addition, GATA3 may regulate c-Myc or CD5 expression in an indirect manner, e.g. involving the basic helix-loop-helix factor E2A [16].

In this report, we aimed to identify putative GATA3 targets in DP thymocytes. The reported induction of GATA3 within this cell population upon TCR stimulation, together with our findings that GATA3 overexpression affects DP cell size and induces DP T cell lymphoma, made DP cells the prime subset for the identification of putative GATA3 target genes. We compared gene expression profiles from total sorted DP cells with enforced GATA3 expression from CD2-GATA3 transgenic mice with wild-type cells. To specifically identify targets of GATA3 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 TCR transgene [23].

MATERIALS AND METHODS

Mice

The generation of CD2-GATA3 (FVB) [21] and CD2-GATA3:DO11.10 (BALB/c) have been described [16]. 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).

Flow cytometry, antibodies and cell sorting

The generation of single-cell suspensions and cell labeling for flow cytometry have been described previously [21]. Antibodies were purchased from BD Biosciences (San Diego, CA). FACS sorting of $CD4^+CD8^+7AAD^-$ thymocytes was performed with a FACSVantage VE, equipped with Diva Option and BD FACSdiva software. Purity of fractions was >99%.

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, St. Louis, MO). The quantity and quality of RNA was determined using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE). Samples with a 260/280 nm optical density ratio >1.8 were used. Two μg of total RNA was used for amplification using T7 MEGAscript Kit (Ambion, Austin, TX), whereby aminoallyl-UTP (Ambion) was incorporated into amplified RNA (aRNA). Subsequently, Cy5- or Cy3-dye (Amersham, GE Healthcare, Piscataway, NJ) was coupled to the aminoallyl-modified aRNA. Labeled aRNA 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 thymocytes ($n = 6$) and scanned with a Scanarray Express HT scanner (Perkin Elmer, Boston, MA). Data were extracted using Imagene software 6.0 (Biodiscovery, CA). Each experiment consisted of 2 oligonucleotide arrays, whereby dyes were reversed between aRNA 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 data

were filtered for genes that were differentially expressed in experiments. Hierarchical clustering of genes and experiments was performed using Genesis 1.5.0 [26].

Semi-quantitative and quantitative PCR analysis

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

Semiquantitave 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 Table 1. 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 C_t values of the genes of interest were normalized to the C_t value of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

RESULTS AND DISCUSSION

Identification of differentially expressed genes as a result of enforced GATA3 expression

The DP compartment in the thymus is very 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 T cells, allowing the identification of genes induces or repressed by GATA3 in a heterogeneous DP population containing many cells in which GATA3 is normally not induced. We also compared 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 3 individual 11-week-old mice from the following 4 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 3 individual mice present

Table 1. Primers and probes used for RT-PCR analysis for expression of the indicated genes.

Gene	Forward primer	Reverse primer	Probe
β-actin	TACCACTGGCATCGTGATGGACT	TTTCTGCATCCTGTCGGCAAT	
c-Myc	GCAGCTGTTTGAAGGCTGGAT	GTCGCAGATGAAATAGGGCTGT	CACGACGATGCCCCTCAACGTGA
Ctla-4	CTTGGCCTTTTGTAGCCCTG	CACATGGAAAGCTGGCGACA	AGCCATACAGGTGACCCAACCTTCAGTG
Gapdh	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	TGCATCCTGCACCACCAACTG
GATA3	CATTACCACCTATCCGCCCTATG	CACACACTCCCTGCCTTCTGT	CGAGGCCCAAGGCACGATCCAG
Granzyme A	TTGGACTAAACATGATTTGTGCAG	AGGTGATGCCTCGCAAAATAC	
IL-18R	ATCCAGGACACATGGCTGTATAAG	CGCAGGAGTAGTAGCCCTCATC	
Rag1	AGGCCTGTGGAGCAAGGTA	GCTCAGGGTAGACGGCAAG	ATGGCTGC
Rag2	TGCCAAAATAAGAAAGAGTATTTCAC	GGGACATTTTTGATTGTGAATAGG	GCAGGAAG
Ror-α	CTGGCTTCTTCCCCTACTGTTC	TTTCCAGGTGGGATTTGGATAT	
VldlR	AGCAGGCAATGCAATGGC	TGCACTTGAACTTTCCAGGGC	

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 (Figure 1A). 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 (Figure 1B). 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 up-regulated or ≥1.4 fold downregulated 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.

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 to D, Figure 2) 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 DO.11.10 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 GATA3 transgene.

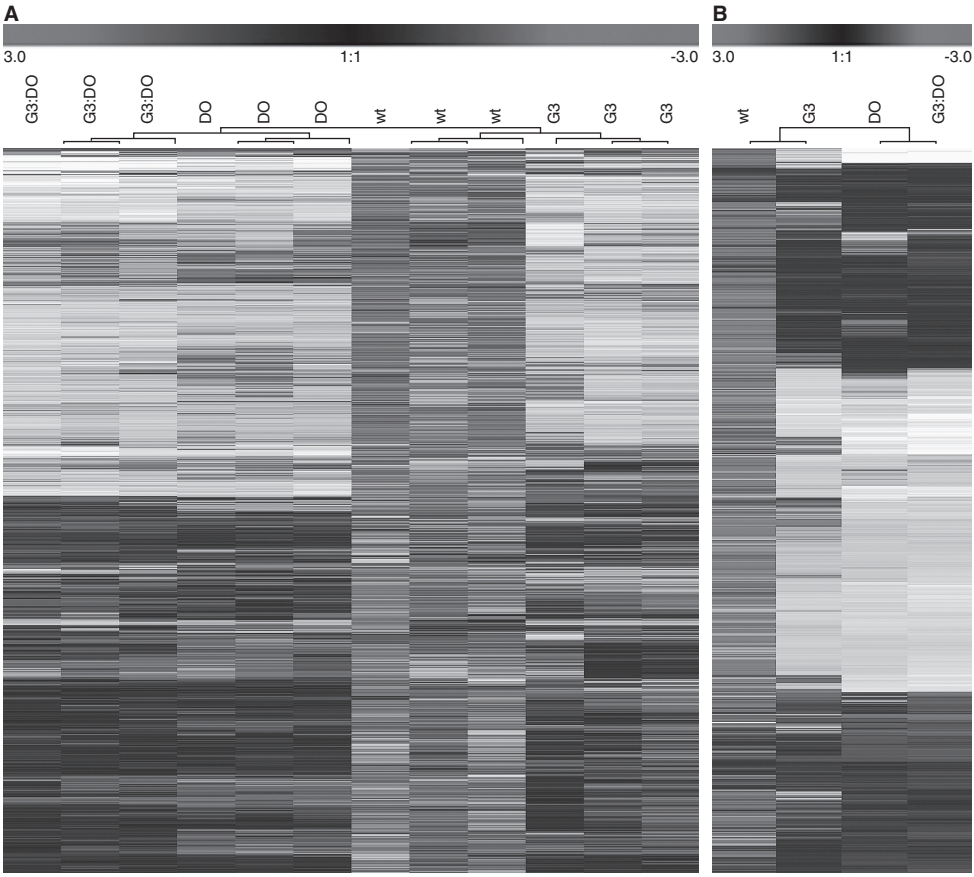

Figure 1. Identification of specific gene expression signatures in DP cells as a result of enforced GATA3 expression. Gene expression profiles were generated from FACS-sorted DP cells from wild-type (wt) mice and CD2-GATA3 transgenic (G3), DO11.10 TCR transgenic (DO) and CD2-GATA3:DO11.10 double transgenic (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 matrixes indicate the average linkage clustering of respectively individual mice and genes. Green and red colors indicate the level of down-regulation or up-regulation 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 values numbers represent ^{2}log values. (See Appendix page 217 for a full-color representation of this figure).

The 276 genes of clusters A to 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 DP cells. The A/D cluster therefore represents genes that were up- or down-regulated by transgenic GATA3

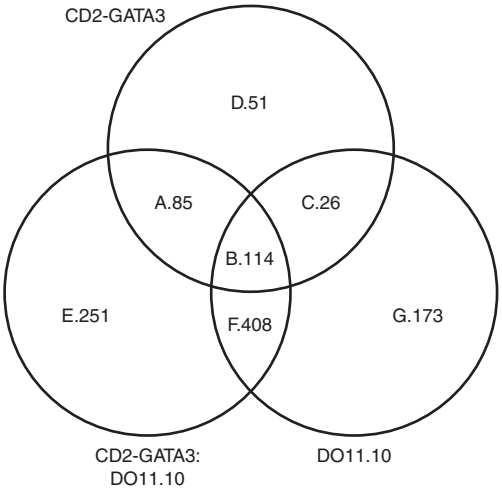

Figure 2. Differentially expressed genes in DP thymocytes from CD2-GATA3 and DO11.10 single transgenic and CD2-GATA3:DO11.10 double transgenic mice, displayed in a Venn diagram. Genes are either up- or down-regulated 1.4 fold (p<0.0001) and are not differentially expressed in a pool of DP cells of wild-type mice. Numbers of differentially expressed genes per cluster are given.

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 up- or down-regulated by transgenic GATA 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 DO.11.10 transgenic DP cells undergoing positive selection towards CD4 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 up- or down-regulated 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 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).

Putative GATA3 target genes that are not implicated in positive selection

First, we focused on cluster A/D containing 136 genes, which were induced or repressed by GATA3 in DP cells, irrespective of their cell fate, but were not implicated in positive selection (Table 2). We found a striking 18.5 fold up-regulation of very low density lipoprotein receptor, which is like apolipoprotein E (2.5 fold up-regulated) involved in lipoprotein metabolism [27]. Together with the up-regulation of multiple

genes associated with structural cellular organization, including thrombospondin-2, 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 targets in lymphomagenesis and cell size regulation [22]. In addition, we found induction of Ctla4, which has been reported to positively regulate activation of DP thymocytes, resulting in their deletion [28] and IL-18 receptor.

Interestingly, we noticed a ~2-fold down-regulation of the transcription factors Tcf1 and Lef1 (Table 2), both of which are regulated by the WNT/beta-catenin signaling pathway and have been implicated in a subset of peripheral T cell lymphomas [29, 30]. 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 [16]. Furthermore, GATA3 expression down-regulated ~1.7 fold the SLAM-associated protein SAP, which is involved in TCR signaling.

Taken together, this gene cluster mainly consists of genes implicated in cell size regulation and lymphomagenesis, including c-Myc and cyclin D2, consistent with the notion that this cluster would mainly reflect the outcome of aberrant GATA3 expression in cells that normally do not receive TCR-mediated signals for positive selection towards the CD4 cell lineage.

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 3). In this group, we surprisingly found upregulation of granzyme A, which is expressed in $CD8^+$ T cells and NK cells and ROR-α, which was recently implicated in Th17 cell differentiation [31]. Interestingly, a natural mutant strain with a disrupted expression of ROR-α, termed *stagger* mice, have reduced thymic and splenic cellularity associated with high a high apoptosis rate of DP thymocytes [32, 33]. However, no defects in thymocyte development were observed in lymphocyte-specific ROR-α-deficient mice, indicating an indirect effect of ROR-α deficiency on thymocyte development [34]. Differential expression of granzyme A and ROR-α was confirmed by semi-quantitative PCR, by which also upregulation of the cluster A/D genes Vldlr and IL-18R could be verified (Figure 3A and 3B). In addition, IL-17BR, IL-12Rβ1 and Stat4 involved in cytokine signaling, and CD44, Lmo4, and Flt3-ligand were induced. Transcriptional regulators, such as, Sp3, Sp4, and Id3 were ~2 fold down-regulated (Table 3). The transcription factor Id3 has been shown to be an important regulator for thymocyte selection, via the regulation of E2A [35]. This in combination with our finding in CD2-GATA3 transgenic DP cells of increased expression of E2A and reduced expression of CD5, might indicate a role of GATA3 in downregulating CD5 via Id3 during thymocyte selection [8, 15, 16].

Interestingly, 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 [36, 37].

Table 2. GATA3 target genes differentially expressed in clusters A and D.

Genes up-regulated ≥1.4 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥1.4 fold	Source*	G3	DO	G3: DO
Very low-density lipoprotein receptor (VldlR)	P98156	18.5	0.5	9.0	Matrix metalloproteinase 14	P53690	0.4	0.9	0.5
Thrombospondin 2	Q03350	13.9	0.8	8.6	Sodium/nucleoside cotransproter 2	O88627	0.4	1.1	0.5
Alpha-actinin 2	Q9JI91	10.8	2.3	7.7	Epithelial membrane protein-1	P47801	0.5	0.7	0.4
Protein tyrosine phosphatase. non receptor type 13	NM_011204	9.9	0.7	6.3	5'-TG-3' interacting factor	P70284	0.5	0.8	0.6
Neuroendocrine convertase 1	P21662	9.8	1.7	6.5	Calcitonin receptor-like	NM_018782	0.5	0.6	0.4
ODD OZ/TEN-M homolog 4	NM_011858	7.7	0.8	4.3	Protein tyrosine phospatase 4A3	NM_008975	0.5	0.8	0.6
Collagen α 2(I) chain	Q01149	6.1	1.6	6.6	GTP-binding protein GEM	P55041	0.5	0.4	0.3
Reduced expression 3	NM_009052	6.1	0.7	3.6	Lymphoid enhancere binding factor 1 (Lef1)	P27782	0.5	0.7	0.7
Interleukin 18 receptor 1	Q61098	5.0	1.6	9.2	Solute carrier family 37	NM_020258	0.5	0.7	0.3
Cytotoxic T-lymphocte protein 4 (Ctla4)	P09793	4.7	0.9	2.8	T cell surface glycoprotein CD5	P13379	0.5	1.4	0.8
Revesrion induce LIM gene	NM_019417	4.5	2.0	6.5	Tristetraproline	P22893	0.5	0.8	0.5
Sam domain and HD domain-containing protein 1	Q60710	4.1	0.7	3.2	Sodium/potasium-transporting atpase beta-1 chain	P14094	0.5	0.9	0.4
Transferrin	Q921I1	4.1	0.8	3.7	T cell specific transcription factor (Tcf1)	Q00417	0.5	0.6	0.5
Interferon consensus sequence binding protein	P23611	3.9	1.3	3.4	Similar to circadian oscillatory protein	Q8QZU8	0.5	0.8	0.7
MYC proto-oncogene (c-Myc)	P01108	3.8	1.1	3.9	Immune associated nucleotide family member 6	NM_153175	0.5	1.4	0.6
Similar to DKFZP564O0823 protein	NM_145562	3.8	0.7	2.5	T cell transduction molecule SAP	O88890	0.5	0.8	0.6
Trans acting T-cell specific transcription factor GATA3	P23772	3.6	1.0	3.1	Brain-muscle-ARNT-like protein2	NM_172309	0.5	3.1	1.4
ERO1-like	NM_015774	3.0	1.2	2.7	Phosphoribosyl pyrophosphate synthetase 2	NM_026662	0.5	0.7	0.4
Cerebellar degeneration-related 2	NM_007672	2.8	1.2	2.1	Similar to diaglycerol kinase	Q91YS0	0.6	0.8	0.6
Myosin IF	P70248	2.8	1.0	1.9	Dead ringer homolog 2	NM_019689	0.6	0.7	0.6
Glucosamine-fructose-6-phospahte aminotransferase	P47856	2.8	1.3	1.7	Inducible 6-phosphofructo-2-kinase	NM_172976	0.6	0.7	0.6
Apolipoprotein E (Apo-E)	P08226	2.7	1.6	6.1	NDRG3 Protein	Q9QYF9	0.6	1.0	0.5
Cyclin D2	P30280	2.6	0.8	3.3	C-X-C chemokine receptor type 4 (Cxcr4)		0.6	0.8	0.5
Glucocorticoid receptor	P06537	2.3	1.3	2.2	EH-domain containing protein 3	Q9QXY6	0.6	0.8	0.4
Probable G protein-coupled receptor GPR72	P30731	2.3	0.8	2.2	C-ETS-2 protein	P15037	0.6	0.7	0.3
Phosphatidylinositol-4-phospahte 5-kinase	NM_008846	2.2	0.8	0.6	Makorin 1	Q9QXP6	0.6	0.8	0.6
Calcium/calmodulin-dependent protein kinase II	NM_023813	2.2	0.9	2.1	EMU1 protein	NM_080595	0.6	0.8	0.6
Insig-1 membrane protein	NM_153526	2.2	0.9	1.6	ELAV-like protein 4	P70372	0.6	0.6	0.7
Similar to LD47277P	Q8K2Q6	2.2	0.7	2.0	Cyclin I	Q9Z2V9	0.6	0.8	0.9
Cytosol aminopeptidase	Q9CPY7	2.2	1.0	3.1	D15WSU75E protein	NM_134095	0.6	0.7	0.5
Splicing factor U2AF 65 kda subunit	P26369	2.1	1.7	2.3	Interleukin 16	NM_010551	0.6	0.8	0.6
Matrilin-2	O08746	2.1	0.8	1.9	Solute carrier family 12 member 6	Q924N4	0.6	0.6	0.5
Thrombin receptor	P30558	2.0	0.8	1.6	G protein kinase 2	Q99MK8	0.6	0.8	0.7
Spermidine synthase	Q64674	2.0	0.9	2.1	Lymphocye specific formin related protein	NM_019679	0.6	0.8	0.7
Acetyl-coa carboxylase 265	Q925C4	1.9	0.8	1.8	Putative phospatase	NM_008916	0.6	0.9	0.7
Annexin A11	P97384	1.9	0.8	1.6	Glucosamine-6-phosphate isomerase	O88958	0.6	0.7	0.7
SH3 domain-binding protein 5	Q9Z131	1.9	1.7	2.1	HASH	NM_026470	0.6	0.9	0.6
TOB1 protein	Q61471	1.9	1.4	2.1	RAS-related protein RAB-33B	O35963	0.6	0.8	0.7
CDNA sequence BC019755	NM_145395	1.8	1.1	2.5	Inosine triphosphatase	NM_025922	0.6	0.7	0.6

Table 2. GATA3 target genes differentially expressed in clusters A and D (continued).

Genes up-regulated ≥1.4 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥1.4 fold	Source*	G3	DO	G3: DO
ND1-S	NM_028582	1.8	1.8	2.0	Microsoamal glutathione S-transferase 3	NM_025569	0.6	0.9	0.5
Mitochondrial import inner membrane translocase	Q9WV98	1.8	1.3	1.9	Myosin regulatory light chain 2	P97457	0.6	0.9	0.6
RAC/CDC42 guanine nucleotide exchange factor	NM_152801	1.8	1.5	1.7	P150 target of rapamycin (Tor)	Q8K4Q0	0.6	0.8	0.6
Galectin-3	P16110	1.7	1.3	2.3	RHO GTPase activating protein 4	NM_138630	0.7	1.1	0.8
Arginine/serine-rich splicing factor 10	Q15815	1.7	1.7	1.7	RAS-related protein RAL-A	P05810	0.7	0.8	0.7
Centrin2	Q9R1K9	1.6	1.4	1.7	Thyroid hormone receptor associated protein		0.7	0.7	0.8
Splicing factor. arginine/serine-rich 1	NM_173374	1.6	1.5	1.7	Mitxantrone resistance protein 1	NM_011920	0.7	0.9	0.6
Phosphoserine aminotransferase	Q99K85	1.6	1.0	2.0	Glycerol-3-phosphate dehydrogenase (NAD^+)	P13707	0.7	0.8	0.9
Cyclin-dependent kinase inhibitor 1B (P27KIP1)	P46414	1.6	1.5	1.5	Leukocyte common antigen precursor (CD45)	P06800	0.7	0.7	0.6
WW domain binding protein 5	Q9DD24	1.6	0.8	1.2	Splicing factor 3b subunit	Q99NB9	0.7	0.7	0.5
40S ribosomal protein S11	P04643	1.6	1.4	1.7	Copine 1	NM_170588	0.7	0.7	0.6
Similar to ribosomal protein S20	Q921M2	1.6	1.4	1.6	Ubiquitin-like 5	NM_025401	0.7	0.8	0.7
Similar to dendritic cell protein	NM_145380	1.5	1.3	1.2	ADP-ribosylation-like factor 6 interacting protein 2	NM_019717	0.7	0.8	0.4
14-3-3 protein zeta/delta	P35215	1.5	1.4	1.5	Cystinosin	P57757	0.7	0.8	0.6
Guanine nucleotide binding protein	NM_025277	1.5	1.2	1.5	F-actin capping protein alpha-1 subunit	P47753	0.7	0.7	0.7
Protein kinase C and casein kinase substrate 2	Q9WVE8	1.5	1.0	1.7	Kinesin-associated proetein 3	P70188	0.7	0.8	0.8
ADP-ribosylation factor 1	P32889	1.5	1.6	1.6	ATP-dependent DNA helicase II. 70 kda subunit	P23475	0.7	0.7	0.5
Ubiquitin carboxyl-terminal hyydrolase isozyme L5	Q9WUP7	1.5	1.4	1.6	Ornithine decarboxylase antizyme	P54369	0.7	0.9	0.7
Transmembrane protein 4	NM_019953	1.5	1.3	1.6	Histone 1	NM_023422	0.7	0.7	0.7
ADP. ATP carrier protein. isoform T1	P48962	1.5	1.2	1.3	SH2-B PH domain containing signaling mediator 1	NM_011363	0.7	0.9	0.7
Fyn binding protein	O35601	1.4	1.2	1.4	Similar to histone H2A.F/Z variant	Q8R029	0.7	1.0	0.7
Similar to ubiquitin-conjugating enzyme E2 variant 1	Q8VEB5	1.4	1.3	1.6	Axin 1 up-regulated gene 1	P59054	0.7	0.7	0.6
Bone morphogenetic protein 7	P23359	1.4	0.6	1.6	Fibulin-2	P37889	0.7	0.7	0.5
Cytochrome B-245 light chain		1.4	1.3	1.4	Protein translation factor SUI1 homolog	P48024	0.7	0.9	0.7
Hyaluronidase 2		1.4	1.1	1.6	Enhancer of zeste homolog 2	Q61188	0.7	0.8	0.7
LUC7 homolog	NM_025881	1.4	1.7	1.5	Lysosomal-associated multitrasnmembrane protein	Q61168	0.7	0.8	0.7
Complement component 1	O35658	1.4	1.0	1.5	ACYL-COA-bindin protein	P31786	0.7	0.8	0.7
Mitochondrial import inner membrane translocase	Q9WVA2	1.4	1.0	1.5	Similar to programmed cell death 6 interacting protein	Q8K2Q7	0.7	0.8	0.6

Genes were sorted on the gene expression level in CD2-GATA3 transgenic mice. Numbers indicate the relative gene expression level compared to wild-type mice.
* RefSec. Swissprot or Sptrembl. G3 = CD2-GATA3. DO = DO11.10. G3:DO = CD2-GATA3:DO11.10

Table 3. GATA3 target genes differentially expressed in clusters B and C.

Genes up-regulated ≥1.4 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥1.4 fold	Source*	G3	DO	G3: DO
Granzyme A	P11032	5.0	1.6	5.2	Ephrin type-A receptor 2	Q03145	0.3	0.1	0.1
Nuclear receptor Ror-α	P51448	4.8	1.7	6.6	Pleckstrin homology-like domain. family A. member 1	NM_009344	0.3	0.5	0.4
Flt3 ligand	Q9JIP3	4.4	2.4	6.8	M-phase inducer phospatase	P30306	0.4	0.6	0.6
Tumor necrosis factor ligand superfamily member 14	Q9QYH9	3.8	1.9	2.3	Death associated protein kinase 1	NM_029653	0.4	0.4	0.1
CD44	P15379	3.3	2.6	3.9	Protein-tyrosine phosphatase epsilon	P49446	0.4	0.5	0.2
RNA-binding protein FUS	P56959	2.9	4.6	2.8	Lymphocyte antigen 108	NM_030710	0.4	0.7	0.3
Alpha-2.8-sialyltransferase	Q64687	2.8	1.7	2.3	Solute carrier family 15 (H^+/peptide transporter)	NM_021301	0.4	0.0	0.0
Elav-like protein 4	Q61701	2.4	0.2	0.3	Ziinc ring finger protein 1	NM_133206	0.4	0.5	0.3
Natural resistance-associated macrophage protein 2	P49282	2.4	0.6	1.1	Nuclear protein 95	NM_010931	0.4	0.4	0.5
Putative 40-2-3 protein	NM_027226	2.4	2.1	2.8	Terminal deoxynucleotidyltransferase (TdT)	P09838	0.4	0.2	0.1
Prolin-rich protein 7	O35327	2.4	2.4	3.3	Retinal short-chain dehydrogease/reductase	NM_011303	0.4	0.2	0.2
Type II 65kd keratin	Q99M73	2.3	2.1	2.9	Dual specificity protein phosphatase	O09112	0.5	0.3	0.3
Protein kinase C. zeta type	Q02956	2.2	1.6	2.1	Glypican	NM_016696	0.5	0.4	0.3
Phosphatidylinositol transfer protein beta isoform	P53811	2.2	1.7	1.8	Interleukin-10 receptor alpha chain	Q61727	0.5	0.6	0.6
Camp-dependent protein kinase	P05206	2.2	2.1	2.1	F-box and WD-40 domain protein 7	NM_080428	0.5	0.5	0.5
Voltage-dependent anion-selective channel protein 3	Q60931	2.1	1.8	2.5	Channel-interacting PDZ domain protein	NM_007704]	0.5	0.4	0.3
Flt3 ligand	P49772	2.0	1.8	2.5	Endothelial monocyte-activating peptide	P50543	0.5	1.5	1.0
Embigin	P21995	2.0	2.3	2.8	Protein 4.1	P48193	0.5	0.5	0.3
Prostaglandin E2 receptor EP4 subtype	P32240	2.0	2.0	2.4	DNA methyl transferase 3A	O88508	0.5	0.7	0.7
Egl nine homolog 3	NM_028133	2.0	1.5	1.9	Transcription factor Sp3	O70494	0.5	0.5	0.5
Surfeit locus protein	P09926	1.9	1.6	1.9	Diaglycerol kinase gamma	Q91WG7	0.5	0.4	0.3
5'-methylthioadenosine phosphorylase	Q9CQ65	1.9	1.8	2.7	Ribinucleoside-diphosphate reductase	P11157	0.5	0.6	0.6
SAM-domain protein SANSN-1	P57725	1.9	2.2	2.3	Lymphoid-restricted membrane protein	NM_008511	0.6	0.6	0.6
Adrenodoxin	P46656	1.9	1.6	1.9	H-2 class I histocompatibility antigen	P01898	0.6	0.3	0.2
LIM domian transcription factor Lmo4	O00158	1.8	2.4	2.9	G2/mittotic-specific cyclin B1	P24860	0.6	0.6	0.7
Trans-acting transcription factor Sp4	NM_009239	1.8	2.5	2.0	Sphingolipid delta 4 desaturase/C-4 hydroxylase	NM_027299	0.6	0.4	0.4
Cell cycle related kinase	NM_053180	1.8	0.6	0.9	Similar to REV/REX activstion domain binding protein	NM_145566	0.6	0.5	0.4
Bifunctional aminoacyl-tRNA synthase	Q9CRF9	1.8	2.1	2.9	Transgelin 2	Q9WVA4	0.6	0.7	0.7
Signal and transducer and activator of transcription 4	P42228	1.8	3.0	3.0	Death associated protein kinase 1	NM_029653	0.6	0.5	0.3
Regulatory protein Tsc22	Q00992	1.7	2.5	2.1	Lipin 1	Q91ZP3	0.6	0.7	0.6
X-linked lymphocyte-regulated 4	NM_021365	1.7	1.7	1.2	Coiled-coil transcriptional coactivator	NM_026192	0.6	0.5	0.4
Exosome complex exonuclease Rrp42	Q9D0M0	1.7	1.3	1.6	Caspase 2	P29594	0.6	0.5	0.5
BCR downstream signalling 1	NM_019992	1.7	1.7	2.1	Transient receptor potential cation channel	NM_011706	0.6	0.7	0.5
Pre-mRNA splicing factor Srp55	Q9CSJ3	1.7	1.6	1.6	Camp regulated phosphoprotein 21	Q9DCB4	0.6	0.6	0.4
Cyclin ANIA-6A	NM_019937	1.7	1.8	1.5	Placental protein 11 related	NM_008902	0.6	0.5	0.4
Signal recognition particle 54 kda protein	P14576	1.6	2.4	2.1	Neurogenic locus notch homolog protein 1 (Notch1)	Q01705	0.6	0.7	0.7
Metal response element binding transcription factor 2	Q02395	1.6	1.7	1.8	RNA-binding protein EWS	Q61545	0.6	0.7	0.7
Calcineurin B subunit isoform 1	Q63810	1.6	2.1	1.8	Retinitis pigmentosa gtpase regulator interacting protein 1	NM_023879	0.6	0.3	0.3
EURL protein homolog	Q9D7G4	1.6	2.0	1.4	Histone H1.2	P15864	0.6	0.6	0.4
Hematopoietic cell signal transducer	NM_011827	1.6	1.5	2.0	Interferon-activatable protein 204	P15092	0.6	0.4	0.2

Table 3. GATA3 target genes differentially expressed in clusters B and C (continued).

Genes up-regulated ≥1.4 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥1.4 fold	Source*	G3	DO	G3: DO
Dual specificity protein phosphatase 2	Q05922	1.6	1.9	1.7	High mobility group nucleosomal binding domain 3 isoform	NM_026122	0.6	0.4	0.2
Regulator of g-protein signalling 10	Q9CQE5	1.6	2.0	2.3	Cytidine monophospho-N-acetylneuraminic acid hydroxylase	NM_007717	0.6	0.5	0.3
28S ribosomal protein S18B	Q99N84	1.6	1.6	1.9	Similar to kinesin light chain KLCT	NM_146182	0.6	0.6	0.3
AU RNA-binding enoyl-coenzyme A hydratase	NM_016709	1.6	1.5	1.5	G protein-coupled receptor 69	NM_021295	0.6	0.5	0.4
Nucleolysin TIA-1	P52912	1.6	1.8	1.8	Treacle	NM_011552	0.6	0.5	0.5
TRIF	NM_019706	1.5	1.5	1.8	Pericentiolar material 1	NM_023662	0.6	0.6	0.6
Interleukin-12 recepeptor beta-1 chain	Q60837	1.5	1.9	2.3	SWI/SNF related regulator of chromatin	NM_053124	0.6	0.6	0.6
Membrane spanning 4-domains. subfamily A. 6B	NM_027209	1.5	2.8	3.3	Cytochrome B5	P56395	0.6	0.7	0.5
Protein-tyrosine phosphatase. non-receptor type 2	Q06180	1.5	1.5	2.0	DNA-binding proetein inhibiter ID-3	P41133	0.6	1.7	0.8
Pyruvate kinase. M2 isoenzyme	P52480	1.5	2.0	2.0	Polyhomeotic-like 2	NM_018774	0.6	0.7	0.7
Tumor susceptibility gene 101 protein	Q61187	1.5	1.8	1.6	Pituitary tumor transforming 1	NM_013917	0.6	0.7	0.6
Putative SP100-related protein	Q99388	1.5	2.0	1.9	BCL2/adenovirus E1B 19-kda	O54940	0.6	0.8	0.6
Nucleoporin 50kda	Q9JIH2	1.5	1.6	1.7	Similar to retinoblastoma binding protein 8	Q8VE67	0.6	0.7	0.7
Lysosomal-associated protein transmembrane 4B	NM_033521	1.5	0.6	0.8	DNAJ homolog subfamily B. member 9	Q9QYI6	0.6	0.6	0.5
Methionine aminopeptidase	O08663	1.5	1.3	1.5	Alpha-1 catenin	P26231	0.6	0.3	0.3
Dihydrolipoamide dehydrogenase	O08749	1.5	1.6	1.5	Etoposide-induced protein 2.4	Q61070	0.6	0.5	0.6
TFIIH basal transcription factor complex P52 subunit	O70422	1.4	2.7	2.1	Similar to neurofilament heavy polypeptide 200kd	Q8R3A0	0.6	0.6	0.5
Actin related protein 2/3 complex. subunit 5	NM_026369	1.4	1.6	1.4	Similar to proline-rich protein 48	Q8R5A3	0.7	0.7	0.5
Zinc finger protein 265	Q9R020	1.4	1.3	1.4	Zinc finger protein 147	Q61510	0.7	0.6	0.5
H-2 class I histocompatibility antigen	P14432	1.4	2.7	2.1	Solute carrier family 23. member 1	Q9EPR4	0.7	0.6	0.5
Zinc finger protein 313	Q9ET26	1.4	1.7	1.4	Protein tyrosine phosphatase alpha	P18052	0.7	0.5	0.5
Cell division protein kinase 4	P30285	1.4	1.6	1.9	Pptassium voltage-gated channel subfamily H member 2	O35219	0.7	0.6	0.4
Solute carrier family 25	NM_172436	1.4	2.2	2.7	CGMP-gated cation channel alpha 1		0.7	0.6	0.3
Protein-L-isoaspartate (D-aspartate)		1.4	1.7	1.9	Caspase 8 associated protein 2	NM_011997	0.7	0.6	0.6
SWI/SNF related regulator of chromatin. subfamily B1	Q9Z0H3	1.4	1.8	1.7	TOB2 protein	Q9JM55	0.7	0.7	0.6
60S acidic ribosomal protein P0	P14869	1.4	1.4	1.7	BH3 interacting domain death agonist	P70444	0.7	0.7	0.8
Alsin	NM_028717	1.4	0.4	0.6	TBC1 domain family protein C22ORF4 homolog	Q8R5A6	0.7	0.5	0.5
					Similar to microsomal glutathionine S-transferase 2	Q8R032	0.7	1.7	1.3
					Similar to spermatogenesis associated 2	NM_170756	0.7	0.7	0.6
					Er degradation enhancing alpha mannosidase-like	NM_138677	0.7	0.7	0.6
					Leucine-rich repeats and immunoglobulin-like domains 1	NM_008377	0.7	0.3	0.3
					Protein phosphatase 4. regulatory subunit 1	NM_146081	0.7	0.8	0.7
					WW domain binding protein	NM_021714	0.7	0.6	0.6

Genes were sorted on the gene expression level in CD2-GATA3 transgenic mice. Numbers indicate the relative gene expression level compared to wild-type mice.
* RefSec. Swissprot or Sptrembl. G3 = CD2-GATA3. DO = DO11.10. G3:DO = CD2-GATA3:DO11.10

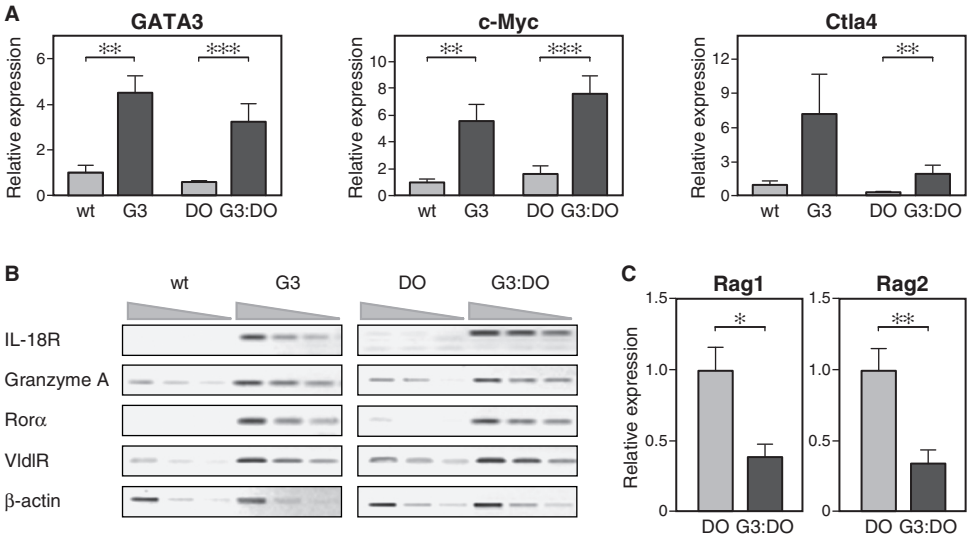

Figure 3. Quantitative gene expression analysis of genes up-regulated as a result of enforced Gata3 expression in DP thymocytes. (A) Quantitative RT-PCR analysis of GATA3, c-MYC and CTLA4 expression in sorted DP cells from wild-type (wt), CD2-GATA3 (G3), DO11.10 (DO) and CD2-GATA3:DO11.10 (G3:DO) transgenic mice. Average values ± SEM are displayed and values indicate the ratio between the expression of the indicate gene and gapdh. Expression levels in wild-type DP cells were set to 1. For statistical analysis a t-test was performed (** = p<0.01 and *** = p<0.001). Data are from 4-10 mice per group. (B) Semi-quantitative PCR analysis for IL-18R, Granzyme A, ROR-α, VldlR and β-actin on serial 3-fold cDNA dilutions prepared from sorted DP cells of the indicated mice. Data are representatives of 4 mice analyzed per group. (C) Quantitative RT-PCR analysis of Rag1 and Rag2 expression in sorted DP cells from DO11.10 (DO) and CD2-GATA3:DO11.10 (G3:DO) transgenic mice. Average values ± SEM are displayed and values indicate the ratio between the expression of the indicate gene and Gapdh. Expression levels in wild-type DP cells were set to 1. For statistical analysis a t-test was performed (* = p<0.05 and *** = p<0.01). Data are from 5-8 mice per group.

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 [12, 21].

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

Cluster E contained genes that were up- or down-regulated 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 at the CD4 SP stage [16]. 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 4). Among the up-regulated genes, Mad4 a suppressor of c-Myc [38], G protein G-alpha13 and CD62L were present. In agreement with upregulation of CD62L transcription, we previously noticed increased surface expression of CD62L on CD4 SP cells by flow-cytometric analysis in CD2-GATA3 transgenic mice [39]. Enforced GATA3 expression also upregulated the expression of the Cop9 signalosome subunit SGN2, which has the ability to activate the basic region-leucine zipper transcription factor c-Jun that regulates gene expression and cell function [40]. Recently, the Cop9 signalosome was found to be essential for T cell differentiation in the thymus, as conditional deletion of the CSN5/JAB1 catalytic subunit of Cop9 signalosome resulted in impaired transition of DN4 to DP cells that was associated with massive apoptosis [41]. Likewise, conditional deletion of the CSN subunit Csn8 in peripheral T lymphocytes established its essential function for peripheral T cell homeostasis and antigen receptor-induced entry into the cell cycle from quiescence [42].

Interestingly, transgenic GATA3 expression up-regulated transcription of the peptidyl-prolyl-isomerase Pin1. This is an important regulator of cell proliferation and differentiation in the thymus, as it is required for the timely activation of p53 and is a negative regulator of intracellular Notch1 levels [43]. Thus, it is very well possible that GATA3 has the capacity to terminate Notch1 signaling at the DP to SP transition, by two separate mechanisms: downregulation of Notch1 expression (see above) and induction of Pin1. An important function for GATA3 as a negative regulator of Notch1 signaling would be supported by transcriptome analysis of laser-dissected GATA3-deficient hair follicles, which revealed overrepresentation of the Notch signaling pathway [44]. Interestingly, transgenic mice expressing intracellular Notch1 demonstrated that Notch activity inhibits the DP to SP transition by abrogating TCR signaling [45], demonstrating that termination of Notch1 signaling is essential for the generation of SP cells. As both GATA3-deficiency and expression of activated Notch inhibit the DP to SP transition, we propose that GATA3 is essential for termination of Notch signaling in developing CD4 SP thymocytes.

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 4). Reduced gene expression of Rag1 and Rag2 in was verified by PCR analysis (Figure 3C). In the promoter of the Rag2 gene a GATA-3 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 [46]. 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 TCR gene rearrangement.

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

Table 4. GATA3 target genes differentially expressed in cluster E.

Genes up-regulated ≥1.6 fold	Source*	G3	DO	G3: DO	Genes down-regulated ≥1.6 fold	Source*	G3	DO	G3: DO
Max-interacting transcriptional repressor Mad4	Q60948	3.8	1.3	8.9	Secreted modular calcium-binding protein 1	NM_022316	0.5	0.4	0.1
Trb2	NM_144551	1.9	0.9	4.7	H-2 class I histocompatibility antigen (H-2K(B))	P01901	0.6	0.4	0.3
Ubiquitin carboxyl-terminal hydrolase 14	Q9JMA1	2.6	1.1	4.0	DNA nucleotidylexotransferase (TdT)	P09838	0.6	0.7	0.3
3110009E13RIK protein	Q9CXT1	1.6	1.4	3.9	Glutathionine S-transferase 5.7	P24472	0.6	0.6	0.3
Melanin concentrating hormone receptor interacting	NM_026021	3.3	1.3	3.7	Tyrosine protein kinase Tec	P24604	0.6	0.7	0.3
3'-5' Exoribobuclease Csl4 homolog	Q9DAA6	2.4	1.5	3.6	L-lactate dehydrogenase B chain	P16125	0.7	0.5	0.3
TRNA-nucleotidyltransferase	Q920N6	1.6	1.3	3.2	V(D)J recombination activating protein 2 (Rag2)	P21784	0.7	0.6	0.3
Cornichon homolog	O35372	2.0	1.6	3.0	Camello-like 2	NM_053096	0.9	0.6	0.3
Similar to GTP binding protein 5	Q8R3J9	1.3	2.2	3.0	Transcription factor Mafb	P54841	0.6	1.0	0.4
L-selectin (CD62L)	P18337	1.9	1.3	2.9	Oligodendrocyte transcription factor 3	NM_053008	0.8	0.5	0.4
Cop9 signalosome complex subunit 2	Q15647	1.1	2.5	2.9	Voltage dependent l-type calcium channel alpha-1C	Q01815	0.7	0.4	0.4
Guanine nucleotide-binding protein. α-13 (Gα13)	P27601	1.3	2.3	2.8	T cell surface glycoprotein CD4	P06332	0.6	0.5	0.4
Vesicle transport v-snare protein VTI1-like	O88384	1.5	2.3	2.5	Vomeronasal 1 receptor I5	NM_134222	0.9	0.6	0.4
Ethanol induced gene product EIG180	NM_133237	1.7	0.9	2.5	Synaptic vesicle membrane protein Vat1 homolog	Q62465	0.5	0.5	0.4
Thioredoxin dependent peroxide reductase	P20108	1.8	0.6	2.5	Single stranded DNA-binding protein 2	Q9CYZ8	0.8	0.6	0.4
Cell growth regulating nucleolar protein	Q08288	1.0	1.7	2.5	Stress induced protein	NM_021897	0.6	0.7	0.4
Porphobilinogen deaminase	P22907	1.7	0.9	2.5	Protein 4.1	P48193	0.6	0.6	0.4
Eukaryotic translation initiating factor 3 subunit 11	Q9DBZ5	0.9	2.4	2.4	V(D)J recombination activating protein 1 (Rag1)	P15919	0.7	0.5	0.4
Similar to autoantigen Ngp1	NM_145552	0.8	2.2	2.4	Mitogen activated protein kinase4-2	NM_009006	0.5	0.6	0.4
Coatomer zeta1 subunit	Q9Y3C3	1.6	1.5	2.4	Zinc finger protein 39	Q02525	1.1	0.6	0.4
Huntigton's disease protein homolog	P42859	1.2	2.7	2.4	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	Q64518	0.5	0.6	0.5
U6 snRNA-associated sm-like protein Lsm4	Q9QXA5	0.9	2.4	2.3	Amyloid-like protein 2	Q06335	0.8	0.5	0.5
T lymphocyte activated protein	P17950	2.1	1.6	2.2	Expressed sequence	NM_134133	0.8	0.7	0.5
Zinc finger protein BC027407	NM_153063	1.2	1.5	2.2	Lim/homeobox protein Lhx2	Q9Z0S2	0.6	0.4	0.5
Adipocyte derived leucine aminopeptidase	Q9EQH2	1.9	2.3	2.2	Tripartite motif protein Trim11	Q99PQ2	1.0	0.6	0.5
Ring finger protein 8 Q8VC56	Q8VC56	1.3	2.0	2.2	Calpain 3 large subunit	Q64691	0.7	0.6	0.5
Peptidylprolyl isomerase like 3	NM_027351	1.6	2.1	2.2	F-box and leucine-rich repeat protein 12	NM_013911	0.8	0.8	0.5
POU domain. class 2. transcription factor 1	P25425	1.6	1.5	2.2	Signal transducer and activator of transcription (Stat5b)	P42232	0.6	0.7	0.5
Thy1 membrane glycoprotein	P01831	1.4	1.7	2.1	Ig gamma-1 chain C region	P01869	0.7	1.0	0.5
Muted	NM_139063	2.0	2.1	2.1	Glycerol kinase	Q64516	0.8	0.6	0.5
Sex-lethal interacting homolog	NM_025298	1.4	1.6	2.0	Phosphatidylinositol-4-phosphate 5-kinase type 1	NM_008846	1.6	0.6	0.5
MO25-like protein	Q9DB16	1.5	0.9	2.0	Polyposis locus protein 1 homolog	Q60870	0.7	0.8	0.5
DNA-3-methyladanine glycolsylase	Q04841	1.1	1.6	2.0	Zinc transporter 4	O35149	0.9	0.7	0.5
Dynamin 1 like	NM_152816	1.2	1.6	2.0	DNAJ homolog subfamily B member 1	Q9QYJ3	1.1	0.8	0.5
Cyclin dependent kinase 2 interacting protein	NM_026048	1.4	1.7	2.0	Swi/snf related actin dependent regulator of chromatin D2	NM_031878	0.7	0.6	0.5
Eukaryotic translation initiating factor 3 subunit 10	P23116	1.4	1.2	2.0	Ccr4-not transcription complex. subunit 7 (Caf1)	Q60809	0.8	0.7	0.5
Flavohemoprotein B5/B5	Q99P29	1.3	2.0	2.0	Ubiquitin conjugating enzyme E2D1	NM_145420	0.7	0.7	0.5
WD-repeat protein Bing4	Q9Z0H1	1.4	1.6	2.0	Widely interspaced zinc finger motifs	NM_011717	0.8	0.7	0.6
T-complex protein 1. theta subunit	P42932	1.4	1.1	1.9	Lysosomal acid lipase/cholesteryl ester hydrolase	Q9Z0M5	0.8	0.8	0.6
Placenta specific 8	NM_139198	1.9	1.6	1.9	B Cell translocation gene 1 (Btg1)	P31607	0.7	0.7	0.6
S-adenosylmethionine decarboxylase proenzyme 1	P31154	1.7	1.1	1.9	Interleukin 21 receptor (IL-21R) Q9JHX3	Q9JHX3	1.1	0.7	0.6
Similar to zinc finger protein 157	NM_145612	1.5	1.0	1.9	Nuclear factor of activated T cells. cytoplasmic 1 (Nfatc1)	O88942	0.9	0.7	0.6
PRO1853 homolog	NM_028611	1.2	1.5	1.9	High mobility group protein 1(Hmg1)	P07155	0.7	0.7	0.6

Table 4. GATA3 target genes differentially expressed in cluster E (continued).

Genes up-regulated ≥1.6 fold	Source*	G3	DO	G3:DO	Genes down-regulated ≥1.6 fold	Source*	G3	DO	G3:DO
Lathosterol oxidase	O88822	1.5	1.2	1.9	T cell activation rho GTPase activating protein	NM_145968	0.7	0.8	0.6
Kinesin like protein Kif2	P28740	1.7	1.7	1.9	Tripartite motif protein 30	P15533	0.6	0.8	0.6
Bcl2/adenovirus E1B 19-kda interacting protein 2	O54940	0.9	1.7	1.9	Tyrosine protein kinas Itk/Tsk	Q03526	0.8	0.8	0.6
Maguk P55 subfamily member 6	Q9JLB0	1.5	1.4	1.9	CUG Triplet repeat. RNA binding protein 2	NM_010160	0.7	0.7	0.6
RNA 3'-terminal phosphate cyclase-like protein	Q9JJT0	1.1	1.6	1.9	Lysosamal acic lipase/cholesteryl ester hydrolase	Q9Z0M5	1.1	0.8	0.6
Melanoma antigen. family D2	NM_030700	1.3	1.6	1.9	Lymphocye antigen Ly-6E	Q64253	0.7	0.6	0.6
Gene trap rosa 26 antisense	NM_008188	1.4	1.9	1.9	Nuclear factor of activated T cells 5 (NFAT5)	Q9WV30	0.8	0.7	0.6
Similar to zinc finger protein 118	Q91VP4	0.8	1.8	1.9	MHC class II transactivator CIITA	P79621	0.9	0.7	0.6
Elongation factor G1	Q8K0D5	1.5	1.5	1.9	Pat-12 protein	Q64154	0.7	0.7	0.6
Protein phosphatase 2. regulatory subunit b	NM_009358	1.4	1.8	1.8	Similar to rearranged L-Myc fusion sequence	Q922U7	0.7	0.8	0.6
Band 4.1-like protein 2	O70318	0.7	1.4	1.8	Signal transducer and activator of transcription 2 (Stat2)	Q9WVL2	0.7	0.8	0.6
Apoptosis antagonizing transcription factor	NM_019816	1.3	1.6	1.8	Mitogen activated protein kinase 6 (Erk3)	Q61532	0.6	0.7	0.6
RD protein (WL623)	P19426	1.6	1.1	1.8	Hypoxia inducible factor 1 α	Q61221	0.8	0.7	0.6
Probable mitochondrial import receptor subunit tom40	Q9QYA2	1.4	1.5	1.8	Ras-related protein rab-3D	P35276	0.8	0.7	0.6
Armadillo repeat protein	P98203	1.4	1.8	1.8	Cyfip2	NM_133769	0.7	0.8	0.6
Septin 1	P42209	1.6	1.5	1.7	Glcnac-t I	P27808	0.7	0.8	0.6
Degenerative spermatocyte homolog	NM_007853	1.1	1.8	1.7	Extacellular superoxide dismutase	O09164	0.9	0.7	0.6
DNA-binding protein Ikaros	Q03267	1.0	1.3	1.7	6-phosphofructokinase. type c	Q9WUA3	0.7	1.0	0.6
Mitogen activated protein kinase 14	P47811	1.3	1.5	1.7	Nuclear Lim interacting factor 2	NM_146012	0.7	0.8	0.6
Galectin 9	O08573	1.1	1.3	1.7	P300/Cbp associated factor	NM_020005	0.8	0.8	0.6
Tyrosyl-DNA phodhodiesterase 1	NM_028354	1.4	1.4	1.7	Cell division cycle 37 homolog	NM_025950	0.8	0.8	0.6
Carnitine deficiency-associated protein	O35594	1.9	1.7	1.7	Similar to CG15168 gene product	Q8R3L0	0.7	0.7	0.6
WD-repeat protein AN11homolog	O15491	1.5	1.5	1.7	Antigen containing epitope to antibody MMS-85/12	O35243	1.0	0.7	0.6
C-ETS-1 protein	P27577	1.5	1.0	1.7	Bromodomain containing 4	NM_020508	0.7	0.6	0.6
Acethylcholine receptor protein. gamma chain	P04760	1.4	1.7	1.7	Gamma-aminobutyric acid (GABA(A))	NM_020590	0.7	0.7	0.6
Telomeric repeat factor 2 interacting protein 1	Q91VL8	1.4	1.3	1.7	TPR-containing. SH2-binding phosphpoprotein	NM_009431	0.6	0.7	0.6
Vesicle transport v-snare protein VTI1-like1	O88384	1.4	1.4	1.7	Signal transducer and activator of transcription 5a (Stat5a)	P42230	0.7	0.8	0.6
Kinesin-like 5	NM_024245	1.5	1.0	1.7	T cell surface glycoprotein CD3 epsilon chain	P22646	0.9	0.7	0.6
Small edrk-rich factor 1	O88892	1.3	1.0	1.7	Morc	NM_010816	0.9	0.8	0.6
Absent in melanoma 1	NM_172393	1.4	1.7	1.7	Myeloid leukemia sequence 1	NM_008562	0.8	0.8	0.6
Heterogeneous nuclear ribonucleoprotein H2	NM_019868	1.3	1.2	1.6	RAL Guanine nucleotide dissociation stimulator-like2	Q61193	0.7	0.8	0.6
Integral membrane protein tmie	NM_146260	1.4	1.5	1.6	Growth factor receptor-bound protein 2	Q60631	0.7	0.7	0.6
D-3-phospoglycerate dehydrogenase	Q61753	1.4	1.3	1.6	ATPsynthase mithochondrial F1 complex assembly factor 2	NM_145427	0.8	0.8	0.6
DNA-binding protein inhibitor ID-2	P41136	1.0	2.0	1.6	Palmitoyl-protein thioesterase 2	O35448	1.1	0.7	0.6
1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	Q9D1E8	1.2	1.3	1.6	Werner syndrome helicase homolog	O09053	0.7	0.6	0.6
Bcl2-like protein 11	O54918	0.7	1.1	1.6	Solute carrier family 39	NM_134135	0.8	0.8	0.6
					Segment polarity protein dishevelled homolog DVL-2	Q60838	0.8	0.7	0.6
					Similar to cell division cycle 2-like 5	Q8K0A4	0.8	0.6	0.6
					Transcription factor E2F2	P56931	0.8	0.8	0.6
					Centaurin. β 1	NM_153788	0.8	0.8	0.6
					Salvador homolog 1	NM_022028	1.0	0.7	0.6
					RHO Guanine nucleotide exchange factor (Gef1)	NM_008488	0.6	0.8	0.7
					Damage-specific DNA-binding protein 2	Q99J79	0.7	0.8	0.7

Genes were sorted on the gene exprression level in CD2-GATA3:D011.10 transgenic mice. Numbers indicate the relative gene expression level compared to wild-type mice.
* RefSec. Swissprot or Sptrembl. G3 = CD2-GATA3. DO = DO11.10. G3:DO = CD2-GATA3:DO11.10

reported to act together in negative regulation of the gene for estrogen receptor α (ERα) [47]. 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 [48]. These data suggest that GATA3 can regulate ERα in a direct manner, but as well indirectly, via Btg1 and Caf1.

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-phospate 5-kinase (PI4P5K-I), and extracellular signal regulated kinase-3 (Erk3). In addition, the tyrosine protein kinases Tec and Itk, which are involved in TCR signaling, were ~2-3 fold downregulated. Furthermore, secreted modular calcium-binding protein-1, sarcoplasmic/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 ~10.

Enforced GATA3 expression also resulted in down-regulated 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 [49]. We did not detect a significant reduction in cell surface protein expression of CD4 by flow cytometric analysis of GATA3 transgenic DP cells, but reduced CD4 expression was found in 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).

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

When comparing the gene expression profiles of non-transformed CD2-GATA3 transgenic DP cells with those from thymic lymphoma samples obtained from CD2-GATA3 transgenic mice [22], 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 down-regulated in both non-malignant DP thymocytes as in GATA3 induced lymphoma cells. Among the up-regulated 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, IL-17BR and granzyme A.

Taken together, by gene expression profiling, we have identified novel putative GATA3 target genes at the DP stage. As both *GATA3*-deficiency and expression of activated Notch inhibit the DP to SP transition, it is attractive to speculate that the most important functional role for GATA3 in developing CD4 SP thymocytes, next to TCR upregulation [48], is the termination of Notch1 signaling. Interestingly, from our list of novel putative GATA3 target genes, it appears that GATA3 is typically involved

in gene regulatory networks in which it has the capacity to directly and indirectly control gene expression through multiple parallel pathways. For example, GATA3 stimulates Th2 cytokine expression directly, by increasing cytokine promoter activity and downregulating IL-12Rβ chain, Stat4 and T-bet. GATA3 limits Notch signaling by decreasing Notch1 expression and by stimulating intracellular Notch degradation by upregulation of Pin1. GATA3 increases ERα transcription directly by increasing promoter activity and indirectly by decreasing transcription of the inhibitory regulators Btg1 and Caf1. Validation of the identified targets in combination with functional experiments will provide improved insight into the essential physiological function of GATA3 during the developmental progression of DP cells that have received TCR-mediated positive selection signals into CD4 SP cells, as well as the role of GATA3 in lymphomagenesis.

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 Heimerikx (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 and the International Association of Cancer Research.

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IV

Cooperation of GATA3, c-Myc and Notch in malignant transformation of double positive thymocytes

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Mol Immunol 2008;45:3085-95

ABSTRACT

GATA transcription factors are critical regulators of proliferation and differentiation implicated in various human cancers, but specific genes activated by GATA proteins remain to be identified. We previously reported that enforced expression of GATA3 during T cell development in CD2-GATA3 transgenic mice induced $CD4^+CD8^+$ double-positive (DP) T cell lymphoma. Here, we show that the presence of the DO11.10 T-cell receptor transgene, which directs DP cells towards the CD4 lineage, resulted in enhanced lymphoma development and a dramatic increase in thymocyte cell size in CD2-GATA3 transgenic mice. CD2-GATA3 DP cells expressed high levels of the proto-oncogene c-Myc but the Notch1 signaling pathway, which is known to induce c-Myc, was not activated. Gene expression profiling showed that in CD2-GATA3 lymphoma cells transcription of c-Myc and its target genes was further increased. A substantial fraction of CD2-GATA3 lymphomas had trisomy of chromosome 15, leading to an increased *c-Myc* gene dose. Interestingly, most lymphomas showed high expression of the Notch targets Deltex1 and Hes1, often due to activating Notch1 PEST domain mutations. Therefore, we conclude that enforced GATA3 expression converts DP thymocytes into a pre-malignant state, characterized by high c-Myc expression, whereby subsequent induction of Notch1 signaling cooperates to establish malignant transformation. The finding that GATA3 regulates c-Myc expression levels, in a direct or indirect fashion, may explain the parallel phenotypes of mice with overexpression or deficiency of either of the two transcription factors.

INTRODUCTION

GATA transcription factors, which bind DNA at a consensus sequence (A/T)GATA(A/G) through conserved zinc-finger domains [1], are important regulators of cellular proliferation and differentiation. GATA1 and GATA2 are expressed in hematopoeietic cells, while GATA4-6 are expressed in non-hematopoetic organs, such as heart, lung and intestine [2]. GATA3 is present in T cells but also in non-hematopoietic tissues, including kidney, central nervous system, skin and mammary gland [3].

The embryonic lethality of *GATA3*$^{-/-}$ mutant mice at day 11 of gestation demonstrated its crucial developmental function [4]. Gene targeting experiments showed that GATA3 is essential in early T cell development [5, 6]. GATA3 is already expressed in the earliest T cell progenitors in the thymus, the $CD4^-CD8^-$ double negative (DN) cells. In these DN cells, gene segments coding for the T cell receptor (TCR) β chain undergo V(D)J recombination, whereby only cells with a functional TCRβ gene rearrangement are selected for further maturation. This β-selection process is characterized by cell size increase and induction of proliferation, followed by upregulation of CD4 and CD8 expression. Analysis of a *GATA3*-lacZ reporter mouse showed that GATA3 is induced in those cells that have passed β-selection [5]. Conditional deletion of the *Gata3* gene

in DN cells revealed that *Gata3*-deficient thymocytes fail to undergo β-selection, indicating that GATA3 is essential for this process [7]. At the $CD4^+CD8^+$ double-positive (DP) stage, functional TCRα gene rearrangement results in expression of a complete αβ TCR, which has the capacity to recognize peptide antigens presented by major histocompatibility complex (MHC) class I and class II molecules. Upon engagement by self-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 [8, 9]. By contrast, strong TCR signals trigger apoptosis, leading to negative selection. GATA3 expression is induced in DP cells after TCR stimulation by MHC class II and probably acts in developing CD4 SP cells in a positive feed-back loop to upregulate TCR expression [5, 10, 11]. By contrast, during development of CD8 SP cells GATA3 is downregulated. In effector T cells, GATA3 acts a master regulator of Th2 differentiation, which is essential for transcriptional regulation of the Th2 cytokine locus and inhibits the differentiation of Th1 and regulatory T cells [12-14].

GATA factors have been implicated in various human cancers. Acquired missense mutations in the GATA1 gene cause acute megakaryoblastic leukemia in humans with Down's syndrome [15]. GATA2 is located near 3q21 breakpoints in acute myeloid leukemia, which is accompanied by increased GATA2 expression. Reciprocal changes in the expression levels of GATA4 and GATA6 have been associated with adrenocortical tumor formation [16-18]. Evidence for the involvement of GATA3 in human cancers came from its aberrant expression in pancreatic cancer [19] and its expression in association with estrogen α receptor in breast cancer [20]. But also somatic mutations resulting in loss of GATA3 function may contribute to breast cancer tumorigenesis [21]. GATA3 is expressed in human T cell acute lymphoblastic leukemias (T-ALL) [22, 23] and capable to form a complex with the transcription factors LIM-only domain protein Lmo2 and the basic-helix-loop-helix (bHLH) protein Tal1, which are often aberrantly expressed in human T-ALL [24].

Direct evidence for a role of GATA3 in T-ALL came from our finding of thymic lymphomas in transgenic mice with enforced GATA3 expression throughout T cell development, driven by the human CD2 promoter and locus control region [25, 26]. These CD2-GATA3 transgenic mice developed monoclonal thymic lymphomas, which were mostly $CD3^+CD4^+CD8^{+/low}$, suggesting a DP origin. However, downstream targets of GATA3 at the DP cell stage that may explain the oncogenic potential of GATA3 have not been identified yet. It is also not known how GATA3 relates to other genes known to play an important role in T-ALL development in human and mice, such as the bHLH proteins c-Myc, Tal1 and E2A, the LIM-only domain proteins Lmo1/Lmo2 and the heterodimeric transmembrane receptor Notch1 [27]. In this report, we characterized the DP compartment of CD2-GATA3 transgenic mice. We found that enforced GATA3 expression converts DP cells into a pre-malignant state characterized by increased cell size and enhanced c-Myc expression. DP lymphomas from CD2-GATA3 transgenic mice manifested a further increase of c-Myc and showed induction of Notch1 signaling, showing cooperation of GATA3, c-Myc and Notch1 in lymphomagenesis.

MATERIALS AND METHODS

Mice

CD2-GATA3/FVB (26), CD2-GATA3:DO11.10 (BALB/c) and CD2-GATA3:HY: Rag2$^{-/-}$ (C57BL/6) mice have been described [11]. Mice were kept under pathogen-free conditions in the Erasmus MC animal care facility. In survival analyses only mice that developed a lymphoma were marked as a case. Mice that died because of other reasons or were used for experiments were marked as censored. Kaplan-Meier analysis was performed using SPSS 11.0.1 statistical software.

Flow cytometry, antibodies and cell sorting

The generation of single-cell suspensions and four-color flow cytometry have been described previously [26]. Antibodies were purchased from BD Biosciences (San Diego, CA), samples were acquired on a FACSCalibur™ flow cytometer; data were analyzed by CellQuest™ software (BD Biosciences). FACS sorting of CD4$^+$CD8$^+$7AAD$^-$ thymocytes was performed with a FACSVantage VE, equipped with Diva Option and BD FACSdiva software. Purity of fractions was >99%.

Spectral karyotyping (SKY)

Cells were treated with 10 ng/ml colcemid (GIBCO-BRL, KaryoMAX Colecemid solution) for 15 minutes to arrest cells at metaphase and subsequently treated with 75 mM KCl and fixed with methanol/acetic acid (3:1). SKY was performed using the Applied Spectral Imaging system (ASI, Migdal Ha'Emek, Israel) following manufacturer's protocols. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) containing DABCO/Vectashield. Chromosomes were analyzed by Zeiss Axioplan 2 microscope equipped with the Spectra Cube system (ASI). Over 10 metaphases from each sample were analyzed, using Skyview analysis software (ASI).

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, St. Louis, MO). The quantity and quality of RNA was determined using a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE). Samples with a 260/280 nm optical density ratio >1.8 were used. Two μg of total RNA was used for amplification using T7 MEGAscript Kit (Ambion, Austin, TX), whereby aminoallyl-UTP (Ambion) was incorporated into amplified RNA (aRNA). Subsequently, Cy5- or Cy3-dye (Amersham, GE Healthcare, Piscataway, NJ) was coupled to the aminoallyl-modified aRNA. Labeled aRNA was purified and concentrated using Microcon YM30 columns (Millipore, Billerica, MA). Oligonucleotide arrays were co-hybridized with purified probes of CD2-GATA3 lymphoma and control pooled

wild-type DP T cells and scanned with a Scanarray Express HT scanner (Perkin Elmer, Boston, MA). Data were extracted using Imagene software 6.0 (Biodiscovery, CA). Each experiment consisted of 2 oligonucleotide arrays, whereby dyes were reversed between aRNA from CD2-GATA3 lymphoma 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 data were filtered for genes that were differentially expressed in experiments with $p<0.01$. Hierarchical clustering of genes and experiments was performed using Genesis 1.5.0 [28].

Quantitative PCR analysis

Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma). One μg of total RNA was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers. PCR primers and probes are shown in Table 1.

Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). 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 C_t values of the genes of interest were normalized to the C_t value of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

Mutation analysis

DNA isolated from CD2-GATA3 lymphoma was used for the amplification of exons encoding the Notch1 heterodimerization and PEST domains. Primers used for the

Table 1. Primers and probes used for quantitative RT-PCR analysis for expression of the indicated genes.

Gene	Forward primer	Reverse primer	Probe
c-Myc	GCAGCTGTTTGAAGGCTGGAT	GTCGCAGATGAAATAGGGCTGT	CACGACGATGCCCCTCAACGTGA
Cyclin D2	CTCCCGCAGTGTTCCTATTTCA	CAGGTAATTCATGGCCAGAGGA	ACATCCAACCGTACATGCGCAGGAT
Deltex	CGCCTGATGAGGACTGTACC	CCCTCATAGCCAGATGCTGT	GCGGCTGG
E47	GGATACTCAGCCGAAGAAGGT	TGAGCTGGGCGGATACAC	CTGGTCTC
Gapdh	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	TGCATCCTGCACCACCAACTG
GATA3	CATTACCACCTATCCGCCCTATG	CACACACTCCCTGCCTTCTGT	CGAGGCCCAAGGCACGATCCAG
Hes1	AAAGATAGCTCCCGGCATTC	TGCTTCACAGTCATTTCCAGA	GAAGGCAG
Notch1	CTCTTCACTGCTTCCTGGT	AATGGAGCCACGGATGTC	GGGAGCTG
preTα	GACAGAACCGGAGCACACA	AAAGCAGCTCCTGGCTGTC	AGCTGGAG

identification of activating Notch1 mutations were: '5-GATGGGACTGAGTGCATCCT-3' and '5-AGGGACACACTGGGAAACAG-3' (exon 26), '5-GAATGACCCCCGCTGAGT-3' and '5-CTGGGATTTGAACCCTTGTC-3' (exon 27), '5-GAGCCTGGTGGTCTAGGATG-3' and '5-'TTCCTTGCTACCACAAGCCA-3' (exon 34-1), '5-TGGCTTGTGGTAGCAAGGA-3' and '5-GCCGTAGTGGGTTGTACTGG-3' (exon 34-2), '5-CCAGTACAACCCACTACGG-3' and '5-GGGGTCAGGAACTGGGTAGT-3' (exon 34-3), '5-ACTACCCAGTTCCTGACCCC-3' and '5-AAAAGGCAGTGTTTCTGTGGA-3' (exon 34-4).

RESULTS

Positive selection towards the CD4 lineage enhances lymphoma induction in CD2-GATA3 mice

Enforced expression of GATA3 during T cell development is associated with the formation of thymic lymphomas with predominantly a DP phenotype [26]. Tumor-free survival analyses performed for two independently generated CD2-GATA3 mouse strains, #a and #b, demonstrated that the effect of the transgene was integration-site independent (Figure 1A).

The DP compartment in the thymus is heterogeneous, containing cells that are positively selected towards the CD4 or CD8 lineage, cells that are negatively selected, as well as cells that are not selected and die by neglect. To investigate which of these categories are the prime target for thymic lymphoma development in CD2-GATA3 mice, we crossed CD2-GATA3#b mice with various TCR transgenic mice and evaluated tumor incidence. Crosses with DO11.10 transgenic mice [29], expressing an MHC class II-restricted TCR$\alpha\beta$ specific for ovalbumine, provided a model for enforced GATA3 expression during CD4 selection. The MHC class I-restricted HY TCR transgene recognizes the male-specific HY antigen, leading to positive selection in female mice and to negative selection in male mice [30]. Thus, HY;CD2-GATA3#b double transgenic mice provide models for enforced GATA3 expression during CD8 selection and negative selection in female and male mice, respectively.

When compared with CD2-GATA3#b single transgenic mice, tumor formation was significantly increased in CD2-GATA3#b:DO11.10 double transgenic mice (p<0.0001), all of which developed lymphoma before 35 weeks of age (Figure 1B). Phenotypic analyses of thymic lymphomas in CD2-GATA3#b:DO11.10 mice by flow cytometry showed that they generally consisted of DP to CD4 SP cells expressing the TCR/CD3 complex (Figure 2 and data not shown), thus resembling the thymic lymphomas observed in CD2-GATA3/FVB transgenic mice previously described [26]. As a control, we incidentally found cases (2/31) of thymic lymphoma in DO11.10 single transgenic mice.

As the presence of the HY TCR transgene does not efficiently prevent V(D)J recombination within endogenous TCR loci, CD2-GATA3#b:HY double transgenic mice were crossed on the recombination activating gene-2 (Rag2-)deficient background. In the absence of the CD2-GATA3#b transgene, HY single transgenic female mice were prone to develop thymic lymphomas with a frequency comparable

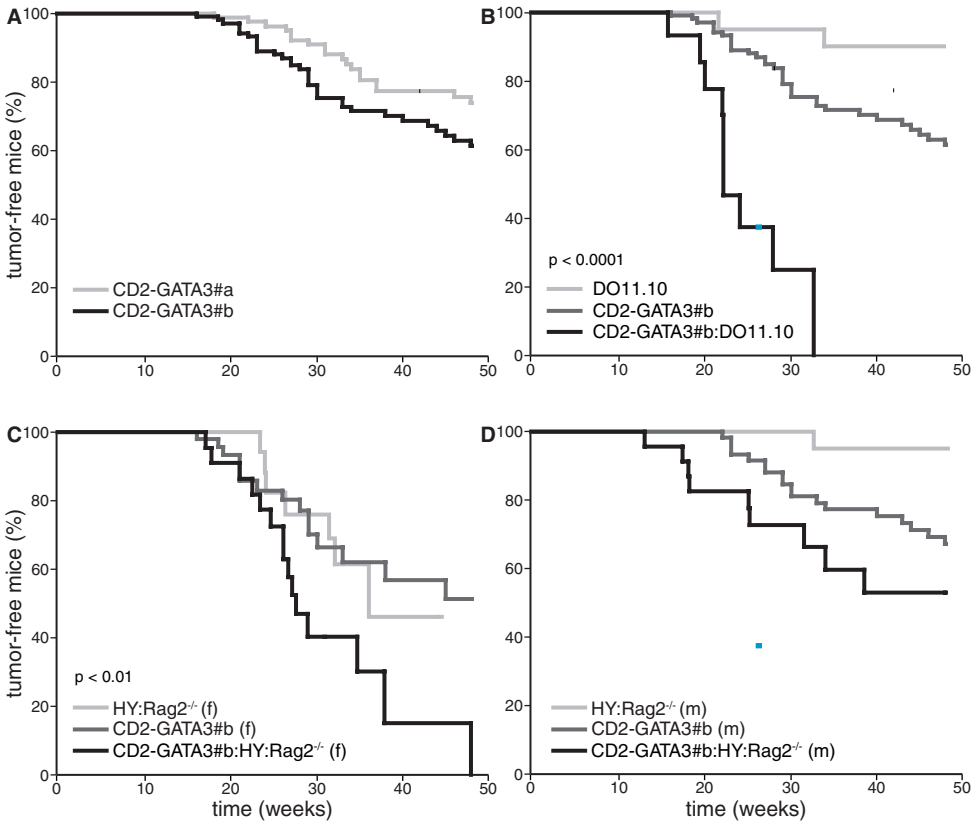


Figure 1. The effect of thymic selection on tumor formation in transgenic mice with enforced GATA3 expression. The tumor-free survival of the indicated transgenic mice was followed up to 48 weeks and displayed as Kaplan-Meier curves. 20-112 mice were used per group. For statistical evaluations the Log rank statistical test was used.

to CD2-GATA3#b single transgenic female mice (Figure 1C). Lymphoma formation in CD2-GATA3#b:HY double transgenic female mice was only moderately enhanced, when compared with the CD2-GATA3#b single transgenic group (p<0.01). The tumor phenotypes in CD2-GATA3#b:HY double transgenic females were variable, but were generally DP to CD8 SP (Figure 2). Thus, HY-mediated positive selection towards the CD8 lineage had a limited effect on lymphoma induction in CD2-GATA3 transgenic mice. The induction of lymphoma in a Rag2-deficient background indicated that tumor formation is independent of the V(D)J recombinase system, both in CD2-GATA3#b and in HY transgenic mice.

Finally, when we compared CD2-GATA3#b single and CD2-GATA3#b:HY double transgenic male mice, we did not detect a significant effect of negative selection (as mediated by the presence of the HY TCR) on tumor-free survival (Figure 1D).

The lymphoma phenotypes in CD2-GATA3#b:HY mice were heterogeneous and included DP, CD8 SP and DN cells (Figure 2).

Taken together, these findings show that induction of thymic lymphoma is specifically enhanced by positive selection towards the CD4 lineage in CD2-GATA3#b:DO11.10 double transgenic mice. Furthermore, tumor formation occurred in the absence of a functional V(D)J recombinase system.

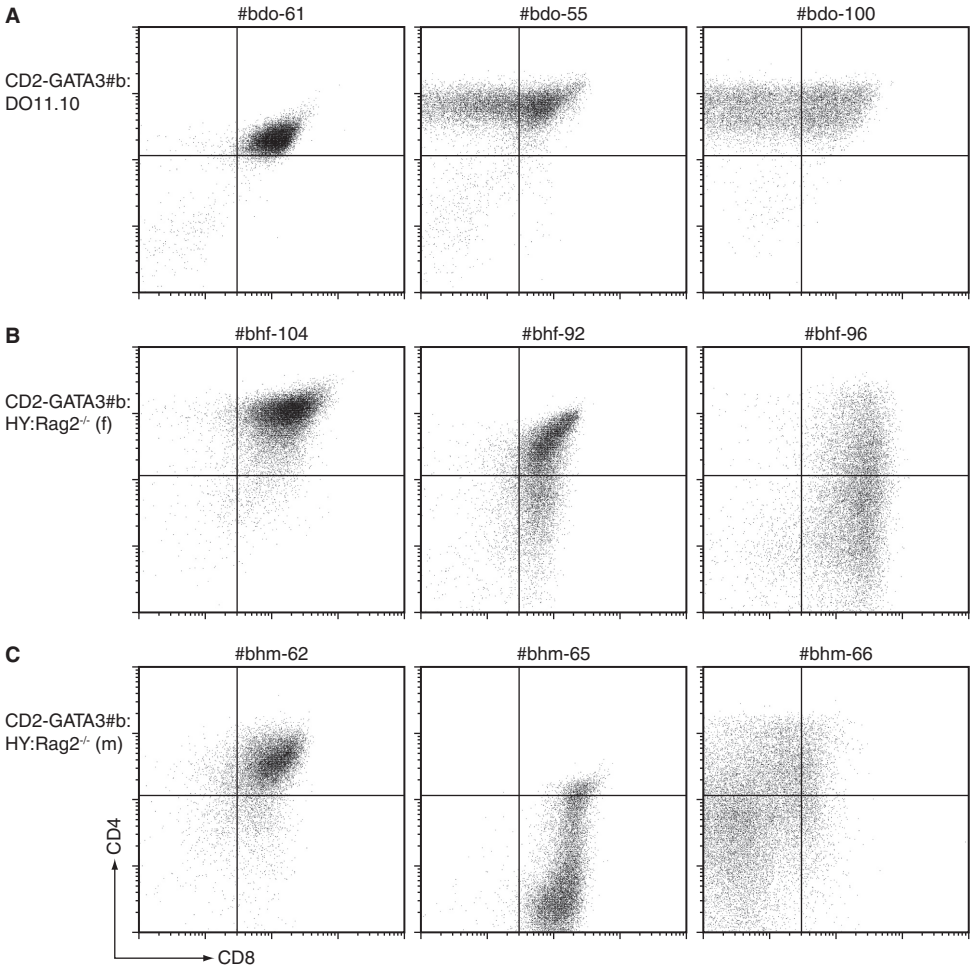


Figure 2. Flow cytometric characterization of thymic lymphomas from CD2-GATA3#b:TCR double transgenic mice, whereby (A) the DO11.10 TCR provides a model for positive selection towards the CD4 lineage, (B) the HY TCR in female (f) mice provides a model for positive selection towards the CD8 lineage, and (C) the HY TCR in male (m) mice provides a model for negative selection. Representative CD4/CD8 expression profiles from various thymic lymphomas are shown.

Enlarged thymocyte cell size in ~14-week-old CD2-GATA3#b:DO11.10 double transgenic mice

To investigate the effect of thymic selection in the context of lymphoma induction, we analyzed the thymic compartments in ~5-week-old and ~14-week-old wild-type and CD2-GATA3#b mice that did or did not carry a DO11.10 or HY TCR transgene.

In the absence of a TCR transgene, wild-type and CD2-GATA3#b mice showed a similar decrease in total thymic cellularity over time (Figure 3A). We previously found that the presence of a TCR transgene in ~5-week-old CD2-GATA3#b mice resulted in reduced thymic cellularity, reflecting increased apoptosis (11). We now found that at the age of ~14 weeks TCR single transgenic and positively selecting CD2-GATA3#b;DO11.10 or female CD2-GATA3#b;HY mice had similar total numbers of thymocytes. Negatively selecting male CD2-GATA3#b:HY mice showed a dramatic

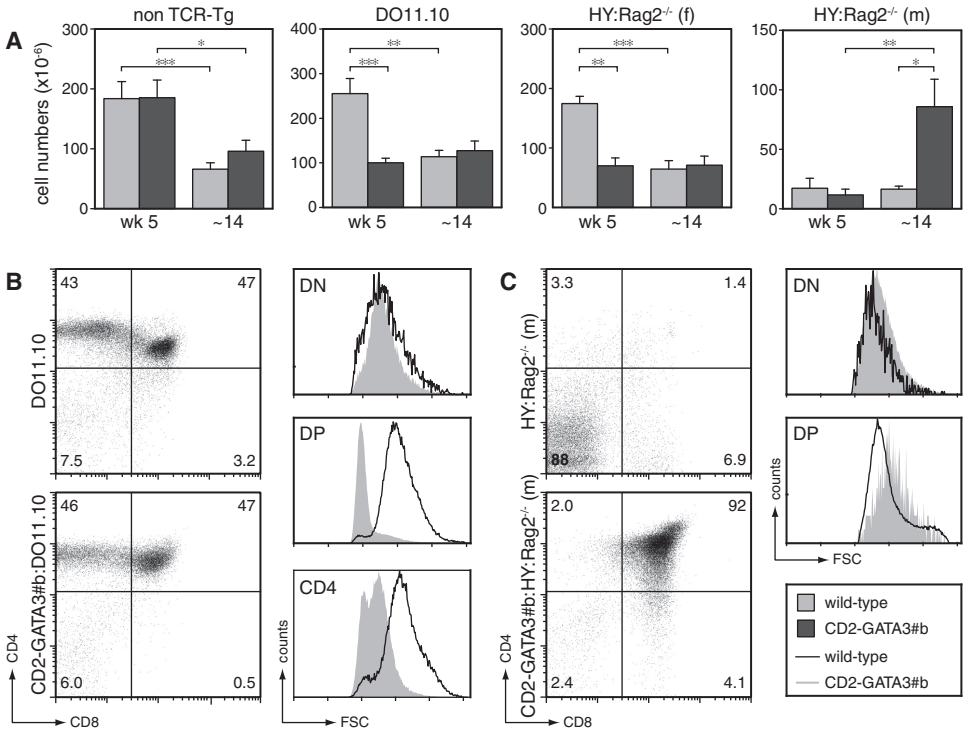


Figure 3. Age-dependent effects of enforced GATA3 expression on T cell development. (A) Total thymocyte number of the indicated mice at the age of 5 weeks and ~14 weeks. Average values ± SEM are displayed. For statistical analysis a t-test was performed (* = p<0.05, ** = p<0.01, *** = p<0.001). (B-C) Flow cytometric CD4/CD8 profiles of total thymocytes of the indicated mice at the age of ~14 weeks. Data are displayed as dot plots. Numbers represent the percentage of cells within a quadrant (left panel). Flow cytometric analysis displayed as histogram plots of forward scatter (FSC) of gated DN, DP or CD4 cells from TCR transgenic (gray area) or CD2-GATA3 / TCR double transgenic (black line) mice of ~14 weeks of age (right panel). Data shown are representatives of 3-14 mice per group.

increase in total thymocyte numbers, when compared with HY single transgenic mice (Figure 3A).

The distribution over the individual thymocyte subpopulations in DO11.10 and CD2-GATA3#b:DO11.10 transgenic mice was not statistically different (Figure 3B). We previously described that at ~5 weeks of age CD2-GATA3#b transgenic mice had a slight increase in DP cell size, which was restored in SP cells [11,25,26]. Remarkably, we observed a dramatic increase in the cell size of both DP and CD4 SP cells in ~14-week-old CD2-GATA3#b:DO11.10 mice (Figure 3B). Apart from the increased cell size, the phenotype of the DP population did not appear to change over time: DP cells from ~14-week-old CD2-GATA3#b:DO11.10 double transgenic mice had decreased levels of CD5 and increased TCR expression, when compared with DO11.10 single transgenic controls (data not shown), consistent with our published findings in 5-week-old mice [11].

The dramatic increase in thymic cellularity of ~14-week-old CD2-GATA3#b:HY male mice was mainly caused by the appearance of a DP cell population (Figure 3C), which expressed CD5 and low TCR levels (data not shown), indicating that enforced GATA3 expression may interfere with the negative selection process.

Taken together, these data show that enforced GATA3 expression resulted in a dramatic increase of the DP cell size over time, specifically in DO11.10 transgenic cells that are positively selected towards the CD4 lineage.

Enforced GATA3 expression results in increased c-Myc expression, specifically in DP cells

A factor known to be crucially involved in lymphocyte cell size regulation is the bHLH transcription factor c-Myc [31]. Similar to enforced GATA3 expression, also enforced expression of c-Myc in thymocytes results in an increase of the average cell size of DP cells and thymic lymphoma formation [32-34]. Because of this intriguing parallel, we determined the expression levels of c-Myc in sorted DP cells from wild-type and CD2-GATA3#b transgenic mice. Using quantitative RT-PCR, we found that both GATA3 and c-Myc expression was increased with a factor of ~5 in CD2-GATA3#b DP cells, when compared with wild-type (Figure 4A). This was also the case on the DO11.10 transgenic background (Figure 4B). Transcription of Cyclin D2, a c-Myc target gene [35], was slightly increased in CD2-GATA3#b DP cells and, more strikingly, in CD2-GATA3#b:DO11.10 DP cells (Figure 4B). In contrast to DP cells, CD4 single positive thymocytes did not exhibit any significant upregulation of GATA3, c-Myc or Cyclin D2 expression in CD2-GATA3#b single or CD2-GATA3#b:DO11.10 double transgenic mice (data not shown). In summary, these findings demonstrate that enforced GATA3 expression correlated with increased c-Myc expression, specifically at the DP cell stage.

Chromosome 15 abnormalities and increased c-Myc expression in CD2-GATA3 lymphomas

To investigate whether thymic lymphomas from CD2-GATA3 transgenic mice had common chromosomal abnormalities, spectral karyotyping analysis (SKY) was

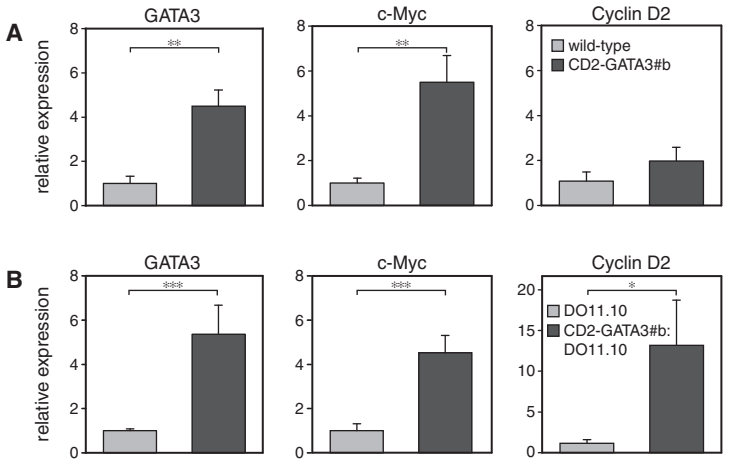


Figure 4. Enforced GATA3 expression results in c-Myc upregulation in DP thymocytes. Quantitative RT-PCR analysis of GATA3 en c-Myc expression in sorted DP cells from wild-type and CD2-GATA3 single transgenic mice (A) or DO11.10 and CD2-GATA3:DO11.10 double transgenic mice (B). Average values ±SEM are displayed and values indicate the ratio between the expression of the indicate gene and Gapdh. Expression levels in wild-type DP cells were set to 1. For statistical analysis a t-test was performed (* = p<0.05, ** = p<0.01, *** = p<0.001). Data are from 4-10 mice per group.

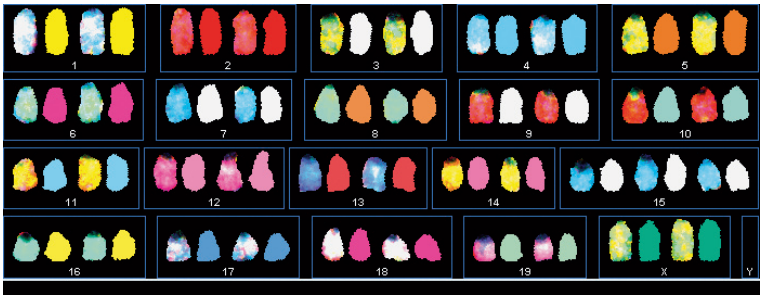
performed in primary tumors and growth factor-independent cell lines established from primary lymphomas. Such cell lines resembled the phenotype of primary lymphoma cells in terms of TCR, CD3, CD4, CD5, CD8 and CD69 expression (data not shown). In 6 out of 9 lymphoma samples chromosomal abnormalities were identified, in particular abnormalities involving chromosome 15, which harbors the c-Myc locus. Three primary lymphoma samples contained a trisomy of chromosome 15 (Figure 5A and 5B), which is often observed in c-Myc transgenic mice and associated with aberrant c-Myc expression [36-38]. Therefore, we investigated GATA3 and c-Myc expression in a large panel of lymphomas, originating from CD2-GATA3#a and #b single transgenic mice, as well as CD2-GATA3#b:DO11.10 double transgenic mice. In these lymphomas, GATA3 expression was ~2-3-fold increased, compared to wild-type or DO11.10 transgenic DP cells (Figure 5C). Remarkably, c-Myc expression levels were dramatically increased (~10-20-fold; Figure 5D), which was significantly more than the ~5-fold c-Myc upregulation observed in non-transformed CD2-GATA3 transgenic DP cells (Figure 4).

Collectively, these results indicate that transformation of CD2-GATA3 transgenic DP cells is associated with secondary events, e.g. a trisomy of chromosome 15, that further increase c-Myc expression.

Increased expression of c-Myc target genes in CD2-GATA3 lymphomas

To investigate, whether increased c-Myc expression in CD2-GATA3 lymphoma cells was accompanied by increased expression of known c-Myc targets, a gene

A



B Sky analysis of CD2-GATA3 lymphoma

Case	Numerical	Structural
#b-9	+15	
#a-11*	-	-
#a-13*	+18, +19	+t(2;16), del(8)
#bhf-50	+15	der(9)
#b-38	-Y, +14	+1, dic(10), der(12;15)
#b-41	-	-
#a-45	+15	-
#bhf-53	-	-

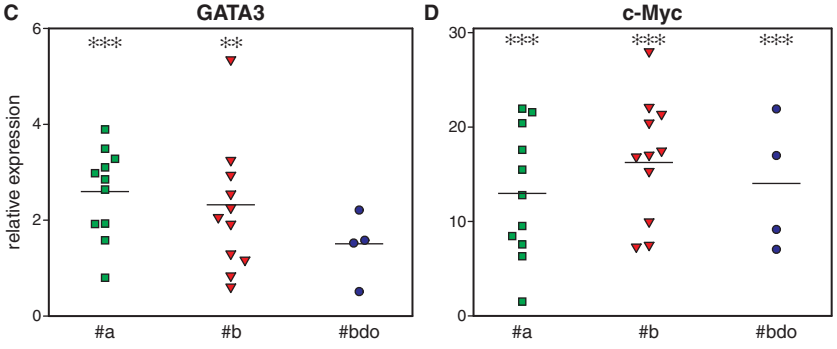
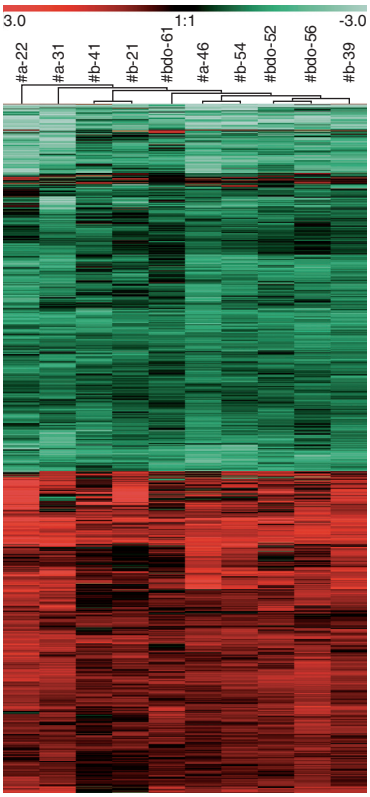


Figure 5. Chromosome 15 abnormalities and increased c-Myc expression in CD2-GATA3 lymphomas. (A) A representative metaphase of SKY analysis of a CD2-GATA3 lymphoma, indicating trisomy of chromosome 15 as a sole chromosomal abnormality (#b = CD2-GATA3#b mice). (B) Summary of SKY analysis performed on CD2-GATA3 lymphoma samples (* = lymphoma derived cell line, #a = CD2-GATA3#a and #bhf = female CD2-GATA3#b:HY:Rag2$^{-/-}$ mice). (C-D) Quantitative RT-PCR analysis of GATA3 (C) and c-Myc (D) expression in thymic lymphoma samples from the indicated mice. Average values and individual data points indicate the ratio between the expression of the indicated gene and Gapdh. Expression levels in wild-type DP cells were set to 1. For statistical analysis a t-test was performed, comparing gene expression in the indicated lymphoma to wild-type DP cells (* = p<0.05, ** = p<0.01, *** = p<0.001). (See Appendix page 218 for a full-color representation of this figure).

expression profiling study was performed. Expression profiles of a panel of ten lymphoma samples (three from CD2-GATA3#a, four from CD2-GATA3#b, and three from CD2-GATA3#b:DO11.10 mice) were compared with a control pool of FACS-sorted DP cells from 11-week-old wild-type mice. After performing a hierarchical clustering, a random clustering was obtained between lymphomas from the three groups of mice, indicating a common gene expression signature of these lymphomas (Figure 6A). To be able to identify upregulated c-Myc targets, 10 gene clusters were specified, which were differentially expressed in at least 2 out of 10 lymphomas

A



B

Selection of differentially expressed genes in CD2-GATA3 lymphoma and their mean expression level

Gene	Mean	Gene	Mean
cluster 9: (down ≤ -1.2 fold, n =261 genes)		*cluster 8:* (up ≥ -1,4 fold, n = 54 genes)	
Rag2	-3.31	Apo E	2.83
Rag1	-2.48	c-Myc	2.22
Ror gamma	-2.27	Ccl8	2.16
Ets2	-2.22	Methyltransferase-like 1	2.01
Ccr9	-2.15	Gadd45 gamma	1.80
Itk	-2.11	Transferrin	1.78
c-Myb	-2.00	Ccl9	1.68
CD5	-1.96	Granzyme A	1.63
CD4	-1.96	Asparagine synthetase	1.63
Bc11B	-1.82		
Nfat-c3	-1.70	cluster 6:	
Cbl	-1.61	(up 1.0 -1.7 fold,	
Tcf1	-1.59	n = 177 genes)	
TEC	-1.55		
IL-16	-1.54	Transferrin receptor	1.63
Socs1	-1.54	spermidine synthase	1.59
Stat5B	-1.54	Heat shock 70 kda-like	
Ifn gamma		protein 1	1.56
receptor alpha	-1.43	IL-17BR	1.53
IL-21R	-1.40	Cyclin D2	1.51
Slam	-1.40	Muc2	1.43
Cxcr4	-1.34	Hes1	1.26
CD3 zeta	-1.31		
IL-4R alpa	-1.29		
Nfat5	-1.25		
JunD	-1.22		

Figure 6. Gene expression profiles of CD2-GATA3 induced lymphoma. (A) Hierarchical clustering of both genes (rows) and thymic lymphoma (columns). Green and red colors indicate the down-regulation or up-regulation of the levels of genes expressed in lymphoma compared to sorted wild-type DP cells. The color scale above the matrix correlates with gene expression and the given values numbers represent ^{2}log values. In total 2845 genes were found to be differentially expressed (p<0.01) in at least 2 lymphoma samples. Gene expression profiles were analyzed for 10 lymphoma samples from CD2-GATA3#b (n = 4, #b), CD2-GATA3#a, (n = 3, #a) and CD2-GATA3#b:DO11.10 mice (n = 3, #bdo). The dendogram above and on the left side of the matrix indicate the average linkage clustering of respectively lymphomas and genes. (B) From the differentially expressed genes shown in A, 3 clusters were specified. For each cluster a selection of genes and their mean expression level is given. Numbers represent ^{2}log values.
(See Appendix page 219 for a full-color representation of this figure).

(p<0.05). Cluster 6 and cluster 8 contained 177 and 54 genes that were moderately and highly upregulated, while cluster 9 consisted of 261 genes that were downregulated (Figure 6B).

C-Myc was present among the genes, which were highly upregulated (~5 times). Furthermore a large number of differentially expressed c-Myc target genes [39] were present in these specified clusters (Figure 6B), including Cyclin D2, transferrin receptor, the enzymes spermidine synthase and asparagine synthetase, inhibitors of which are often used as antiproliferative agents in leukemia. We also found upregulation of Muc2, which may represent an important target of GATA3 as it was recently found that GATA3 acts as a Muc1 transcriptional regulator in breast cancer cells (Figure 6B).

Among the downregulated genes, we found genes involved in V(D)J recombination, including Rag1, Rag2 and Tdt. This would be in agreement with completed V(D)J recombination in CD3/TCR$\alpha\beta^+$ lymphoma cells, whereas the DP thymic compartment contain large populations of cells that are in the process of TCRα rearrangement.

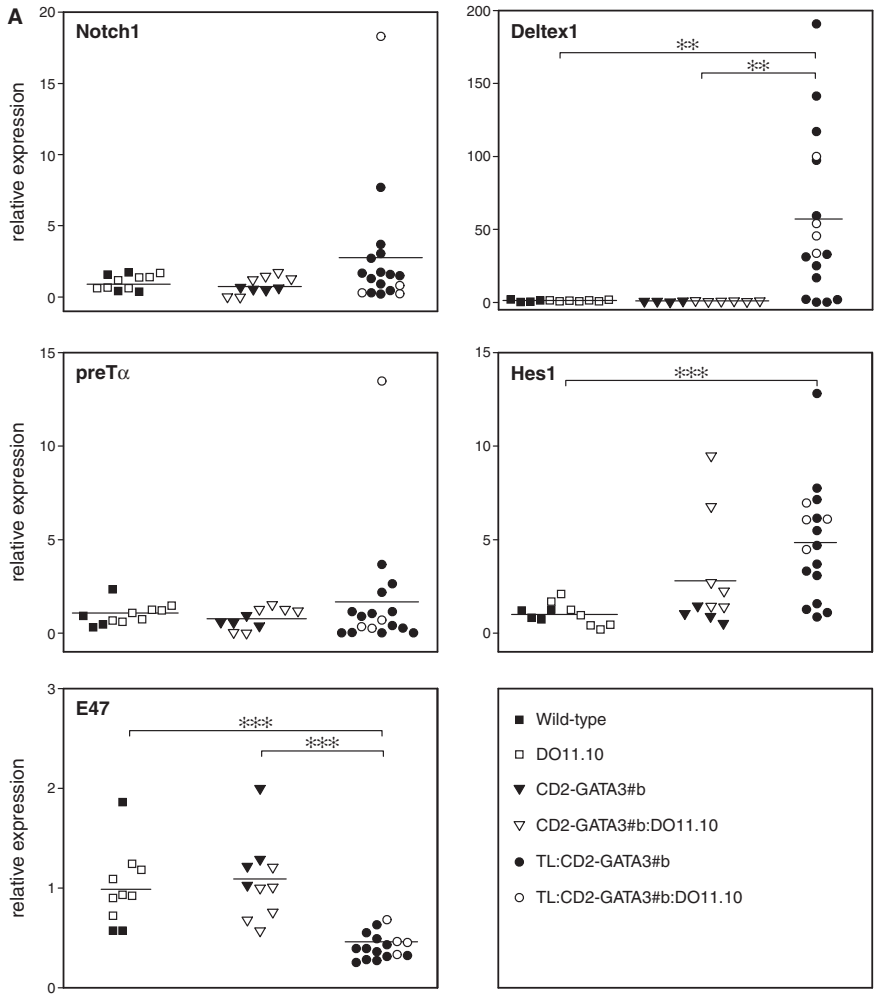
In addition, transcription factors, such as Ets2, c-Myb, RORγ, Bcl11B, Tcf1, Nfatc3, Nfat5 and JunD, genes involved in TCR signaling including CD4, CD5, Itk, Tec, Cbl, Slam and CD3ζ, as well as cytokine and chemokine signaling-related genes, such as Ccr9, Stat5b, Socs1, IL-16, IFNγRα, IL-4R, IL-21R and Cxcr4 were down-regulated (Figure 6B).

Taken together, from these findings we conclude that c-Myc and c-Myc target genes were upregulated in CD2-GATA3 lymphoma cells. Expression of genes involved in TCR signaling was low, suggesting that survival and proliferation of CD2-GATA3 lymphoma cells is independent of TCR signaling.

Increased expression of Notch1 targets in CD2-GATA3 lymphomas

Interestingly, another gene that was found upregulated in lymphoma cells was the bHLH factor Hes1 (Figure 6B), which is a known target of Notch1 signaling. Notch1 is essential for T cell development and regulates cell growth, proliferation, differentiation and apoptosis [40]. Importantly, more than 50% of human T-ALL harbor Notch1 mutations and recently c-Myc was identified as a direct target of Notch1 in T-ALL [41-43]. Therefore, we hypothesized that activation of Notch1 signaling may contribute to malignant transformation of CD2-GATA3 lymphoma cells, using c-Myc as an essential mediator of oncogenic signaling.

To investigate this, we determined the expression levels of Notch1 and various targets in sorted non-malignant DP cells (from wild-type, DO11.10 and CD2-GATA3#b single and double transgenic mice) and CD2-GATA3#b and CD2-GATA3#b:DO11.10 lymphoma cells by quantitative RT-PCR. All four groups of non-malignant DP cells expressed similar levels of Notch1 and its direct targets Deltex1, a modulator of Notch function, and preTα, a type I transmembrane glycoprotein that binds with TCRβ to form the pre-TCR (Figure 7A). Also expression of Hes1 was similar, except that it was found slightly increased in two out of six CD2-GATA3#b:DO11.10 DP cell fractions (Figure 7A). In lymphoma samples, expression was quite heterogeneous, whereby transcription of Notch1 and preTα was slightly increased. Remarkably, Hes1



B Notch1 exon 34 activating mutations in CD2-GATA3 lymphoma

Case	Mutation	Nucl./residue	Protein change
#b-9	G to CCCCC	#7434/2380	frameshift
#b-12	Insert A	#7323/2361	frameshift
#b-18	Deletion TGTGCA CACCATTCTGCC	#7521/2426	CTG Leu to CCC Pro
#b-21	GG to CCCC	#7322/2361	frameshift
#b-30	Insert T	#7322/2361	frameshift

Figure 7. Increased expression of Notch1 targets in CD2-GATA3 lymphoma. (A) Quantitative RT-PCR analysis of indicated genes in sorted wild-type and CD2-GATA3 DP cells and in lymphoma samples, as indicated. Average values and individual data points are displayed. Values indicate the ratio between the expression of the indicated gene and Gapdh, whereby the expression levels in wild-type DP cells were set to 1. For statistical analysis a t-test was performed (** = $p<0.01$, *** = $p<0.001$). (B) Activating mutations in exon 34 of Notch1 were identified in CD2-GATA3 lymphoma.

and Deltex1 were significantly upregulated in lymphoma cells, when compared with non-malignant DP cells (~6 and ~50-fold, respectively; Figure 7A). Only 4 out of 17 lymphoma samples showed expression of Hes1 and Deltex1 within the normal ranges. All lymphomas manifested low expression of the bHLH gene E47, which inhibits cellular proliferation and functions as a tumor suppressor [44].

In summary, quantitative RT-PCR experiments showed that CD2-GATA3 lymphomas have increased expression of Notch1 targets, indicating activation of Notch1 signaling in these transformed cells.

Activating Notch1 mutations CD2-GATA3 lymphomas

T-ALL in human and mouse is often associated with activating Notch1 mutations, inducing ligand-independent activation of the receptor or increased stability of intracellular Notch1 in the nucleus [45-49]. Therefore, we performed DNA sequence analysis of exons 26/27 and 34, encoding the heterodimerization domain and the PEST degradation domain, respectively, in a panel of 20 CD2-GATA3 lymphomas. No mutations were found in exons 26/27, but we identified 5 activating mutations in exon 34, predicted to result in the expression of truncated forms of Notch1 lacking the C-terminal PEST domain (Figure 7B). Thus, in a substantial fraction of CD2-GATA3 lymphomas the occurrence of activating Notch1 mutations contributed to malignant transformation.

DISCUSSION

Enforced GATA3 expression during T cell development induces thymic lymphoma, but the downstream targets and events, which can explain the oncogenic potential of GATA3, are unknown. In this report, we provide evidence for a cooperation of c-Myc, Notch1 and GATA3 at the DP T cell stage, eventually resulting in lymphomagenesis.

GATA3 is specifically required during CD4 selection and involved in a positive feedback loop that increases TCR surface expression [7, 11]. We found that enforced GATA3 expression resulted in high c-Myc expression and increased DP cell size, particularly in cells undergoing positive selection into the CD4 lineage. Striking functional parallels exist between GATA3 and c-Myc during T cell development: (1) Deletion or inhibition of GATA3 or c-Myc in early T cell development both result in a block at the DN3 stage [7, 50]; (2) Enforced expression of GATA3 or c-Myc are both associated with increased lymphocyte cell size [26, 31, 34]; (3) Inhibition of GATA3 or c-Myc both result in decreased efficiency of positive selection [7, 51]; (4) Both GATA3 and c-Myc activation result in enhanced positive selection and in a bypass of negative selection (see Figure 3C; [51-53]; (5) Like GATA3 [26], also enforced expression of c-Myc results in DP thymic lymphoma [32, 33, 54].

As we did not find evidence for activation of Notch signaling in non-transformed CD2-GATA3 DP cells, we conclude that upregulated c-Myc expression in these cells is independent of Notch1. It is not very likely that c-Myc is a direct target of GATA3. Although it was shown that the GATA3 family member GATA1 directly interacts

with the c-Myc promoter *in vivo* [55], GATA1 serves as a repressor of c-Myc and thus does not have a parallel function to GATA3 in the regulation of c-Myc expression. Also, we were unable to detect a direct occupation of the c-Myc promoter by GATA3 with chromatin immunoprecipitation. We therefore conclude that GATA3 probably regulates c-Myc expression in an indirect fashion, but independent of Notch1.

When compared to non-transformed CD2-GATA3 transgenic DP cells, in the lymphomas GATA3 expression was slightly decreased and c-Myc expression was significantly enhanced, suggesting secondary events. These included trisomy 15, which is often found in c-Myc induced lymphoma [37, 38], and activation of Notch signaling. In several cases activation of Notch signaling could be attributed to an activating mutation in the Notch1 gene PEST domain. In one lymphoma case we found evidence for a contribution of both trisomy 15 and Notch1 mutation to increased c-Myc expression (#b-9; Figure 5B and 7B).

Although GATA3 can form a complex with transcription factors Lmo2 and Tal1 [24], these factors were not aberrantly expressed in the lymphomas (data not shown). On the other hand, important parallels exist between CD2-GATA3 DP lymphoma and lymphoma induced by aberrant expression of Tal1/Lmo1 or E2A deficiency, including differential expression of Cyclin D3, Rorγ, Rag1,2, CD3, CD4, CD5, CD6 and IL-4R [56]. In addition, E2A-deficient lymphomas aberrantly express high levels of c-Myc with secondary events, including activating mutations of Notch1 and a trisomy 15 [36,57]. Thus, it appears attractive to speculate that GATA3 induces c-Myc expression by reducing E2A activity, which would then lead to secondary Notch1 mutations. However, our finding in CD2-GATA3 transgenic DP cells of increased expression of E2A and reduced expression of CD5, which is negatively regulated by E2A [5,10,11] excludes this possibility. Rather, the observed reduction of E2A appears a secondary event in DP cells expressing high c-Myc levels.

Finally, it has been shown that GATA3 is a target of Notch1 signaling, as the Notch1 pathway controls the lineage commitment of early thymic precursors by altering GATA3 levels [58] and determines T helper differentiation potential by direct regulation of GATA3 expression [59]. Because activation of Notch 1 does not result in higher GATA3 levels in lymphoma cells, further experiments are required to investigate if this capacity of Notch 1 is inhibited or dependent on a co-factor that is not expressed in these lymphoma cells. Likewise, we found differentiation stage-dependent effects of GATA3: it is well established that GATA3 is a key regulator of Th2 cytokines in effector T cells, but our DNA micro array analyses did not provide evidence for the induction of Th2 cytokines in thymic DP cells or in leukemias in CD2-GATA3 transgenic mice (Suppl. Table 1; van Hamburg *et al.*, unpublished). In this context, we found that enforced GATA3 affected c-Myc levels in DP cells, but not in Th2 effector cells (van Hamburg *et al.*, unpublished).

In summary, our findings revealed an oncogenic potential of GATA3 in that it has the capacity to enhance expression of c-Myc, thereby converting DP thymocytes into a pre-malignant state, in which subsequent induction of Notch1 signaling cooperates to establish malignant transformation. Interestingly, the relatively low GATA3 expression observed in the resulting lymphomas masks the primary role of GATA3

overexpression in lymphomagenesis in our CD2-GATA3 lymphoma model. This may have implications for human cancers, such as T-ALL and breast cancer, in which GATA3 may also cooperate in transformation with c-Myc and Notch1. The finding that GATA3 regulates c-Myc expression levels may explain the parallel phenotypes of mice with overexpression or deficiency of either of the two transcription factors. Although GATA3 was shown to be regulated by Notch1 in Th2 cells [59, 60] and Notch1 is a direct regulator of c-Myc in T cell leukemia [41-43], we now show for the first time that GATA3 takes part in the c-Myc/E2A/Notch network by regulating c-Myc expression in a direct or indirect fashion. Since the c-Myc/E2A/Notch network is not only involved in T-lineage malignancies in mouse and man, but also orchestrates T cell development, our findings do not only have implications for GATA3 as an oncogene in the T cell lineage, but also provide inside into the role of GATA3 in T cell development in the thymus.

ACKNOWLEDGEMENTS

We would like to thank T. Schonewille, E. de Haas, E. Splinter and E. van Drunen (Erasmus MC, Rotterdam) for assistance at various stages of the project. This work was partly supported by the Dutch Cancer Society and the International Association of Cancer Research.

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V

GATA3 controls the expression of CD5 and the T cell receptor during CD4 T cell lineage development

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Eur J Immunol 2007;37:1043-52

ABSTRACT

Transcription factor GATA3 is essential at multiple stages of T cell development, including the earliest double negative stages, β-selection and CD4 single positive (SP) thymocytes. Here, we show that in CD2-GATA3 transgenic mice, with enforced GATA3 expression driven by the CD2 promoter, thymocytes have reduced levels of CD5, which is a negative regulator of TCR signaling participating in TCR repertoire fine-tuning. Reduction of CD5 expression was most prominent in $CD4^+CD8^+$ double positive (DP) cells and was associated with increased levels of the transcription factor E2A. Conversely, GATA3-deficient DP thymocytes showed consistently higher CD5 levels and defective TCR upregulation during their development towards the $CD4^{lo}CD8^{lo}$ subpopulation. CD2-GATA3 transgenic mice carrying the MHC class II-restricted TCR DO11.10 also manifested decreased CD5 levels. As in these TCR transgenic mice reduced CD5 expression cannot result from an effect of GATA3 on repertoire selection, we conclude that enforced GATA3 interferes with the developmentally regulated increase of CD5 levels. Enforced GATA3 expression in DO11.10 transgenic mice was also accompanied by enhanced TCR expression during CD4 positive selection. Because GATA3 is induced by TCR signaling in DP thymocytes, our findings indicate that GATA3 establishes a positive feedback loop that increases TCR surface expression in developing CD4-lineage cells.

INTRODUCTION

Both CD4 and CD8 SP thymocytes differentiate from a common precursor pool of CD4/CD8 DP thymocytes through a process termed positive selection [1, 2]. One of the transcription factors indispensable for CD4 lineage development is the zinc-finger transcriptional regulator GATA3, which was originally identified as a protein that binds to the TCRα gene enhancer [3]. GATA3 is indispensable for early T cell lineage development and also plays a crucial role in the differentiation of mature Th2 effector cells by regulating transcription and chromatin configuration of the IL-4, IL-5 and IL-13 Th2 cytokine genes (Reviewed by Ansel *et al.*, 4).

A role for GATA3 in CD4 thymocyte development was first implied from its expression pattern in the thymus, as identified in *Gata3-LacZ* knock-in reporter mice. Low *lacZ* expression was found in DP and CD8 SP thymocytes, whereas high *lacZ* expression was found in the transitional $CD4^{hi}CD8^{lo}$ population and in CD4 SP cells [5, 6]. Importantly, RT-PCR and western blotting experiments demonstrated that GATA3 is upregulated in response to TCR stimulation during positive selection of CD4 but not CD8 thymocytes [7]. GATA3 is also expressed in early $CD4^-CD8^-$ double negative (DN) thymic T cell progenitors, whereby analysis of the *Gata3-LacZ* reporter mouse indicated that GATA3 is induced in those cells that have passed β-selection [5].

The embryonic lethality of *Gata3*$^{-/-}$ mutant mice at day 11 of gestation [8] and the failure of *Gata3*$^{-/-}$ ES cells to contribute to the T cell compartment in *Rag-2*$^{-/-}$ or WT chimeric mice [5, 9], precluded the analysis of GATA3 function in murine T cell development *in vivo*. Conditional deletion of the *Gata3* gene at the DN stage

using the *Cre*-loxP system, whereby the *Cre* transgene was driven by the proximal *Lck* promoter, resulted in a developmental arrest at the $CD25^+CD44^-$ DN3 stage, implicating GATA3 in β-selection [10]. Deletion of the *Gata3* gene after β-selection, using CD4-*Cre* transgenic mice, resulted in a profound specific deficiency of CD4 SP cells. These findings demonstrated the absolute requirement of GATA3 for survival or development of CD4-committed thymocytes *in vivo*. However, in the absence of GATA3, MHC class II-restricted T cells were not diverted into the CD8 lineage [10]. Conversely, sustained overexpression of GATA3 in fetal thymic organ cultures favored selection of CD4 over CD8 SP cells, but did not divert MHC class I-restricted precursors into the CD4 lineage [7]. On the basis of these findings it has been concluded that GATA3 is necessary for post-commitment CD4 generation, rather than for commitment to the CD4 lineage [7, 11]. Nevertheless, a possibility remains that GATA3 promotes CD4 lineage choice, as it is conceivable that MHC class II-restricted $CD8^+$ T cells or MHC class I-restricted $CD4^+$ T cells die as they fail to undergo MHC-TCR and CD4/CD8 co-engagement required for their survival [1].

We have previously shown that enforced expression of GATA3 in transgenic mice, driven by the *CD2* gene promoter and locus control region, inhibited maturation of CD8 SP cells, enhanced Th2 cell development in the periphery and induced thymic lymphoma in aging mice [6, 12]. We also identified a small increase in DP cell size and TCRαβ/CD3 expression levels in $CD69^+$ DP cells, suggesting that enforced GATA3 expression may influence the kinetics of positive selection.

In contrast to the important progress that has been made in understanding the role of GATA3 in the transcriptional regulation of the Th2 cytokine locus [4], downstream targets of GATA3 in T cell development are unknown. By analysis of CD2-GATA3 transgenic and *Gata3* conditonal knock-out mouse models, as well as CD2-GATA3 transgenic mice which were crossed into various TCR transgenic mice, we show in this report that GATA3 modulates the surface expression level of the glycoprotein CD5 and enhances TCR expression during CD4 T lineage development *in vivo*. The regulatory mechanisms that control CD5 expression are key to lymphocyte development and function, because CD5 is a negative regulator of TCR signaling during thymocyte development and therefore participates in the fine-tuning of the TCR repertoire [13-16]. Interestingly, because GATA3 is induced by TCR signaling [7], our finding that GATA3 controls TCR upregulation and CD5 downregulation implicate GATA3 in a positive feedback loop that increases TCR surface expression during CD4 T lineage development.

MATERIALS AND METHODS

Mice

CD2-GATA3 (FVB) transgenic mice have been described [6]. CD2-GATA3 x DO11.10 transgenic mice [29] were backcrossed to Balb/c ($H\text{-}2^d/I\text{-}A^d$) for 9 generations. HY/*Rag2*$^{-/-}$ (C57BL/10) mice, purchased from Taconic Europe A/S (Denmark), were crossed with CD2-GATA3 transgenic mice and backcrossed until *Rag2*$^{-/-}$ and homozygous for $H\text{-}2^b$. CD4-*Cre* mice [23] were a kind gift of Dr. C. Wilson (University

of Washington, Seattle, USA). The generation of *Gata3*$^{f/f}$ mutant mice has been described [22]. Similar to the *Gata3*$^{f/f}$ mice reported independently [10], we also failed to obtain the expected Mendelian transmission, suggesting significant lethal developmental defects originating from the floxed *Gata3* alleles. Occasionally *Gata3*$^{f/f}$ mice were very small and therefore excluded from analysis. CD4-Cre x *Gata3*$^{f/f}$ x CD2-GATA3 transgenic mice were on a mixed FVB x C57BL/6 background. Mice were bred and maintained in the Erasmus MC animal care facility under specific pathogen-free conditions and analyzed at 5-12 weeks. Experimental procedures were reviewed and approved by the Erasmus University committee of animal experiments.

Mouse genotyping

For mouse genotyping, tail DNA was analyzed by PCR. Primers for determination of the presence of the CD2-GATA3 transgene were 5'-CAGCTCTGGACTCTTCCC-AC-3' and 5'-CAGCTCTGGACTCTTCCCAC-3', and for the CD4-Cre transgene 5'-ACCCTGTTAC-GTATAGCCGA-3' and 5'-CTCCGGTATTGAAACTCCAG-3'. Genotyping for DO11.10 and HY was performed according to protocols obtained from Jackson laboratory and Taconic Europe A/S, respectively. Myogenin (primers: 5'-TTACGTCCATCGTGGACAGC-3' and 5'- TGGGCTGGGTGTT-AGTCTTA-3') was used as an internal control. PCR-based typing of *Gata3*$^{f/f}$ mice, and analysis of *Gata3* locus deletion was as described previously [22]. Mouse MHC haplotypes were confirmed by FACS analysis of peripheral blood using MHC class I-specific mAb.

Flow cytometry, mAb, and cell sorting

Preparation of single-cell suspensions, flow cytometry and mAb have been described previously [6]. Anti-human E2A and PE-labeled annexin V were purchased from BD Biosciences (San Diego, CA). Anti-HY TCR (T3.70) and anti-DO11.10 TCR (KJ1-26) mAb were from eBioscience and Caltag Laboratories, respectively. FACS sorting was performed with a FACSVantage VE equipped with Diva Option and BD FACSDiva software (BD Biosciences). The purity of the obtained fractions was >99%.

In vivo injection of CD2-GATA3 (non-)transgenic *Rag2*$^{-/-}$ mice was done i.v. with 50 µg a rat anti-mouse CD3 mAb (145-2C11; BD Biosciences) in 0.5 ml PBS.

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted from 5 x 10^6 sorted cells, using the GenElute™ mammalian total RNA miniprep system (Sigma-Aldrich, St. Louis, MO). One µg of total RNA was used as a template for cDNA synthesis using Superscript II RT (Invitrogen) and random hexamer primers. cDNA was diluted serially 3-fold before PCR amplification. Primers for CD5 were: 5'-AAGCATCATCCTGACCCTTG-3' and 5'-AGATCGGTGTAGGGCTCCTT-3' and primers for β-actin were: 5'-TACCACTGGCATCGTGATGGACT-3' and 5'- TTTCTGCATCCTGTCGGCAAT-3'. RT-PCR products were separated by standard agarose electrophoresis and visualized by ethidium bromide staining.

Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) and standard thermocycling conditions. Threshold levels were set and further analysis was performed using the

SDS v1.9 software (Applied Biosystems). The obtained C_t values for GATA3 were normalized by the C_t values for glycereraldehyde-3-phosphate dehydrogenase (GAPDH). Primers detecting both endogenous and transgenic GATA3 were: 5'-CATTACCACCTATCCGCCCTAT-3' and 5'-CACACACTCCCTGCCTTCTGT-3' with 5'-CGAGGCCCAAGGCACGATCCAG-3' as a probe. GAPDH primers were 5'-TTCACCACCATGGAGAAGGC-3' and 5'-GGCATGGACTGTGGTCATGA-3' with 5'-TGCATCCTGCACCACCAACTG-3' as a probe.

RESULTS

Enforced GATA3 expression is associated with reduced CD5 surface expression

As a strategy to identify downstream targets of GATA3, we performed DNA micro-array analyses, comparing expression profiles of sorted WT and CD2-GATA3 transgenic DP thymocytes (van Hamburg *et al*., manuscript in preparation). In these analyses, enforced GATA3 expression correlated with low levels of CD5 transcripts in DP thymocytes. RT-PCR experiments confirmed that CD5 mRNA levels were consistently lower in CD2-GATA3 transgenic DP cells (Figure 1A).

Next, we compared CD5 surface expression in WT and CD2-GATA3 transgenic thymocytes by flow cytometry (Figure 1BC). Early DN cells initiate low-level CD5 expression independently of TCRβ rearrangement [14]. Then CD5 expression levels are upregulated in response to pre-TCR signaling, increase progressively as cells develop into immature single positive (ISP) and DP cells, and further increase after TCR engagement. The expression levels on mature SP thymocytes correlate to the avidity of the TCR, whereby CD4 SP cells generally express higher CD5 levels than CD8 SP cells [14]. The presence of the CD2-GATA3 transgene did not affect CD5 expression on DN cells (Figure 1C). Detailed analysis of DN1-DN4 subfractions, as defined by surface CD25 and CD44, also did not reveal significant differences between WT and CD2-GATA3 transgenic cells (data not shown). However, at the ISP stage and in particular at the DP stage surface CD5 levels were consistently lower in CD2-GATA3 transgenic thymocytes, when compared with WT thymocytes (median fluorescence values were decreased with a factor ~5 in DP cells; Figure 1C). During the multistage positive selection process involving $CD4^{lo}CD8^{lo}$ and $CD4^{hi}CD8^{lo}$ stages [17], upregulation of CD5 levels occurred both in WT and in CD2-GATA3 transgenic thymocytes. Nevertheless, CD5 levels were consistently lower in CD2-GATA3 transgenic thymocytes (Figure 1C). This decrease in CD5 expression persisted in CD4 SP thymocytes and mature CD4 T cells in blood and spleen (Figure 1D, E). CD5 expression on CD8 SP thymocytes and mature peripheral CD8 T cells was only marginally decreased in CD2-GATA3 transgenic mice, when compared with WT controls.

Collectively, these results show that enforced expression of GATA3 results in decreased CD5 surface levels, in particular at the DP stage in the thymus and in mature $CD4^+$ peripheral T cells.

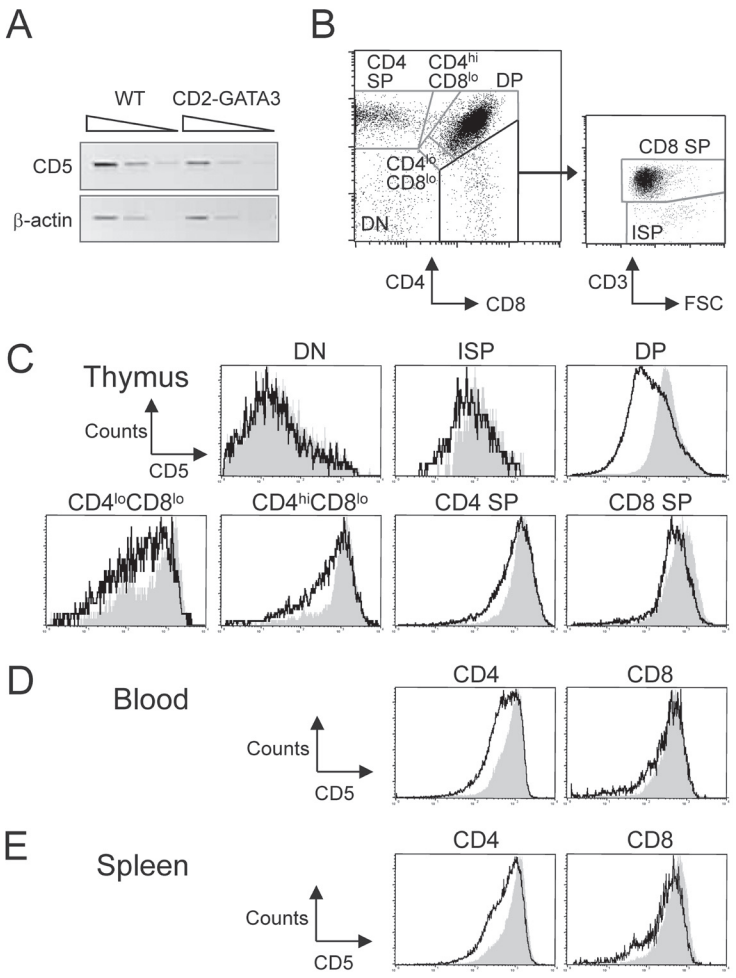

Figure 1. Enforced GATA3 expression results in reduction of surface CD5 levels. (A) RT-PCR analysis of CD5 expression in DP cells from WT and CD2-GATA3 transgenic mice. cDNA templates were 3-fold serially diluted and β-actin was used as a loading control. Data shown are representative of four mice analyzed per genotype. (B) Flow cytometric analysis, defining the indicated subsets of developing thymocytes with respect to CD4, CD8 and CD3 expression. (C, D and E) Flow cytometric analysis for CD5 surface expression on the indicated subpopulations in thymus (C), blood (D) and spleen (E) from WT (shaded histograms) and CD2-GATA3 mice (line overlays). Data shown are representative of 4-6 mice analyzed per group.

Enforced GATA3 expression is associated with increased levels of E2A

The reduced surface expression of CD5 in CD2-GATA3 transgenic DP cells was paralleled by reduced intracellular expression levels and was associated with increased expression of the transcription factor E2A (Figure 2A). As it has been

reported that CD5 expression is negatively regulated by interaction of E2A with the CD5 regulatory promoter [18], it is very well possible that GATA3 reduces CD5 levels by upregulation of E2A.

Because the majority of DP cells will not be selected and die "by neglect", the finding of decreased surface CD5 levels in CD2-GATA3 transgenic ISP and DP cells suggested that GATA3 has the capacity to regulate CD5 expression at the DP stage in a TCR-MHC interaction-independent fashion. In agreement with this notion, we found that DP cells that were induced by stimulation of CD2-GATA3 transgenic *Rag2*$^{-/-}$ DN3 cells with anti-CD3 mAb *in vivo* expressed significantly reduced CD5 levels in mice, when compared with induced DP cells from non-transgenic *Rag2*$^{-/-}$ mice (Figure 2B). In Rag-deficient mice, where T cell development is arrested at the DN3 stage, injecting anti-CD3 mAb has been shown to mimic pre-TCR signaling: it overcomes the developmental block, increases thymic cellularity, allows DN3 cells to differentiate into DN4 and then subsequently to the DP stage, but not to SP stages [19-21].

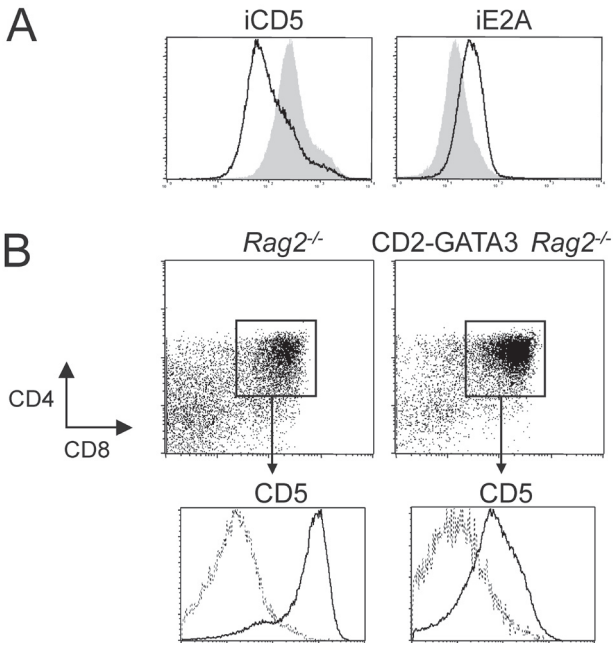

Figure 2. Enforced GATA3 expression is associated with increased E2A and with low CD5 levels upon *in vivo* induction of *Rag-2*$^{-/-}$ DPcells by anti-CD3. (A) FACS analysis for intracellular CD5 (iCD5) and E2A (iE2A) expression in gated DP cells from WT (shaded histograms) and CD2-GATA3 mice (line overlays). (B) FACS analysis of thymocytes from non-transgenic and CD2-GATA3 transgenic *Rag-2*$^{-/-}$ mice that were injected with 50 μg of anti-CD3 mAb. Expression profiles of CD4 and CD8 are displayed as dot plots. DP cell fractions were gated and analyzed for the expression of CD5, displayed as histograms from uninduced total thymocytes (broken lines) with overlays from anti-CD3 induced (day 3) DP cells (line overlays). Data shown are representative of 2-4 mice analyzed per group.

Taken together, these findings show that enforced expression of GATA3 is correlated with increased E2A levels and interferes with the progressive increase in CD5 expression that takes place when *Rag-2*$^{-/-}$ DN3 cells differentiate into DP cells *in vivo*.

GATA3-deficiency is associated with defective regulation of CD5 and TCR during CD4 T cell lineage development

To further investigate the relevance of the finding of reduced CD5 expression in CD2-GATA3 transgenic mice, we analyzed the expression of CD5 during T cell development in mice with a conditional deletion in the *Gata3* locus. *Gata3*$^{f/f}$ mice, harboring *Gata3* alleles containing two *loxP* sites [22] (see Figure S1), were crossed with CD4-Cre transgenic mice [23], resulting in conditional inactivation of the *Gata3* gene in the T cell lineage. To verify Cre-mediated deletion of the *Gata3* gene, DP, CD4 SP and CD8 SP cells were FACS-sorted, and floxed and deleted *Gata3* alleles were identified by PCR assays (see Figure S1). We found that in CD4-*Cre Gata3*$^{f/f}$ mice essentially all DP cells had undergone *Cre*-mediated deletion, since we were not able to amplify floxed *Gata3* alleles from thymic DP, CD4 or CD8 fractions (Figure 3A). Only, in CD4$^+$ and CD8$^+$ cell fractions from spleen we detected low levels of PCR products from floxed *Gata3* alleles (Figure 3A), which might be derived from rare CD4$^+$ or CD8$^+$ T cells that have escaped deletion [23] or alternatively from CD4$^+$ or CD8$^+$ dendritic cells.

Whereas thymic T cell development was found to be normal in *Gata3*$^{f/+}$ and *Gata3*$^{f/f}$ mice, all CD4-*Cre Gata3*$^{f/f}$ mice analyzed showed a specific defect in the development of CD4 lineage cells, consistent with previously reported findings [10] (see Figure S2). In contrast to Pai *et al*., 10, we observed a ~3 4-fold reduction in total thymocyte numbers in our CD4-*Cre Gata3*$^{f/f}$ mice. Both ISP and DP total cell numbers were diminished (see Figure S2), indicating that (i) the *Cre* recombinase is already expressed at significant levels at the ISP stage, and (ii) that GATA3 is important for the production and/or the survival of ISP and DP cells.

We analyzed CD5 levels in the various T cell subpopulations in the GATA3 conditional knock-out mice by flow cytometry. As shown in Figure 3C, CD4-*Cre Gata3*$^{f/f}$ mice manifested consistently higher CD5 levels in ISP cells and all subsequent stages including CD4 and CD8 SP cells, when compared with littermate controls. Next, we investigated other positive selection parameters, including cell size (forward light scatter), surface TCR and CD69 expression levels [17, 24-26]. Remarkably, in *Gata*3-deficient CD4^{lo}CD8^{lo} and CD4^{hi}CD8^{lo} cells upregulation of TCR and CD69 was hampered. Those few CD4 SP cells present had low TCR and CD69 expression, whereas CD8 SP fractions had essentially normal expression of TCR and CD69 (Figure 4), in agreement with the analyses of Pai *et al*., 10.

Therefore, we conclude that GATA3 is involved in modulation of CD5 levels from the ISP stage onwards and in upregulation of TCR expression during positive selection of CD4 lineage cells.

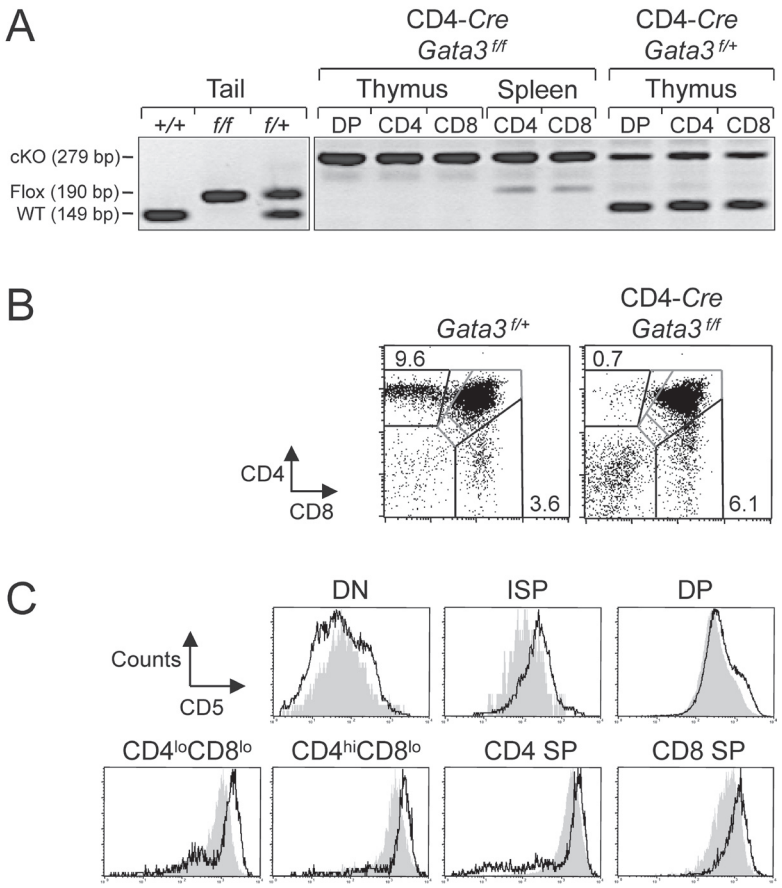

Figure 3. GATA3-deficiency is associated with increased CD5 surface expression. (A) PCR analyses of conditional deletion of the *Gata3* gene in tails, thymus and spleen samples. The Gata3 floxed allele (190bp PCR product), present in tail DNA, is deleted in CD4-*Cre Gata3*$^{f/f}$ or *Gata3*$^{f/+}$ DP, CD4 SP or CD8 SP thymocytes. Since CD4-*Cre Gata3*$^{f/f}$ essentially do not have splenic CD4 cells, these samples were from CD2-GATA3 transgenic CD4-*Cre Gata3*$^{f/f}$. (B) Flow cytometric analysis for surface CD4 and CD8 on thymocytes from the indicated mice. The percentages of gated CD4 and CD8 SP cells are given. (C) Flow cytometric analysis for surface CD5 expression on the indicated thymocyte subpopulations from *Gata3*$^{f/+}$ control mice (shaded histograms) and CD4-*Cre Gata3*$^{f/f}$ mice (line overlay). Results are representative of 3-5 mice per group.

Enforced GATA3 expression does not affect CD4/CD8 lineage choice

We previously observed that enforced GATA3 expression inhibited maturation of CD8 cells, but did not appear to affect the CD4 versus CD8 lineage fate decision [6]. However, in the CD2-GATA3 transgenic mice analyzed, differences in levels of endogenous GATA3 between developing CD4 and CD8 cells were maintained. Therefore, we crossed CD2-GATA3 transgenic mice with the conditionally deleted

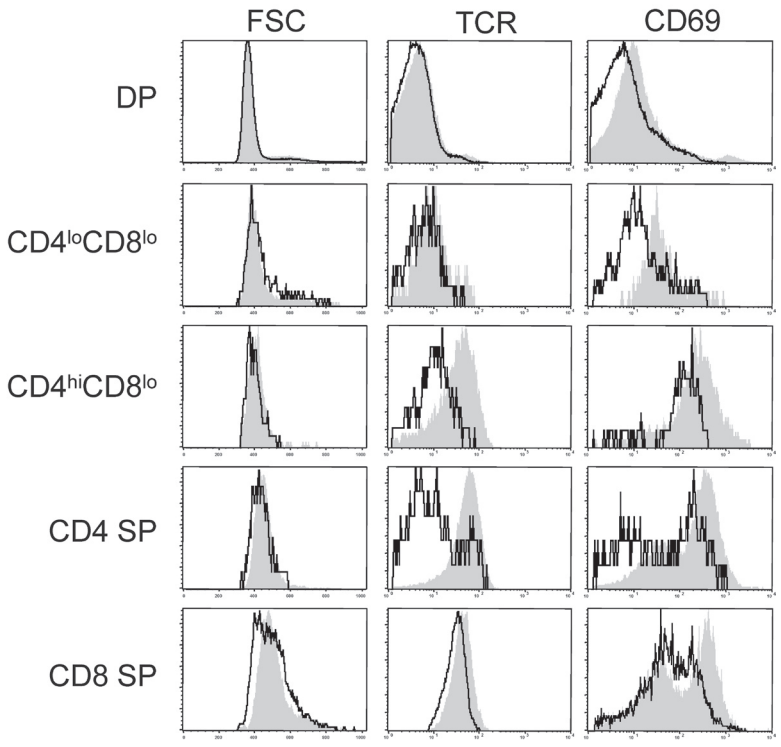

Figure 4. Defective TCR and CD69 upregulation in GATA3-deficient CD4-lineage development. Flow cytometric analysis for forward light scatter (FSC), TCR and CD69 expression on the indicated thymocyte subsets from *Gata3*$^{f/+}$ controls (shaded histograms) and CD4-*Cre Gata3*$^{f/f}$ mice (line overlays). Results are representative of 3-5 mice per group.

CD4-*Cre Gata3*$^{f/f}$ mice, to generate mice in which developing CD4 SP and CD8 SP thymocytes express the same level of GATA3, contributed exclusively by the CD2-GATA3 transgene, irrespective of their developmental choice. Quantitative RT-PCR experiments confirmed that in the CD2-GATA3 transgenic CD4-*Cre Gata3*$^{f/f}$ mice GATA3 expression was increased to similar levels in DP, CD4 SP and CD8 SP cells (see Figure S2).

We found that the presence of the CD2-GATA3 transgene corrected the defects in CD4-*Cre Gata3*$^{f/f}$ mice: total thymocyte numbers and the sizes of thymocyte subpopulations, including ISP, DP and CD4, were comparable to those in control littermates (Figure S2), showing that in this respect the CD2-GATA3 transgene could functionally replace the endogenous *Gata3* gene. Similar to the CD2-GATA3 transgenic mice [6], also the CD2-GATA3 transgenic CD4-*Cre Gata3*$^{f/f}$ mice showed defective maturation of CD8 cells, characterized by reduced numbers of CD8 SP cells (Figure S2), impaired downregulation of CD69, HSA and CD44, and reduced numbers of splenic CD8^{+} T cells (data not shown), similar to the phenotype described for CD2-GATA3 transgenic mice on the FVB background [6].

In spite of this defective CD8 maturation, quantification of thymocyte subpopulations showed that in the absence of differential GATA3 expression between MHC class I- and MHC class II-mediated positive selection both CD4 and CD8 SP cell populations could develop (See Figure S2). We did not find evidence for differential modulation of GATA3 activity in CD4 and CD8 SP cells by co-factors, because RT-PCR analyses of CD2-GATA3 transgenic CD4-*Cre Gata3*$^{f/f}$ CD4 and CD8 SP cell fractions did not reveal differences in the expression of the GATA3 co-factors FOG (friend of GATA) or ROG (repressor of GATA) [27, 28] (data not shown).

Collectively, these findings support earlier conclusions [6, 7, 11] that CD4/CD8 lineage choice is independent of GATA3 expression levels during positive selection and that GATA3 selectively controls developmental progression of committed CD4 T lineage cells.

Enforced GATA3 expression results in defective CD5 and enhanced TCR upregulation during CD4 positive selection

The finding of reduced CD5 levels in the presence of enforced GATA3 might either reflect an effect of GATA3 on repertoire selection or alternatively result from defective CD5 upregulation. To distinguish between these possibilities, we crossed the CD2-GATA3 transgene into TCR transgenic mice, which express TCR of only a single specificity.

The DO11.10 transgene encodes an ovalbumin peptide-specific TCRαβ that is MHC class II-restricted, and is recognized by the specific antibody KJ1-26 [29]. In the I-A^d background DO11.10 thymocytes are positively selected towards the CD4 lineage (Figure 4A). Consistent with the function of GATA3 as a transcription factor essential for CD4 thymocyte development, the presence of the CD2-GATA3 transgene did not affect DO11.10 CD4 lineage-restriction (Figure 5A). However, in the presence of the CD2-GATA3 transgene total thymocyte and splenic CD4 T cell numbers were significantly reduced (Figure 5AB). CD2-GATA3 DO11.10 double transgenic mice manifested a considerable reduction of thymic DN and, in particular, of the DP subpopulation (Figure 5B). The reduction of the DP population in CD2-GATA3 DO11.10 double transgenic mice was accompanied by an enhanced apoptosis susceptibility of these cells, as evidenced by their increased annexin V positivity (35% ± 4, n = 3), compared with 8.4% ± 1.7 (n = 4) in DO11.10 single transgenic DP cells (Figure 5C). Nevertheless, the total numbers of positively selected CD4 SP cells in the thymus appeared unaffected (Figure 5B).

As a next step, we compared DO11.10 single transgenic and CD2-GATA3 DO11.10 double transgenic thymocytes for the expression of CD5, TCR (total and KJ1-26$^+$) and CD69. DO11.10 single transgenic thymocytes increased their CD5 expression levels at the DN to DP transition, whereas TCR and CD69 were upregulated at the DP to CD4 SP transition (Figure 5). Compared with DO11.10 single transgenic thymocytes, CD2-GATA3 DO11.10 double transgenic thymocytes showed an altered phenotype, characterized by (i) increased FSC values and TCR expression levels at the DP stage and (ii) decreased CD5 expression, both at the DP and the CD4 SP stage. Also the splenic CD4 T cell population in the CD2-GATA3 DO11.10 double transgenic

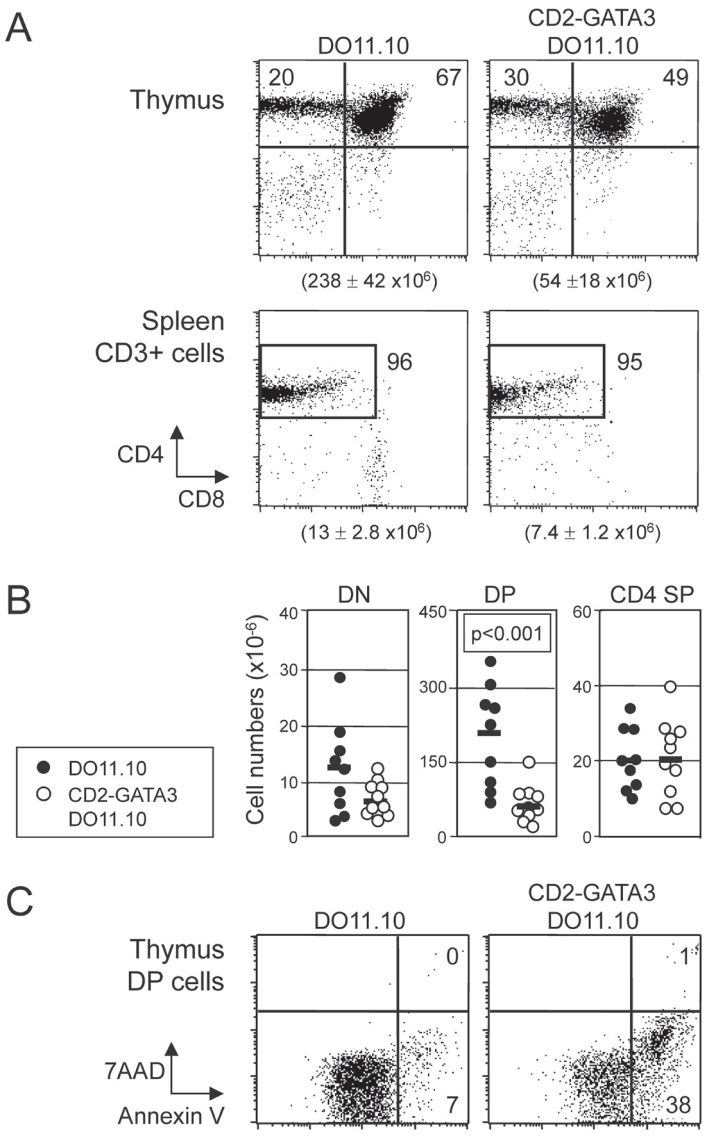

Figure 5. Reduced cell survival in CD2-GATA3 DO11.10 double transgenic DP thymocytes. (A) Flow cytometric analysis for surface CD4 and CD8 expression on total thymocytes and gated $CD3^+$/KJ1-26^+ splenocytes from DO11.10 transgenic and CD2-GATA3 DO11.10 double transgenic mice. Numbers in the dot plots indicate the proportions of CD4 SP and DP thymocytes (*upper part*) or the proportions of $CD4^+$ cells (*lower part*). Total numbers of thymocytes and $CD4^+$ splenocytes are given in parentheses. (B) Thymocyte subpopulations in the indicated mouse groups. Black lines represent average values. A significant difference was only observed in the DP subpopulations, by t-test. (C) Detection of apoptotic cells by flow cytometric analysis for 7AAD and Annexin V on gated DP thymocytes from the indicated mice. The proportions of DP thymocytes within the indicated quadrants are given.

mice showed decreased CD5 expression levels (data not shown). Interestingly, CD2-GATA3 DO11.10 double transgenic DP cells manifested elevated TCR and CD69 levels, which were already close to those of CD4 SP cells (Figure 5).

Taken together, these findings show that enforced expression of GATA3 results in impaired upregulation of CD5 and enhanced TCR upregulation during positive selection of DO11.10 transgenic CD4 cells.

Enforced GATA3 expression also affects CD5 levels during CD8 positive selection

Next, we crossed our CD2-GATA3 transgenic mice with HY αβTCR transgenic mice on a *Rag2*$^{-/-}$ background. The MHC class I-restricted HY TCR is specific for the male-specific HY antigen peptide [30].

In the H-2^{b} class I female background, HY-specific thymocytes were positively selected towards the CD8 lineage (Figure 7A). Parallel to our observations in the DO11.10 CD4 positive selection model, we found that the presence of the CD2-GATA3 transgene reduced DP cellularity, but numbers of thymic CD8 SP and splenic CD8^{+} cells were comparable between the HY single transgenic and CD2-GATA3 HY double transgenic littermates (Figure 7AB). The CD2-GATA3 transgene did not affect CD8-lineage restriction by the HY transgene, as CD4 SP cells were not found in the CD2-GATA3 HY double transgenic female thymus or spleen (Figure 7A). Yet, we observed defective CD4 silencing in the CD2-GATA3 HY double transgenic CD8 population in the spleen (Figure 7A).

In CD2-GATA3 HY double transgenic female DP cells, additional CD5^{lo} and TCR^{hi} populations were present and CD69 expression levels were slightly increased. In contrast to the CD2-GATA3 CD4 SP cells in the DO11.10 model, the CD2-GATA3 HY double transgenic CD8 SP cells manifested normal CD5 expression levels (compare Figure 6 and 7C). The effect of the CD2-GATA3 transgene on cell size and TCR expression in CD8 SP cells was negligible. The CD2-GATA3 HY double transgenic CD8 SP cells failed to downregulate CD69 (Figure 7C). This most likely reflected an inhibitory effect of enforced GATA3 on SP cell maturation, as was substantiated by analysis of additional maturation markers including CD44, CD62L and HSA (data not shown) and paralleled our previous findings in CD2-GATA3 single transgenic mice on the FVB background [6].

Analysis of male HY transgenic and CD2-GATA3 HY double transgenic thymocytes by flow cytometry revealed that negative selection was preserved in the presence of enforced GATA3 expression. Hereby a majority of thymocytes had a DN phenotype [30], irrespective of the presence of the CD2-GATA3 transgene. (data not shown).

Taken together these findings show that also in DP cells that are committed to the CD8 lineage, enforced GATA expression results in defective CD5 upregulation and in the appearance of a small but detectable subpopulation with increased TCR expression.

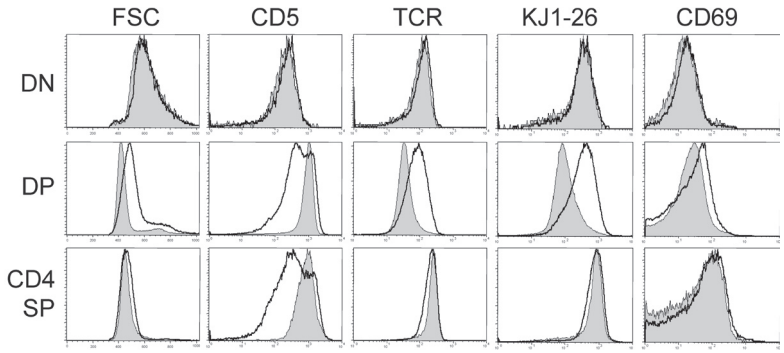

Figure 6. Reduced CD5 and enhanced TCR expression in CD2-GATA3 DO11.10 double transgenic DP thymocytes. Flow cytometric analysis for the indicated parameters of gated thymocyte subpopulations (indicated on the left) from DO11.10 transgenic mice (shaded histograms) and CD2-GATA3 DO11.10 double transgenic mice (line overlays). Histograms are representative for 8-10 mice analyzed per group.

DISCUSSION

The role of GATA3 in the development of the CD4 T cell lineage is largely unknown. In this report, we show that GATA3 controls CD5 and TCR expression during CD4 T cell lineage development.

Our FACS analyses in CD4-*Cre Gata3*$^{f/f}$ mice show that in the absence of GATA3, DP cells have slightly increased CD5 levels, which remain elevated during positive selection towards CD4 SP and CD8 SP subpopulations. Conversely, enforced GATA3 expression resulted in significantly reduced CD5 expression in DP cells, irrespective of their commitment towards CD4 or CD8 lineage (in DO11.10 or HY TCR transgenic mice respectively). Nevertheless, this reduction was maintained in CD4 SP cells, but not in CD8 SP cells. As in the TCR transgenic mice reduced CD5 expression cannot result from an effect of GATA3 on repertoire selection, we conclude that enforced GATA3 interferes with the developmentally regulated increase of CD5 levels. We propose that this effect is independent of TCR-MHC interactions, as CD5 levels were also increased when DN3 cells differentiated into DP cells during β-selection induced by anti-CD3 antibodies *in vivo*. In CD4-Cre *Gata3*$^{f/f}$ mice TCR and CD69 are not effectively upregulated during positive selection of CD4 lineage cells. Conversely, enforced GATA3 expression was associated with increased cell size, enhanced or accelerated TCR (and CD69) expression during CD4 T cell lineage development in MHC class II-restricted DO11.10 TCR transgenic mice. Taken together, we conclude that GATA3 is essential for appropriate TCR upregulation and CD5 modulation, selectively in developing CD4 T lineage cells. As GATA3 expression is specifically upregulated during development of CD4 and not CD8 lineage cells [7], our findings also imply that the molecular mechanisms of the modulation of TCR and CD5 expression levels during positive selection are different between the CD4 and the CD8 lineage.

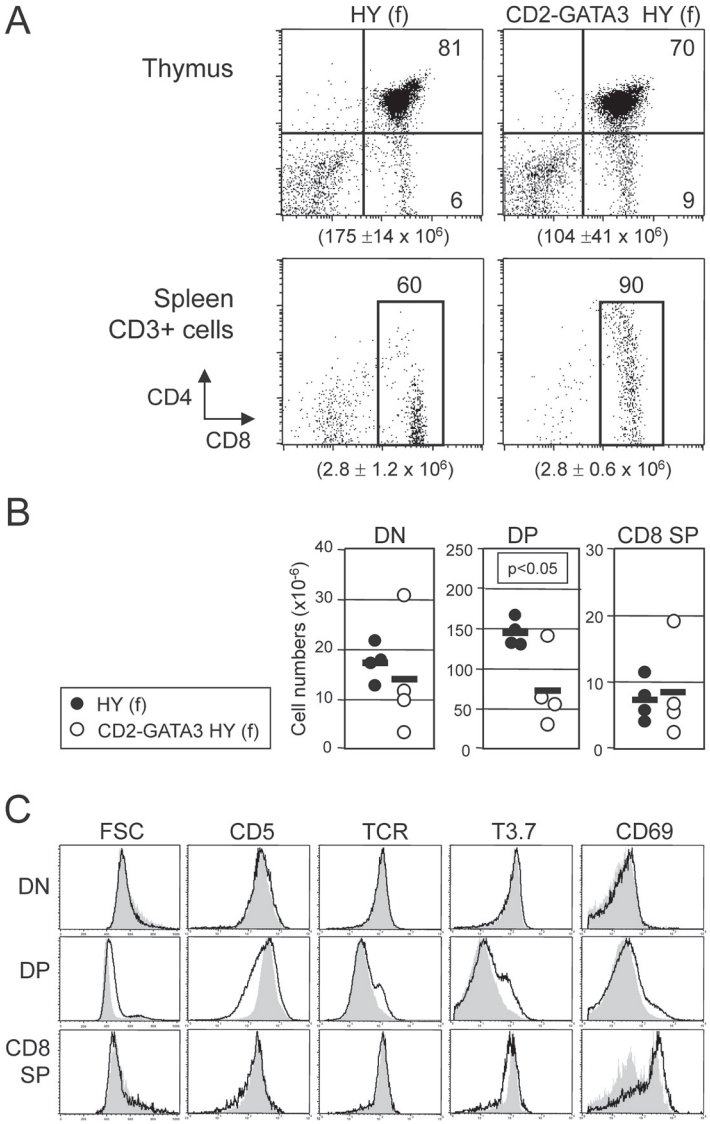

Figure 7. Aberrant phenotype of female CD2-GATA3 HY double transgenic DP cells. (A) Flow cytometric analysis for surface CD4 and CD8 expression on total thymocytes and gated T3.7^{+}CD3^{+} splenocytes from HY and CD2-GATA3 HY double transgenic female (f) mice. Numbers in the dot plots indicate the percentages of DP and CD8 SP thymocytes (*upper part*) or the proportions of CD8^{+} cells (*lower part*). Total numbers of thymocytes and CD8^{+} splenocytes are given in parentheses. (B) Thymocyte subpopulations in the indicated mouse groups. Black bars represent average values. A significant difference was only observed in the DP cell populations, by t-test. (C) Flow cytometric analysis for the indicated parameters of gated thymocyte subpopulations (indicated on the left) from HY transgenic female mice (shaded histograms) and CD2-GATA3 HY double transgenic female mice (line overlays). Histograms are representative for 4 mice analyzed per group.

It has been proposed that GATA3 contributes to linking TCR signal strength to the distinct differentiation programs of CD4 and CD8 thymocytes, implicating GATA3 in CD4/CD8 lineage choice [7]. Here, we substituted the endogenous *Gata3* gene expression by the CD2-GATA3 transgene (which is not subjected to regulation by TCR signals during positive selection). As a result, DP thymocytes containing MHC class I- and class II-restricted TCR expressed equivalent levels of GATA3, as contributed by the CD2-GATA3 transgene only. We found that both CD4 and CD8 SP cells were generated (Figure S2), ruling out that GATA3 expression levels are instrumental in CD4/CD8 lineage choice.

Enforced expression of GATA3 was also associated with survival defect of DP cells, both in the DO11.10 (I-A^d) and HY (female) positive selection model. As targeted deletion of CD5 has been shown to reduce DP cellularity in various TCR transgenic models, including HY and DO11.10 [16,31], it is likely that the reduction of DP cell numbers in the CD2-GATA3 transgenic models is a result of decreased CD5 expression. As CD5 acts as a negative regulator of TCR-mediated signal transduction [13, 16], reduction of CD5 expression levels in CD2-GATA3 expressing DP cells is expected to increase TCR signal strength, which will result in negative selection and thus deletion. However, it cannot be excluded that the impaired thymocyte expansion and the partial developmental arrest found in thymocytes with premature TCRαβ expression and signaling [32], may also contribute to the observed reduction in the DP cell population in TCR CD2-GATA3 double transgenic mice.

Parallel to our observations in mice with enforced GATA3 expression, *CD5*$^{-/-}$ DP cells are also larger and show increased levels of TCR and CD69 [13, 16, 18]. In the absence of CD5, DO11.10 (I-A^d) transgenic mice also show enhanced deletion of DP cells, accompanied by efficient positive selection towards CD4 SP cells [31]. Conversely, the residual $CD4^{hi}CD8^{lo}$ cells present in mice in which the *Gata-3* gene is conditionally targeted showed a $CD5^{hi}TCR^{lo}CD69^{lo}$ phenotype, thereby apparently mimicking a “death by neglect” cell fate. Because in GATA3 deficient DP cells the TCR-proximal signal transduction pathways, including ZAP70, Lck and Erk, are intact [11], it is attractive to hypothesize that GATA3 is required as a downstream target of TCR signaling, whereby GATA3 function is essential for CD4-lineage development to initiate TCR upregulation.

In this context, our finding that CD2-GATA3 DP cells have increased expression levels of the basic helix-loop-helix transcription factor E2A, which is a negative regulator of CD5 expression during thymocyte development [18], is interesting. Although by RT-PCR and DNA microarray analyses we did not detect significant differences in E2A transcript levels between CD2-GATA3 and WT DP cells (Ling *et al.*, unpublished), it is very well possible that GATA3 acts by stabilizing E2A protein levels, as in erythrocytes E2A has been shown to be part of a large DNA-binding complex containing the GATA-family member GATA1 [33], and the related helix-loop-helix transcription factor Tal-1/Scl has been shown to interact directly with GATA1 and GATA3 [34, 35]. A role for GATA-3 as a negative regulator of CD5 gene expression would also be supported by our observation in micro array experiments that enforced GATA3 expression is associated with downregulation of CD6 (van Hamburg *et*

al., unpublished), which is closely linked to CD5 in the genome and has a similar pattern of expression (ref. 31 and references therein). Nevertheless, as transgenic overexpression of CD5 does not result in complete arrest of development of CD4 T-lineage cells [16], it is clear that appropriate reduction of CD5 expression levels is not the only function of GATA3 during positive selection and maturation of CD4 T cells.

As GATA3 is induced by TCR signaling [7], our finding that enforced GATA-3 expression in developing DO11.10 transgenic CD4 lineage cells is associated with premature TCR upregulation (Figure 4) implicates GATA3 as a key regulator in a positive feedback loop. Induction of GATA3 by TCR signaling will increase TCR expression and thereby enhance the induction of GATA3. Moreover, GATA3 has the ability to increase TCR signal strength in an independent parallel pathway by downregulating CD5, leading to an efficient mode of signal amplification in this positive regulatory loop. As a result, during development of DP to CD4 SP cells the expression levels of the TCR and GATA3 will increase. It may be possible that GATA3 directly regulates TCR α and β transcription, as binding sites have been identified in these loci [3, 36]. As GATA3 expression is not induced in the CD8 cell lineage [5, 7], it is clear that different nuclear factors should be responsible for the regulation of the expression level of CD5 and TCR molecules in the CD8 lineage.

ACKNOWLEDGEMENTS

We acknowledge E. de Haas, J. van de Wees, F. Grosveld, H. Kuipers, B. Lambrecht and the people from the EDC Animal Facility (Erasmus MC, Rotterdam) for assistance at various stages of the project. We also thank Dr. C. Wilson (University of Washington, Seattle, USA) for providing CD4-Cre mice. This work was supported by the Association for International Cancer Research and the Dutch Cancer Society.

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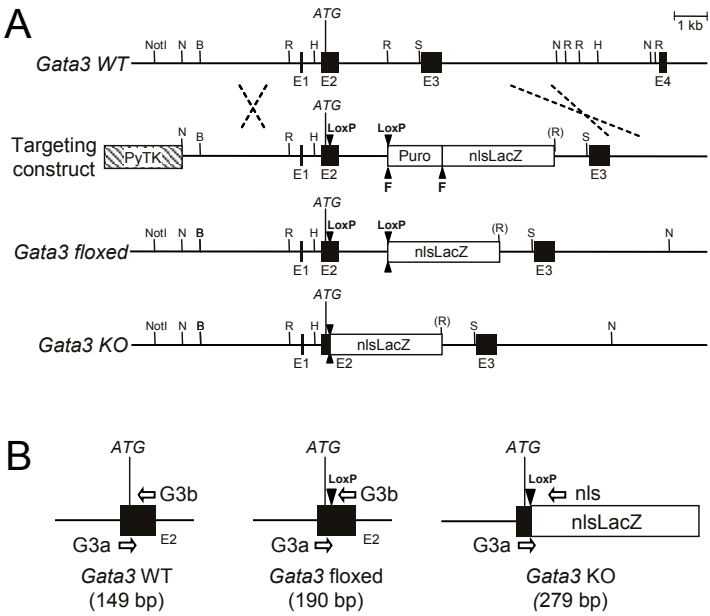

Figure S1. Conditional deletion of the Gata3 gene. (A) The targeting vector for conditional deletion of the Gata3 gene contained (i) a polyoma thymidine kinase gene to select against random integration events, (ii) part of the *Gata3* locus, in which LoxP sites were introduced in the start codon in exon 2 (E2) and in the second intron, (iii) a Puro cassette, flanked by FLP recognition target sites and coupled to an nlslacZ gene, which was introduced in *Gata3* intron 2 as well [22]. The wild-type Gata3 locus, targeting construct, floxed Gata3 (Puro cassette was removed by crossing with a Flp recombinase transgenic mouse) as well as the Gata3 KO allele generated by Cre-mediated deletion are shown. (B) Position of the primers used to specifically amplify wild-type exon 2, floxed exon two, containing a single LoxP site, and the targeted *Gata3* allele (consisting of the 5' part of exon 2, a loxP and a FLP recognition target site and the inserted nlslacZ cassette). See ref. 22 for details.

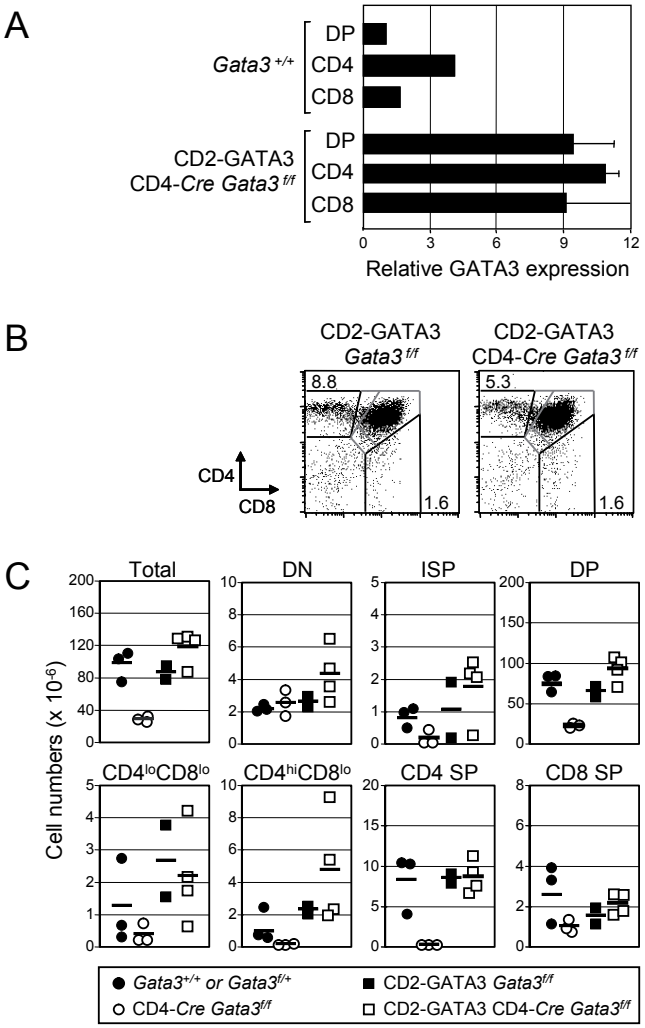

Figure S2. The CD2-GATA3 transgene rescued CD4 T cell development in mice with conditional deletion of the Gata3 gene. (A) Quantitative GATA3 RT-PCR analyses of the indicated sorted thymocyte fractions from a wild-type mouse and from CD2-GATA3 CD4-*Cre GATA3*$^{f/f}$ mice (n = 3). Obtained C_t values were normalized by the C_t value of glycereraldehyde-3-phosphate dehydrogenase (GAPDH), whereby the expression in WT DP cells was set to one. (B) CD4/CD8 flow cytometry profiles of thymocytes from the indicated mice. (C) Total number of thymocytes and the indicated thymocyte subpopulations in the four mouse groups. Every symbol represents an individual mouse. Black horizontal line = average value. Values in conditionally deleted CD4-*Cre GATA3*$^{f/f}$ mice were significantly reduced for total thymocytes (p<0.05), ISP (p<0.05), DP (p<0.002) and CD4 cells (p<0.02), when compared with wild-type controls, by t-test. Likewise, values in CD2-GATA3 transgenic CD4-*Cre GATA3*$^{f/f}$ mice were significantly increased for total thymocytes (p<0.001), ISP (p<0.05), DP (p<0.001), CD4 (p<0.001) and CD8 cells (p<0.05), when compared with the conditionally deleted CD4-*Cre GATA3*$^{f/f}$ mice.

VI

Enforced expression of GATA3 allows differentiation of IL-17 producing cells, but constrains Th17-mediated pathology

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Eur J Immunol 2008: in press

ABSTRACT

The zinc-finger transcription factor GATA3 serves as a master regulator of T helper-2 (Th2) differentiation by inducing expression of the Th2 cytokines IL-4, IL-5 and IL-13 and by suppressing Th1 development. Here, we investigated how GATA3 affects Th17 differentiation, using transgenic mice with enforced GATA3 expression. We activated naive primary T cells *in vitro* in the presence of TGFβ and IL-6, and found that enforced GATA3 expression induced co-expression of Th2 cytokines in IL-17 producing T cells. Although the presence of IL-4 hampered Th17 differentiation, TGFβ/IL-6 cultures from GATA3 transgenic mice contained substantial numbers of IL-17$^+$ cells, partially because GATA3 supported Th17 differentiation by limiting IL-2 and IFNγ production. GATA3 additionally constrained Th17 differentiation *in vitro* through IL-4-independent mechanisms, involving downregulating transcription of STAT3, STAT4, NFATc2 and the nuclear factor RORγt, which is crucial for Th17 differentiation. Remarkably, upon myelin oligodendrocyte glycoprotein immunization *in vivo*, GATA3 transgenic mice contained similar numbers of IL-17-producing T cells in their lymph nodes as wild-type mice, but were not susceptible to autoimmune encephalomyelitis, possibly due to concomitant production of IL-4 and IL-10 induction. We therefore conclude that although GATA3 allows Th17 differentiation, it acts as an inhibitor of Th17-mediated pathology, through IL-4-dependent and IL-4-independent pathways.

INTRODUCTION

T helper cells are classically divided into two functionally distinct subsets, termed T helper-1 (Th1) and Th2 (See for review: ref. 1, 2). Th1 cells, producing IFNγ and lymphotoxin-α are associated with the elimination of intracellular pathogens. Two major signaling pathways facilitate Th1 development, one involving IL-12/STAT4 and the other involving IFNγ/STAT1/T-bet. Th2 cells, producing IL-4, IL-5 and IL-13, are critically important for the eradication of parasitic worms, but are also implicated in allergic responses. Th2 differentiation is dependent on IL-4-induced activation of STAT6, leading to expression of the zinc finger transcriptional regulator GATA3 [3, 4]. GATA3 auto-activates its own expression and increases the accessibility of the Th2 cytokine cluster containing the genes coding for IL-4, IL-5 and IL-13 [5-7]. Furthermore, GATA3 suppresses Th1 development by downregulating STAT4 and IL-12Rβ2 chain expression [8-10]. Only if T-bet is sufficiently induced in naive cells, such GATA3 suppression is counteracted, permitting Th1 differentiation to occur. Conditional gene targeting experiments showed that GATA3 deficiency was sufficient to induce Th1 differentiation in the absence of IL-12 and IFNγ, demonstrating that GATA3 serves as a principal Th1/Th2 switch [11].

In differentiating CD4$^+$ effector T cells loss of GATA3 results in a substantial reduction in the generation of IL-4-, IL-5-, and IL-13-producing cells [11, 12]. GATA3 binds to multiple sites within the 200-kb Th2 cytokine locus in resting T cells; binding to the

IL-5 and IL-13 promoters increases upon T cell activation. Introduction of GATA3 into *in vitro*-cultured T cells generates Th2-specific DNAse I hypersensitive sites independently of STAT6, implicating GATA3 in the process of chromatin remodeling [6]. In activated Th2 cells GATA3, STAT6, c-Maf and the chromatin-remodeling enzyme Brg1 and RNA polymerase II are all bound across the Th2 locus, whereby densely looped, transcriptionally active chromatin is packaged by the special AT-rich sequence binding protein (SATB1) [13]. Furthermore, GATA3 activity is required during embryonic development [14] and at multiple stages of thymocyte development, including the earliest double negative (DN) stages [15, 16], β-selection [17] and CD4 single positive cell development [17-19].

In recent years, effector T cells distinct from the Th1 or Th2 subsets have been described, which produce IL-17 and are therefore termed Th17 cells [20, 21]. IL-17 is a pro-inflammatory cytokine involved in the control of a wide range of infections at mucosal surfaces and has been implicated in the pathogenesis of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis, autoimmune myocarditis and psoriasis (Reviewed in ref. 22, 23). In the mouse, transforming growth factor-β (TGFβ) and IL-6, an acute phase protein induced during inflammation, act together to induce Th17 differentiation [24-26]. Because TGFβ is also a critical factor for the generation of anti-inflammatory regulatory T (Treg) cells, there is a dichotomy in the generation of pathogenic Th17 T cells that induce autoimmunity and Tregs that inhibit autoimmune disease [25, 26]. The orphan nuclear receptor RORγt has been identified as a key regulator of the differentiation program of Th17 cells [27]. Th17 differentiation is regulated by several cytokines: IL-2 and IL-27 are strongly inhibitory, whereas IL-21 and IL-23 have been reported to promote IL-17 expression [27-31].

As Th1, Th2, Th17 and Treg, appear to arise in a mutually exclusive fashion, various mechanisms of counter-regulation exist, at the level of cytokines as well as transcription factors. For example, TGFβ has the capacity to block Th2 development through inhibition of GATA3 expression [32]. In Th1 cells T-bet essentially functions as a negative regulator of GATA3 expression [10, 33]. GATA3 suppresses the induction of T-bet by downregulating STAT4 and IL-12Rβ2 chain [8-10]. In CD8 cells, the Th2 cytokine locus is silenced by expression of repressor of GATA (ROG) [34]. Yet, it is currently not known whether GATA3 activity inhibits or stimulates differentiation of Th17 cells. On the one hand, GATA3 induces IL-4, which has been shown to suppresses Th17 development [20, 21]. But since IFNγ is the main inhibitor of Th17 development, GATA3 activity may on the other hand promote Th17 cell generation because of its capacity to negatively regulate Th1 development and thus IFNγ production [8-10].

In this report, we investigated how GATA3 expression affects Th17 differentiation. We employed our previously characterized CD2-GATA3 transgenic mice, which have enforced GATA3 expression driven by the CD2 locus control region, resulting in enhanced Th2 differentiation of peripheral $CD4^+$ T cells [35]. We isolated naive primary CD4 T cells from CD2-GATA3 mice, activated them *in vitro* under culture conditions supporting Th17 differentiation, and found co-expression of Th2 cytokines in IL-17-producing T helper cells. Interestingly, we identified multiple stimulatory and

inhibitory effects of GATA3 on Th17 differentiation *in vitro*. Although CD2-GATA3 mice contained normal numbers of IL-17$^+$ cells in gut-draining lymphoid tissue, they were not susceptible to Th17-mediated EAE *in vivo.*

MATERIALS AND METHODS

Mice

CD2-GATA3 mice [36] were backcrossed on the C57BL/6 background for at least 8 generations and genotyped by PCR. GATA3 primers were: 5'-CAGCTCTGGACTCTTCCCAC-3' and 5'-GTTCACACACTCCCTGCCTT-3'.

Flow cytometric analyses

Preparations of single-cell suspensions from thymus, spleen and lymph nodes, mAb incubations and four-color flow cytometry have been previously described [60]. Monoclonal antibodies were purchased from BD Biosciences (San Diego, CA), except PE-conjugated anti-Granzyme B (GB12) and biotinylated anti-IL-13 (BAF413), which were from Caltag Laboratories (Burlingame, CA) and R&D Systems (Minneapolis, MN), respectively. For intracellular detection of cytokines, cells were stimulated with plate-bound anti-CD3 mAb (10 μg/ml in PBS) in the presence of GolgiStop™ (BD Biosciences) for 4h. Cells were harvested, extracellularly stained with anti-CD4 or anti-CD8 mAbs, followed by standard intracellular staining using 2% paraformaldehyde and 0.5% saponin. Samples were acquired on a FACSCalibur™ flow cytometer and analyzed using CELLQuest™ (Becton Dickinson, Sunnyvale, CA) or FlowJo™ (Tree Star Inc., Ashland, OR) research software. Live events were collected based on forward and side scatter.

Purification of naïve or activated/memory T cells and *in vitro* T cell cultures

Naive CD62L$^+$ CD4$^+$ or CD8$^+$ T cells were purified from spleens obtained from 8 week-old mice and activated/memory CD62L$^-$ CD4$^+$ were purified from spleens from mice subjected to EAE induction. Cells were sorted, using a FACSVantage VE equipped with Diva Option and BD FACSDiva software (BD Biosciences). Purity of obtained fractions was typically >98%.

CD62L$^+$ CD4$^+$ or CD8$^+$ T cell fractions were cultured at concentrations of 1 x 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker, Walkersville, MD), containing 10% heat-inactivated fetal calf serum (FCS) (Sigma, St. Louis, MO), 5 x 10^{-5} M β-mercapto-ethanol (Merck, Darmstadt, Germany), supplemented with various cytokines (R&D Systems), as described below. Plates were coated with anti-CD3 and anti-CD28 (145-2C11; 37.51, BD Biosciences) at a concentration of 10 μg/ml each in PBS at 4^{o}C overnight. For Th1 polarizing conditions, IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml; 11B11) were added. Th2 polarizing conditions included IL-4 (10 ng/ml, R&D Systems), anti-IFNγ (5 μg/ml; XMG1.2) and anti-IL-12/23 p40 (5 μg/ml; C17.8). Treg and Th17 polarizing conditions included TGF-β (3 ng/ml), anti-IL-4 and anti IFNγ. Th17 polarizing conditions additionally contained IL-6 (20 ng/ml),

and in specified conditions also IL-4 (10 ng/ml), IL-2 (5 ng/ml) or anti IL-2 (10 μg/ml; JES6-1A12, Southern Biotech Associates, Birmingham, AL). For Th0 conditions or CD8 T cells no cytokines or mAbs were added. On day 3 anti-CD3/CD28 activation was stopped and T cell cultures were stained for intracellular cytokine detection or expanded in the presence of the indicated cytokines, supplemented with IL-2 (5 ng/ml) for up to 7 days.

Quantitative PCR analyses

Total RNA was extracted using the GeneElute mammalian total RNA miniprep system (Sigma) and one μg was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers. PCR Primers spanning at least one intron-exon junction were designed manually or using ProbeFinder software (Roche Applied Science, Indianapolis, IN) and probes were chosen from the universal probe library (Roche Applied Science) or designed manually and purchased from Eurogentec (Seraing, Belgium) (Table S1). Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). To confirm the specificity of the amplification products, samples were analyzed by standard agarose gel electrophoresis. Threshold levels were set and further analysis was performed using the SDS v1.9 software (Applied Biosystems). The obtained C_t values were normalized to those of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

EAE induction

EAE was induced in ~10-week-old mice by subcutaneous injection of 200 μg MOG $^{35-55}$ peptide emulsified in complete Freund's adjuvant (Difco), as described [61]. Mice were daily weighed and scored for clinical signs, as follows: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE. Paralyzed mice with EAE scores above 2 were afforded easier access to food and water.

RESULTS

The CD2-GATA3 transgene induces IL-4 and limits IL-2 production in T-helper cells

To investigate how the presence of GATA3 affects differentiation of naive T cells towards the Th17 cell lineage, we used CD2-GATA3 transgenic mice on a C57Bl/6 background. Their phenotype was essentially similar to CD2-GATA3 transgenic FVB mice described previously [35, 36]. These mice manifested a significant reduction of thymic cellularity, affecting most stages of thymic development (see supplementary data, Figure S1), and contained reduced numbers of CD4 T cells and particularly CD8 T cells in spleen and lymph nodes (Figure S1). Peripheral T cells manifested an

increased surface expression of the CD3/TCRαβ-complex and a small but consistent decrease in CD5, which is a negative regulator of TCR signaling (Figure S1). We previously found that when total peripheral CD4 T cell fractions from CD2-GATA3/FVB mice were cultured under Th1 conditions, Th2 cytokine production was increased and IFNγ production was hampered [35]. In these experiments, the presence of antigen-experienced Th2-committed cells might have contributed to the observed increased Th2 differentiation.

Because both IL-4 and IFNγ are negative regulators of Th17 differentiation [20,21,24,37], we first aimed to investigate the effect of GATA3 on Th17 differentiation, independent of its effects on IL-4 and IFNγ production. To this end, we activated sorted splenic naive $CD62L^+CD4^+$ T cells from wild-type and CD2-GATA3/C57BL/6 mice with plate-bound anti-CD3/anti-CD28 under Th17 conditions, i.e. in the presence of TGFβ IL-6, anti-IFNγ and anti-IL-4 for 7 days. We also cultured $CD62L^+CD4^+$ T cells under Th0 (no additional cytokines or mAb), Th1 (with IL-12 and anti-IL-4) and Th2 (with IL-4, anti-IFNγ and anti-IL-12) polarizing conditions to confirm that the CD2-GATA3 transgene had the capacity to induce IL-4 and to limit IFNγ production during differentiation of naive T cells *in vitro*.

In all T cell cultures from CD2-GATA3 transgenic mice, GATA3 expression levels reached values that were up to a factor ~2-3 higher than those in wild-type Th2 cultures (Figure 1A), irrespective of polarization conditions. Enforced GATA3 expression did not significantly affect the expression levels of T-bet in Th0, Th1 and Th2 cultures, but in CD2-GATA3 transgenic Th17 cultures the levels of T-bet were reduced (Figure 1A). Whereas wild-type Th0 cultures contained detectable fractions of IFNγ-producing cells, CD2-GATA3 Th0 cultures contained significant proportions of $IL\text{-}4^+$ cells, as determined by intracellular flow cytometry (Figure 1B). Under Th1 polarizing conditions, a large majority of wild-type T cells produced IFNγ. In CD2-GATA3 transgenic Th1 cultures, IFNγ was produced by a significant proportion (~43%) of the cells, a large fraction of which co-expressed IL-4. Under Th2 conditions, the presence of the CD2-GATA3 transgene resulted in significantly increased proportions of $IL\text{-}4^+$ cells. In wild-type Th17 cultures, $IL\text{-}4^+$ or $IFN\gamma^+$ cells were not detected, but in CD2-GATA3 transgenic Th17 cultures ~16 % of cells produced IL-4 (Figure 1B).

CD2-GATA3 transgenic Th cultures showed decreased production of IL-2, when compared with wild-type (Figure 1C), irrespective of polarization conditions. This reduction of IL-2 appeared (at least partially) independent of GATA3-induced IL-4, as it was also seen under Th1 or Th17 conditions in the presence of anti-IL-4 antibodies. Reduced IL-2 production did not reflect impaired activation of CD2-GATA3 transgenic T cells, as at day 3 expression levels of the activation markers CD25/IL-2R and CD69 were upregulated. CD25 expression was even slightly higher in CD2-GATA3 transgenic T cells, when compared with wild-type (Figure 1D). Nevertheless, CD2-GATA3 transgenic T cell cultures showed increased apoptosis, as determined by Annexin V staining, and reduced proportions of cells in the S/G2/M phase of the cell cycle, but remaining cells showed a normal capacity to go through sequential cell divisions detected by carboxy-fluorescein succinimidyl ester (CFSE)-labeling (Figure 1D and 1E).

Collectively, these data show that enforced GATA3 expression is sufficient to induce IL-4 expression and to reduce IL-2 production in all culture conditions. As a result, Th1 cultures contained many IL-4/IFNγ double positive cells and Th17 cultures contained IL-4$^+$ cells, even in the presence of anti-IL-4 antibodies during cell culture.

Enforced GATA3 expression allows Th17 differentiation *in vitro*

Interestingly, Th17 cultures from wild-type mice and CD2-GATA3 transgenic mice contained equal proportions of IL-17-expressing cells at day 7 (Figure 2A), but the CD2-GATA3 transgenic IL-17-producing cells co-expressed IL-4 and IL-13 (Figure 2B). The proportions of IL-13$^+$ cells in CD2-GATA3 transgenic Th17 cultures were in the same ranges as those in CD2-GATA3 transgenic Th0, Th1 and Th2 cultures (Figure S2). GATA3 also has the capacity to induce the immunosuppressive cytokine IL-10 [35], which was originally described as a product of Th2 cells but is produced by many other cell types. We found a significant induction of IL-10$^+$ cells in CD2-GATA3 transgenic Th0 cells (Figure 2C and 2D). In the presence of the CD2-GATA3 transgene the proportions of IL-10$^+$ cells were generally increased in Th1 and Th2 cultures. We found only a modest increase in CD2-GATA3 transgenic Th17 cultures, when compared with wild-type cultures (Figure 2C and 2D). The presence of GATA3 induced expression of IL-5, irrespective of polarization conditions (Figure S2 and 2E). IL-6, which is also associated with Th2 differentiation [1,2,23] was induced in Th0, Th1 and Th2 but not in Th17 cultures, as determined by quantitative RT-PCR analyses (Figure 2E).

The ability of enforced GATA3 expression to induce Th2 cytokines and to suppress IL-2 production was not unique to T helper cells, as similar observations were made in cultured CD8$^+$ T cells (without additional cytokines or mAb) and Treg cells (in the presence of TGFβ, anti-IFNγ and anti-IL-4) for 7 days (Figure S3).

To further examine the properties of the effector T cells that developed in the CD2-GATA3 transgenic Th17 cultures, we determined expression of cytokines known to be produced by Th17 cells using quantitative RT-PCR. Consistent with the observed presence of IL-17$^+$ cells in the CD2-GATA3 Th17 cultures, we found that they expressed IL-21 and TNFα, albeit at lower levels when compared with wild-type Th17 cultures (Figure 2E). Enforced GATA3 expression allowed normal IL-22 production in Th17 cells.

Taken together, these experiments demonstrate that, in the presence of anti-IL-4 and anti-IFNγ antibodies, enforced GATA3 expression does not significantly affect the generation of IL-17-producing cells *in vitro*. The CD2-GATA3 cells cultured in the presence of TGFβ and IL-6 had characteristics of both Th17 cells (production of IL-17, IL-21 and IL-22, low IL-6 expression) and Th2 cells (production of the Th2 cytokines IL-4, IL-5 and IL-13, low levels of IL-2).

Th17 differentiation in the presence of transgenic GATA3 is associated with downregulation of STAT3, STAT4, SOCS3, NFATc2 and RORγt

In our Th17 culture system, in the presence of anti-IFNγ and anti-IL-4 antibodies, the proportions of IL-17 producing cells in wild-type and CD2-GATA3 transgenic

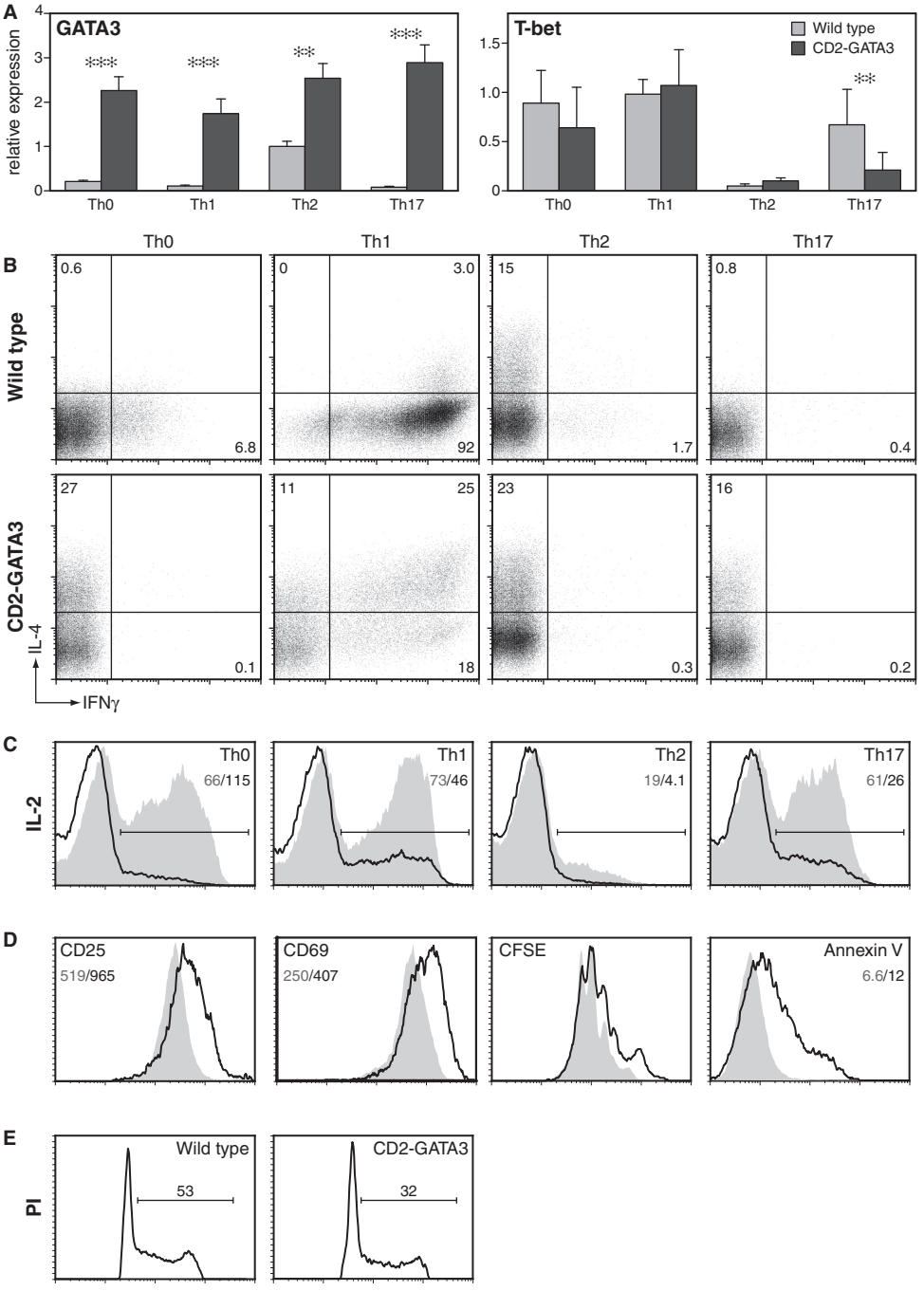

A
GATA3
relative expression
Th0
Th1
Th2
Th17
T-bet
Wild type
CD2-GATA3
B
Wild type
CD2-GATA3
IL-4
IFNγ
C
IL-2
Th0 66/115
Th1 73/46
Th2 19/4.1
Th17 61/26
D
CD25 519/965
CD69 250/407
CFSE
Annexin V 6.6/12
E
PI
Wild type 53
CD2-GATA3 32

Figure 1. The CD2-GATA3 transgene induces IL-4 and limits IL-2 production in Th1, Th2 and Th17 cells. (A) Quantitative RT-PCR analysis of GATA3 and T-bet expression in different T effector cells from wild-type (white bars) and CD2-GATA3 transgenic mice (black bars), cultured for seven days. Expression was normalized to GAPDH and expression levels of GATA3 in wild-type Th2 cells and T-bet in wild-type Th1 cells were set to one. Mean values and SEM are given for 5-7 mice per group (**, $p<0.01$; ***, $p<0.001$; *t*-test). (B) Flow cytometric analysis for intracellular expression of IL-4 and IFNγ in gated $CD4^+$ T cells from day 7 cultures. Numbers indicate percentages of total cells within the quadrants. *C-D*, Flow cytometric analysis for intracellular IL-2 expression (C) CD25 and CD69 expression, Annexin and CFSE (*D*) in the indicated wild-type (gray filled histogram) and CD2-GATA3 transgenic (black line) T cell cultures. Cells were cultured for 3 days. Numbers indicate the percentage of total cells (*C*) or the mean fluorescence intensity (D) from wild-type (gray numbers) or CD2-GATA3 transgenic (black numbers) Th0 cell cultures. (E) Cell cycle distribution by PI (propidium iodide) staining of Th0 cells cultured for 3 days. Numbers indicate the proportions of cells in S/G2/M. Data shown are representatives of four mice analyzed per group.

cultures were similar at day 7 (See Figure 2B), probably reflecting an equilibrium of stimulatory and inhibitory effects of GATA3. This enabled us to analyze the effect of GATA3 on the expression of individual factors involved in regulation of Th17 differentiation in cell populations that contained equal proportions of $IL\text{-}17^+$ cells. Day 7 wild-type and CD2-GATA3 transgenic Th17 cultures were activated by anti-CD3/anti-CD28 stimulation for 4 hours and mRNA expression levels were analyzed by quantitative RT-PCR and compared with those in Th0, Th1, Th2, Treg and CD8 cultures (Figure 3 and Figure S4).

Expression of the CD2-GATA3 transgene resulted in reduced transcription levels of RORγt, which is essential for Th17 cell generation. Also transcription of NFATc2, which stimulates expression of IL-17 in human [38], was reduced. Growth factor independent-1 (Gfi-1) is a STAT6-dependent transcriptional repressor that is induced by IL-4 in activated CD4 T cells and increases Th2 cell expansion by promoting proliferation and preventing apoptosis [39]. Enforced GATA3 expression did not result in significantly increased Gfi-1 levels in Th17 cultures, but was associated with a substantial increase of Gfi-1 expression in Treg cultures (Figure S4). GATA3 expression did not have detectable effects on the expression levels of NFATc1 or IRF4, which was recently shown to be essential for Th17 differentiation [40] (Figure S4). JAK-STAT signaling is crucially involved in pathways integrating cytokine signals into T cell differentiation programs: STAT1 and STAT4 in Th1 cells, STAT5a and STAT6 in Th2 cells, and STAT3 and STAT4 in Th17 cells. In agreement with previous findings in Th1/Th2 polarization [8, 9], we found that GATA3 is capable of down-regulating STAT4 in Th17 cultures (Figure 3). As STAT4 was recently shown to direct Th17 cells [41, 42], this finding indicates that GATA3 might be a negative regulator of Th17 differentiation by STAT4 downregulation.

Enforced GATA3 expression was also associated with reduced expression of STAT1, specifically in Th17 and Th0 cells, and STAT3, specifically in Th17 cells (Figure 3 and S4). The latter finding would implicate GATA3 as a negative regulator of IL-17 differentiation, because STAT3 directly binds to the IL-17A and IL-17F promoters [43]. No significant effects of GATA3 on STAT5a or STAT6 were observed (Figure S4).

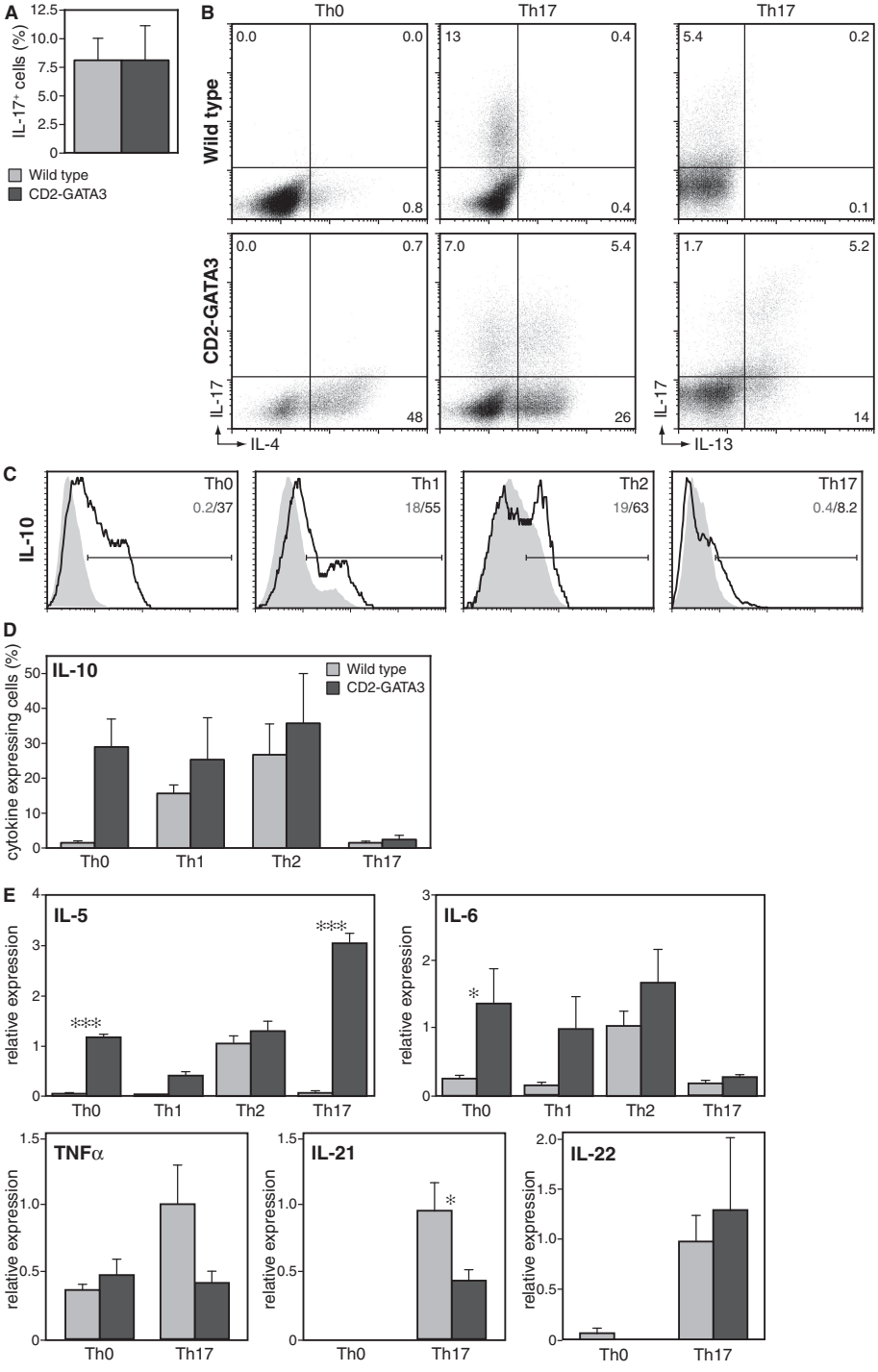

A
IL-17+ cells (%)
12.5
10.0
7.5
5.0
2.5
0
Wild type
CD2-GATA3
B
Th0
Th17
Th17
Wild type
CD2-GATA3
0.0
0.0
0.8
13
0.4
0.4
5.4
0.2
0.1
0.0
0.7
48
7.0
5.4
26
1.7
5.2
14
IL-17
IL-4
IL-17
IL-13
C
IL-10
Th0
0.2/37
Th1
18/55
Th2
19/63
Th17
0.4/8.2
D
cytokine expressing cells (%)
IL-10
Wild type
CD2-GATA3
50
40
30
20
10
0
Th0
Th1
Th2
Th17
E
relative expression
IL-5
***
***
IL-6
*
TNFα
IL-21
*
IL-22
4
3
2
1
0
1.5
1.0
0.5
2.0
1.5
Th0
Th1
Th2
Th17

Figure 2. Enforced GATA3 expression induces Th17 cells with Th2 characteristics. (A) Flow cytometric analysis of IL-17 production of wild-type (white bar) and CD2-GATA3 Tg (black bar) Th17 cells. Mean values and SEM are from 17 mice per group. (B-C) Intracellular flow cytometric analysis of the indicated cytokines in wild-type and CD2-GATA3 transgenic T cell cultures at day 7. Numbers indicate the percentages of total cells within the quadrants (B) or per gate (C). (D) Quantification of flow cytometric analysis for intracellular IL-10 in gated $CD4^+$ T cells from wild-type (white bars) and CD2-GATA3 transgenic mice (black bars). Mean values and SEM are from 4-6 mice per group (**, p<0.01; t-test). (E) Quantitative RT-PCR analysis of cytokine expression in the indicated T cell cultures. Expression was normalized to GAPDH and expression levels of IL-5 and IL-6 in wild-type Th2 and expression levels of TNFα, IL-21 and IL-22 in wild-type Th17 cells, were set to one. Mean values and SEM are for 5-7 mice per group (*, p<0.05;***, p<0.001; t-test).

IL-23-mediated phosphorylation of STAT3, and thus Th17 generation, is negatively regulated by the cytokine-inducible inhibitor SOCS3 [43]. We found that the presence of enforced GATA3 expression is associated with significantly reduced levels of SOCS3 transcripts (Figure 3).

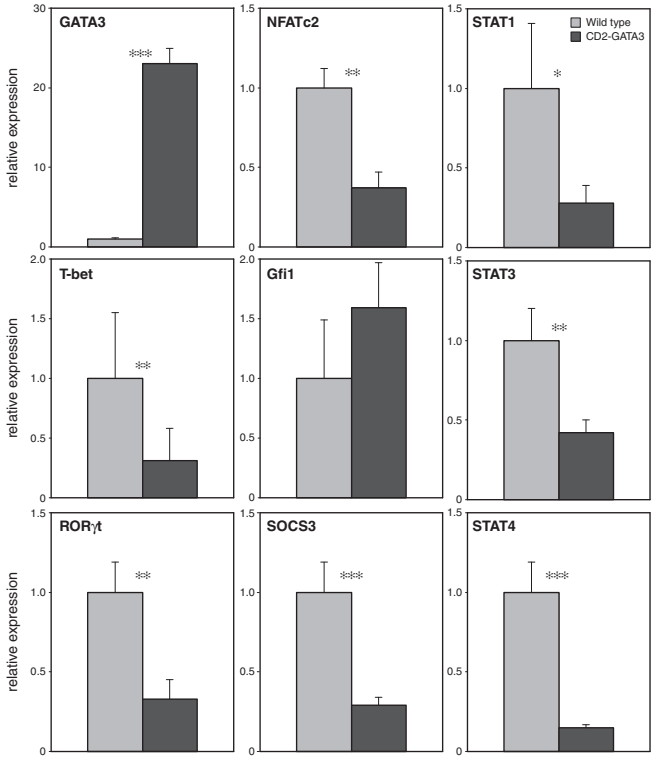


Figure 3. Enforced GATA3 expression affects factors involved in Th17 differentiation. Quantitative RT-PCR analysis of expression of the indicated genes in wild-type (white bars) and CD2-GATA3 transgenic (black bars) Th17 cultures at day 7. Expression levels were normalized for GAPDH and values in Th17 cultures from wild-type mice were set to one. Mean values and SEM are given for 6-11 mice analyzed per group (*, p<0.05; **, p<0.01; ***, p<0.001; t-test).

Taken together, these RT-PCR analyses show that enforced GATA3 expression is associated with significant changes in the expression levels of critical regulators of Th17 development. GATA3 has the capacity to stimulate Th17 cell generation by decreasing expression of STAT1 and SOCS3 and to inhibit Th17 differentiation by limiting expression of RORγt, STAT3, STAT4 and NFATc2.

Effects of GATA3, IL-2 and IL-4 on Th17 differentiation

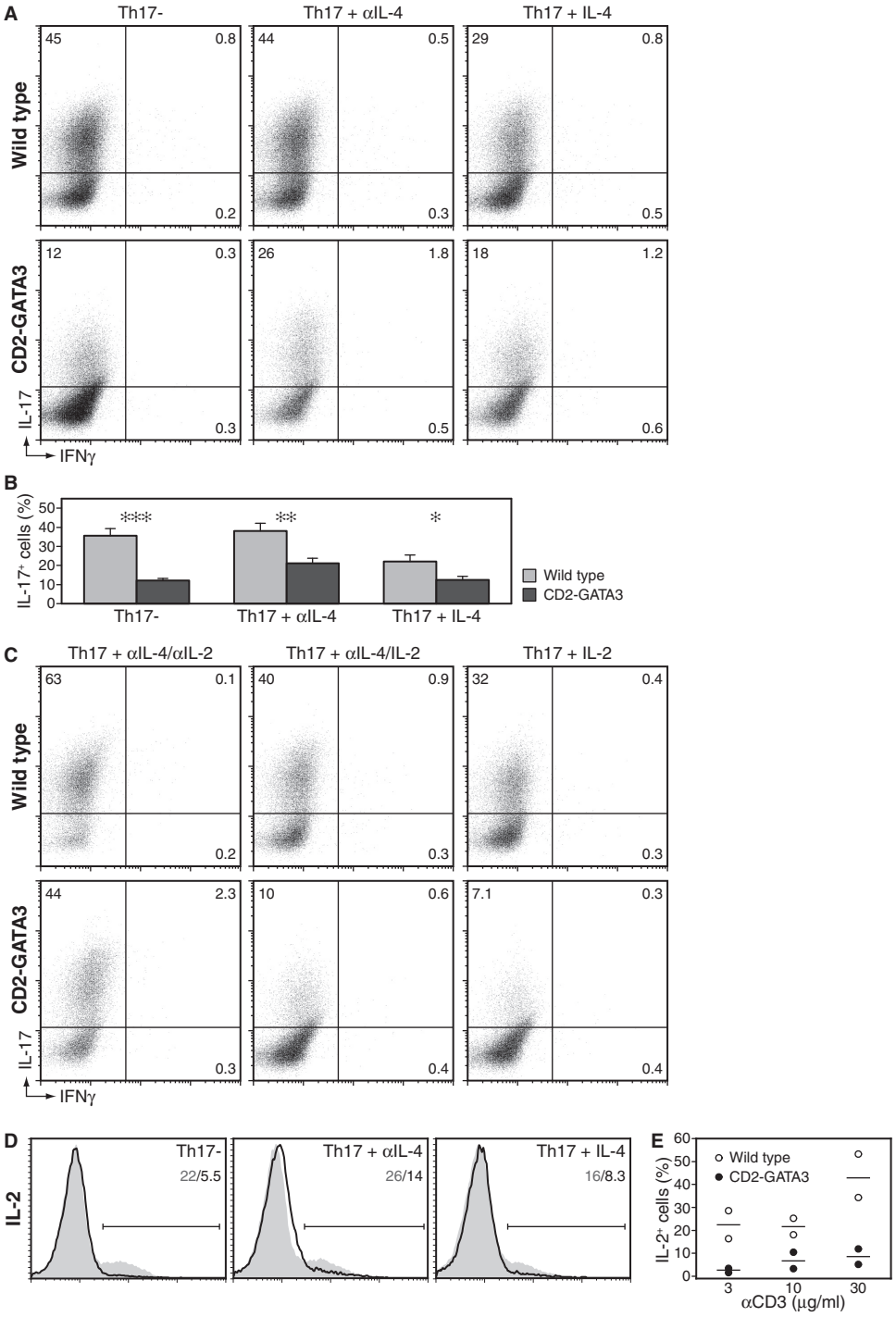
As enforced GATA3 expression resulted in increased IL-4 and reduced IL-2 expression, both of which are negative regulators of Th17 differentiation [24, 28, 37], we explored the effects of GATA3, IL-2 and IL-4 on Th17 cell generation in more detail.

To this end, we stimulated primary CD62L$^+$ CD4$^+$ T cells with plate-bound anti-CD3/anti-CD28 and cultured them with TGFβ, IL-6 and anti-IFNγ in the presence or absence of exogenous IL-2 or IL-4 or antibodies against these cytokines, for 3 days. In these experiments, cells were re-stimulated with PMA/ionomycin for 4 hours before intracellular cytokine staining, which yielded higher proportions of IL-17$^+$ cells, when compared to anti-CD3 restimulation (Compare Figure 2A with Figure 4). Hereby, clearance of IL-4 had only marginal effects on the generation of IL-17$^+$ cells in wild-type cells, probably due to the limited IL-4 expression of differentiating Th17 cells in the presence of TGFβ and IL-6. At day 3, the CD2-GATA3 Th17 cultures contained significantly reduced proportions of IL-17$^+$ T cells: ~12% in the absence and ~22% in the presence of anti-IL-4 (Figure 4A and 4B). Whereas addition of external IL-4 reduced the proportions of IL-17$^+$ cells expression in wild-type Th17 cultures, it had only marginal effects on IL-17 expression in CD2-GATA3 cultures, probably due to substantial IL-4 production of differentiating GATA3-expressing Th17 cells (Figure 4A and 4B).

Addition of anti-IL-2 mAb, next to anti-IL-4, significantly increased IL-17 production in differentiating wild-type and CD2-GATA3 transgenic Th17 cells (Figure 4C). Consistent with this finding, IL-17 production was low in CD2-GATA3 Th17 cultures when exogenous IL-2 was added, irrespective of the addition of anti-IL-4 mAb. Thus, at day 3 CD2-GATA3 Th17 cultures contained fewer IL-17-producing cells than wild-type cultures, even in the presence of anti-IL-4 mAb and independent of clearance of IL-2 or addition of IL-2 (Figure 4C).

To further explore whether the negative effect of GATA3 on IL-2 production in Th17 cells is regulated via IL-4, we investigated IL-2 production in 3-day Th17 cultures in

Figure 4. Enforced GATA3 expression affects Th17 differentiation via IL-4 and IL-2. (A-C) IL-17/IFNγ expression profiles of CD4$^+$ T cells in Th17 cultures from the indicated mice. Cells were cultured for 3 days with the indicated antibodies or cytokines. Numbers indicate percentages of total cells within the quadrants (A, C) or mean values and SEM of the percentage of IL-17$^+$ cells are given (*, p<0.05; **, p<0.01; ***, p<0.001; t-test) (B). Data shown are representatives of 5-11 mice per group. (D) Flow cytometric analysis of intracellular IL-2 expression in wild-type (gray filled histogram) and CD2-GATA3 transgenic (black line) Th17 cells, which were cultured for 3 days with or without αIL-4 or IL-4. Numbers indicate the percentages IL-2$^+$ cells. Data shown are representatives of 2-10 mice per group. (E) Flow cytometric analysis of intracellular IL-2 in Th17 cultures from the indicated mice. Cells were cultured for 3 days and stimulated with indicated concentrations of plate-bound anti-CD3. Mean and individual data points are displayed, indicating the percentage of IL-2$^+$ cells.


A
Th17-
Th17 + αIL-4
Th17 + IL-4
Wild type
CD2-GATA3
IL-17
IFNγ
45
0.8
0.2
44
0.5
0.3
29
0.8
0.5
12
0.3
0.3
26
1.8
0.5
18
1.2
0.6
B
IL-17+ cells (%)
50
40
30
20
10
0
***
**
*
Th17-
Th17 + αIL-4
Th17 + IL-4
Wild type
CD2-GATA3
C
Th17 + αIL-4/αIL-2
Th17 + αIL-4/IL-2
Th17 + IL-2
Wild type
CD2-GATA3
IL-17
IFNγ
63
0.1
0.2
40
0.9
0.3
32
0.4
0.3
44
2.3
0.3
10
0.6
0.4
7.1
0.3
0.4
D
IL-2
Th17-
22/5.5
Th17 + αIL-4
26/14
Th17 + IL-4
16/8.3
E
IL-2+ cells (%)
60
50
40
30
20
10
0
Wild type
CD2-GATA3
3
10
30
αCD3 (μg/ml)

the absence or presence of anti-IL-4 mAb or exogenous IL-4. IL-2 was expressed in ~22-26% of the WT cells, irrespective of clearance of IL-4 (Figure 4D). Expression of IL-2 in CD2-GATA3 transgenic Th17 cultures was very low (~6% of cells), and the proportion of IL-2-producing cells only moderately increased in the presence of anti-IL-4. Thus, low IL-2 expression in the presence of the CD2-GATA3 transgene is partially IL-4-dependent and partially IL-4-independent. The capacity of IL-4 to reduce IL-2 production in Th17 cells was confirmed by the finding of reduced IL-2 expression in WT Th17 cells that were cultured in the presence of IL-4 for 3 days (Figure 4D). Consistent with the reported TCR signal strength-dependency of IL-2 production, and the inverse correlation between GATA3 induction and IL-2 expression in naive T cells [44], we found that in differentiating Th17 cells IL-2 production increased when cells were stimulated with higher doses of anti-CD3 and that in CD2-GATA3 transgenic T cells increasing the anti-CD3 concentration had limited effect (in the presence of anti-CD28 mAb; Figure 4E).

Taken together, we found that enforced GATA3 expression in 3-day Th17 cultures resulted in reduced proportions of IL-17-expressing cells under all conditions analyzed. GATA3 (i) stimulates IL-17 production via IL-2 downregulation, (ii) inhibits IL-17 production via induction of IL-4, and (iii) additionally constrains IL-17 production in an IL-4-independent way.

Inhibitory effect of GATA3 on STAT3, STAT4, SOCS3, NFATc2 and RORγt transcription is independent of IL-4

Our finding that GATA3 has the capacity to limit expression of STAT3, STAT4, SOCS3, NFATc2 and RORγt in Th17 cultures in the presence of antibodies to IL-4 and IFNγ (Figure 3) suggested that these inhibitory effects of GATA3 were IL-4-independent. To analyze this IL-4 independence directly, we performed Th17 cultures in the presence or absence of anti-IL-4 antibodies or exogenous IL-4 and analyzed the expression of STAT3, STAT4, SOCS3, NFATc2 and RORγt at day 7 by RT-PCR. We found that the capacity of GATA3 to reduce transcription of these factors was indeed independent of IL-4 addition or clearance (Figure S5A). The presence of the GATA3 transgene did not significantly affect the *in vitro* proliferative capacity of Th17 cultures, excluding the possibility that the observed effects of GATA3 on gene transcription were unspecific (Figure S5B).

We therefore conclude that the observed inhibitory effect of GATA3 on STAT3, STAT4, SOCS3, NFATc2 and RORγt transcription is independent of IL-4.

IL-17-producing T cells are present *in vivo* in CD2-GATA3 transgenic mice

Next, we investigated whether enforced GATA3 expression would allow the differentiation of Th17 cells *in vivo*. Since Th17 cells have been associated with the mucosal lymphoid system [27], we analyzed IL-17 expression in gut-draining lymphoid tissue, including Peyer's patches, mesenteric and iliacal lymph nodes. In these tissues we found that in the presence of the GATA3 transgene the populations of IL-17-producing T cells were maintained (Figure 5A), but also IL-4 production was induced (Figure 5B). Thus, enforced GATA3 expression allowed differentiation of Th17 cells *in vivo*.

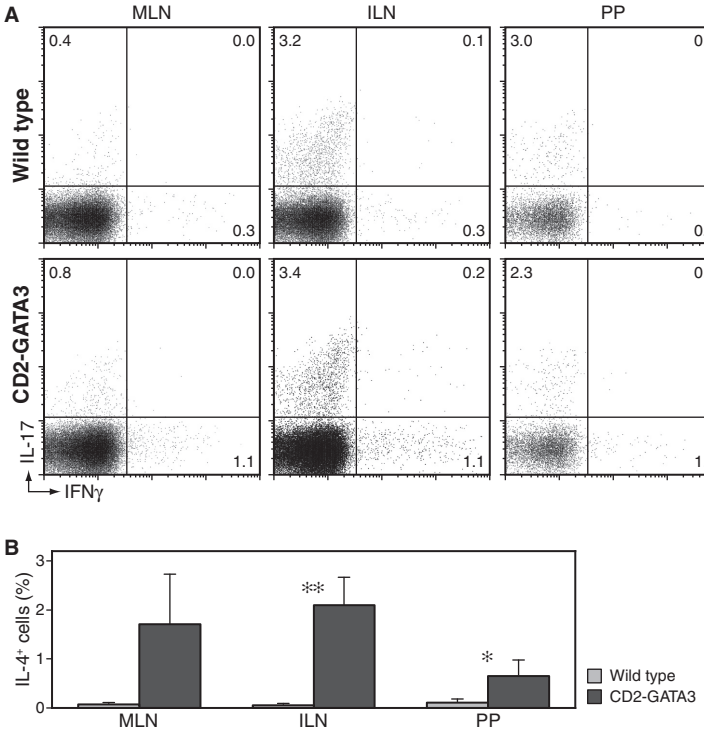


Figure 5. IL-17 producing cells in CD2-GATA3 transgenic mice *in vivo*. (A) Flow cytometric analysis for intracellular IL-17 and IFNγ expression in gated CD4+ T cells from mesenteric lymph nodes (MLN), iliacal lymph nodes (ILN) and Peyer's patches (PP) from wild-type and CD2-GATA3 Tg mice. Numbers indicate the percentage of cells within the quadrants. Data are representatives of 6 mice analyzed per group. (B) Quantification of flow cytometric analysis for intracellular IL-4 in gated CD4+ T cells from the indicated tissues from wild-type (white bars) and CD2-GATA3 transgenic mice (black bars). Mean values and SEM are from four mice per group (*, $p<0.05$; **, $p<0.01$; *t*-test).

CD2-GATA3 transgenic mice show *in vivo* Th17 differentiation but are not susceptible to EAE

Although our analyses in gut-draining lymphoid tissues showed that enforced GATA3 expression allowed differentiation of IL-17+ T cells *in vivo*, the various stimulatory and inhibitory effects of GATA3 on Th17 differentiation identified prompted us to investigate the effect of the CD2-GATA3 transgene on development of EAE, which has been reported to be highly dependent on Th17 cells [22, 23, 29]. After MOG peptide/CFAimmunization, five out of eight wild-type mice developed EAE. In contrast, no clinical disease symptoms were found in ten CD2-GATA3 transgenic mice (Figure 6A). We determined the presence of IL-17-producing cells in a pool of axillary, brachial and inguinal lymph nodes after MOG peptide injection. Importantly, we found similar proportions of IL-17-producing CD4+ T cells in wild-type and in CD2-GATA-3 transgenic mice (Figure 6B), although the values in GATA3 transgenic mice

were slightly higher at day 14, and slightly lower at day 28, as compared to wild-type mice. Importantly, at day 14 CD2-GATA3 transgenic mice had significant numbers of IL-4 and IL-10-producing $CD4^+$ T cells in their lymph nodes (~2.0% and ~2.5%, $n = 5$, respectively), in contrast to wild-type mice (both proportions were <0.2%) (Figure 6C).

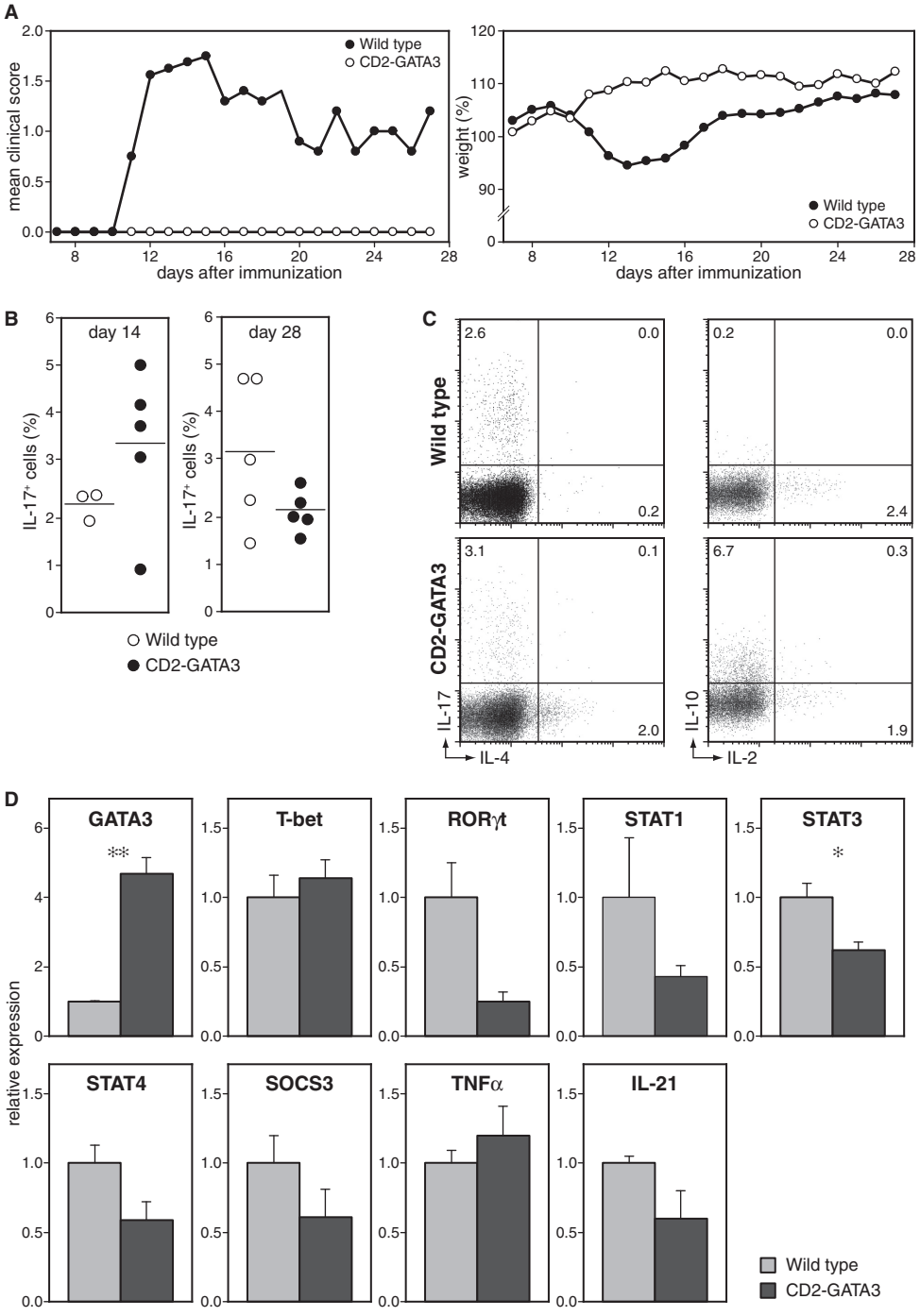
Next, we sorted T helper cells with the $CD62L^-CD4^+$ activated/memory phenotype from spleens from wild-type and CD2-GATA3 transgenic mice at day 14 after MOG peptide injection. Quantitative RT-PCR analyses revealed that increased expression of GATA3 was associated with reduced transcription of RORγt, STAT1, STAT3, STAT4 and SOCS3 (Figure 6D), in agreement with our findings in *in vitro* Th17 cultures (Figure 3). In addition, we found that T-bet and TNFα expression were unaffected by the presence of the CD2-GATA3 transgene *in vivo.*

We therefore conclude that enforced GATA3 expression exerts a marginal effect on the generation of IL-17 producing cells in EAE. Nevertheless, CD2-GATA3 transgenic mice are not susceptible to *in vivo* EAE induction, possibly because concomitant production of IL-4 and the induction of IL-10 may restrain Th17-mediated pathology.

DISCUSSION

In this report, we studied the molecular mechanisms of counter-regulation by which the key regulator of Th2 differentiation, transcription factor GATA3, affects Th17 differentiation. We show that enforced expression of GATA3 from a T cell-specific transgene essentially induces Th2 cytokine production and hampers IL-2 cytokine production in various *in vitro* differentiating effector T cell populations, including Th17 cells. The induction of the Th2 cytokine IL-4, by GATA3, had a negative effect on TGFβ/IL-6-induced Th17 differentiation, but this was partially compensated by the capacity of GATA3 to limit IL-2 production, which functions as a negative regulator of Th17 differentiation [28]. However, the GATA3-induced stimulation of IL-4 production alone could not fully explain the inhibitory effect of GATA3 on Th17 differentiation: also in the presence of anti-IL-4 antibodies CD2-GATA3 transgenic Th17 cultures contained fewer $IL\text{-}17^+$ cells, when compared with WT cultures, at day 3. Therefore, we conclude that GATA3 additionally constrained Th17 differentiation through IL-4-

Figure 6. CD2-GATA3 transgenic mice are not susceptible to EAE induction. (A) Induction of EAE by immunization with MOG35–55 peptide. Mean clinical scores and weight are given for 8-10 mice per group. (B) Flow cytometric analysis of intracellular IL-17 expression in gated LN $CD4^+$ T cells from wild-type and CD2-GATA3 transgenic mice 14 and 28 days after EAE induction. Mean and individual data points are displayed, indicating the percentage of $IL\text{-}17^+$ cells. (C) Flow cytometric analysis for the indicated cytokines in gated LN $CD4^+$ T cells from wild-type mice and CD2-GATA3 transgenic mice 14 days after EAE induction. Numbers indicate the percentage of cells within the quadrants. Data shown are representatives of 3-5 mice per group. (D) Quantitative RT-PCR analysis of expression of the indicated genes in sorted activated/memory $CD4^+CD62L^-$ T cells from wild-type (white bars) and CD2-GATA3 transgenic (black bars) mice 14 days after EAE induction. Expression levels were normalized for GAPDH and values of wild-type mice were set to one. Mean values and SEM are given for 3 mice analyzed per group (*, $p<0.05$; **, $p<0.01$; *t*-test).


A
mean clinical score
days after immunization
Wild type
CD2-GATA3
weight (%)
days after immunization
Wild type
CD2-GATA3
B
day 14
day 28
IL-17+ cells (%)
Wild type
CD2-GATA3
C
Wild type
CD2-GATA3
2.6
0.0
0.2
0.2
2.4
3.1
0.1
2.0
6.7
0.3
1.9
IL-17
IL-4
IL-10
IL-2
D
GATA3
T-bet
RORγt
STAT1
STAT3
STAT4
SOCS3
TNFα
IL-21
relative expression
Wild type
CD2-GATA3

independent mechanisms. Our RT-PCR analyses showed that GATA3 may stimulate Th17 cell differentiation by decreasing expression of STAT1 and SOCS3 and may inhibit this process by limiting expression of RORγt, STAT3, STAT4 and NFATc2. As in CD2-GATA3 transgenic mice IL-17-producing cells were present in normal numbers *in vivo*, both in gut-draining tissue and in lymph nodes during EAE induction, we conclude that the final consequences of the various stimulatory and inhibitory effects of GATA3 were that *in vivo* the presence of enforced GATA3 expression still allows for differentiation of IL-17-producing cells. However, because CD2-GATA3 transgenic mice were not susceptible to EAE, it appears that GATA3 mainly acts as an inhibitor of Th17-mediated pathology, possibly by driving IL-4 and IL-10 production.

In single CD2-GATA3 transgenic T cells, Th2 cytokines were co-expressed together with cytokines that signify differentiation to other subsets, such as IL-17 (in Th17 cells) or IFNγ (in Th1 cells). Therefore, these findings indicate that GATA3 expression is sufficient to induce Th2 cytokine production, irrespective of the presence of critical transcription factors such as RORγt and T-bet in Th17 or Th1 cultures, respectively. Also $CD8^+$ T cell cultures contained substantial proportions of cells producing Th2 cytokines or IL-10 at levels, similar to those found in wild-type Th2 cell cultures. Thus, in CD8 T cells transgenic GATA3 could counteract the repression of Th2 cytokine production by ROG. This finding is in apparent conflict with the report by Omori *et al.* [34], who found that ectopic expression of GATA3 in cytotoxic type 2 cells resulted only in a limited generation of IL-4-producing cells. This discrepancy might be explained by the fact that in our study GATA3 is already overexpressed in naive $CD8^+$ T cells *in vivo*, whereas in their retrovirus gene introduction system GATA3 expression is induced during cell culture.

The finding that GATA3 had the capacity to inhibit IL-2 expression, even in Th1 or Th17 cultures in the presence of anti-IL-4 antibodies, indicates that GATA3 directly regulates IL-2, through an IL-4-independent mechanism. GATA3 expression is sufficient to induce IL-6 in Th0 or Th1 cells, but not in Th17 cells, suggesting that this capacity of GATA3 is either specifically inhibited in Th17 cells or dependent on a co-factor that is not present in Th17 cells. It has been shown that IL-4 suppresses Th17 cell development *in vitro* and that Th2 cytokines are important for preventing or ameliorating EAE; conversely, loss of IL-25 results in accelerated EAE pathology, associated with an increased number of IL-17-producing T cells that invade the central nervous system [20, 29, 45, 46]. The prevention of EAE in CD2-GATA3 mice is consistent with our findings of (i) significant IL-4 production in lymph nodes from CD2-GATA3 mice during EAE induction *in vivo*, and (ii) limited Th17 differentiation of cells from CD2-GATA3 transgenic mice, cultured for 3 days in Th17 conditions in the absence of neutralizing anti-IL-4 antibodies (Figure 5A). Also the observed induction of IL-10 by transgenic GATA3 might well contribute to the resistance of CD2-GATA3 transgenic mice to EAE. In this context, it was very recently found that TGFβ and IL-6 do not only drive Th17 commitment, but they also act together to restrain the pathogenic potential of Th17 cells, by inducing the production of IL-10, which is an important factor in the downmodulation of immune responses and EAE [47-50]. In fact, evidence was provided for the existence of Th17 subsets with effector or regulatory functions that correlate in part with their ability to produce IL-10. Although GATA3 has been

shown to directly remodel the IL-10 locus in CD4$^+$ T cells independently of IL-4 [51], the observed significant proportions of IL-10$^+$ CD4 T cells during *in vivo* EAE induction in CD2-GATA3 transgenic mice (Figure 6C) do not necessarily point to a direct role of GATA3 in upregulating IL-10 production in Th17 cells. On the contrary, we found that transgenic expression of GATA3 had only a modest effect on IL-10 production by Th17 cells generated *in vitro* (Figure 2C). Therefore, it is more likely that the induction of IL-10$^+$ T cells *in vivo* is largely indirect, e.g. through tolerogenic dendritic cells [47]. Further experiments are required to determine whether prevention of EAE induction by enforced GATA3 expression is indeed dependent on the induction of IL-4, IL-10 or both, or whether other molecules are involved. In this context, we found that in the presence of neutralizing antibodies to IL-10 (from day 0 to day 8), EAE can be induced in CD2-GATA3 transgenic mice, albeit with an incidence and severity that is lower than in wild-type mice (van Hamburg *et al.*, unpublished data). Therefore, these preliminary experiments indicate that IL-10 induction may contribute to the effects of transgenic GATA3, but does not completely explain the reduced susceptibility of CD2-GATA3 transgenic mice.

Although in 3-day cultures transgenic GATA3 expression limited Th17 differentiation, 7-day wild-type and CD2-GATA3 transgenic Th17 cultures contained similar proportions of IL-17 producing cells. Apparently at day 7 inhibitory and stimulatory effects of GATA3 are in balance, which made these cultures good tools to investigate the effect of GATA3 activity on the expression of genes involved in Th17 differentiation. We found that enforced GATA3 is associated with reduced expression of RORγt, STAT3, STAT4 and NFATc2, but further experiments are required to establish whether these genes are direct GATA3 targets. Interestingly, NFATc2, which is known to stimulate IL-17 in human T cells and IL-21 in Th2 cells, can be induced by IL-6 in Th2 cells [38, 52, 53] and has the ability to interact with IRF-4 [54], which was recently shown to be a critically involved in Th17 differentiation. But, it is currently not known how NFATc2 functions in Th17 differentiation. Furthermore, GATA3 expression resulted in downregulation of STAT1, known to suppress Th17 differentiation via IL-27 signaling [55] and of SOCS3, which is a negative regulator of STAT3 phosphorylation [43]. The effects of GATA3 on RORγt, SOCS3, STAT3, STAT4 and NFATc2 were not influenced by the addition or clearance of IL-4, indicating that GATA3 regulates these factors in an IL-4-independent manner. This is quite remarkable for SOCS3, since one of the mechanisms by which IL-4 suppresses Th17 cell development may be through induction of SOCS3 expression through IL-4R signaling. This was shown to be the case in B cells by a p38 MAPK-mediated mechanism [56]. Finally, the *in vivo* development of significant numbers of IL-17$^+$ effector cells in CD2-GATA3 transgenic mice could also partially be stimulated by the ability of GATA3 to limit IL-2 production and inhibit the generation of IFNγ producing cells, which suppress development of Th17 cells from naive precursor cells [20, 21]. Taken together, GATA3 has various stimulatory and inhibitory effects on Th17 differentiation (see Figure 7), whereby enforced GATA3 expression still allows the generation of IL-17 producing cells *in vivo*.

It is not surprising that with the identification of two additional effector T cell subsets, Th17 and Treg cells, the molecular mechanisms of the reciprocal interactions between

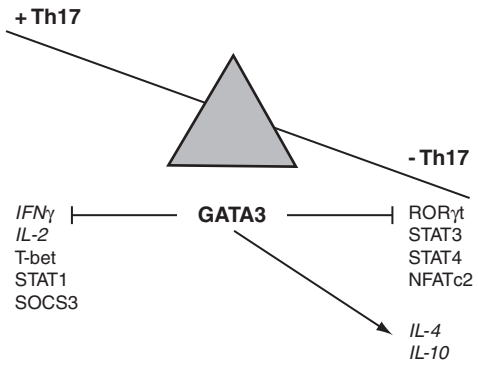


Figure 7. Regulatory effects of GATA3 on Th17 differentiation. Model showing effects of GATA3 on Th17 differentiation. GATA3 has positive effects by limiting IFNγ and IL-2 production and inhibiting expression of T-bet, STAT1, and the Th17 repressor protein SOCS3. On the other hand GATA3 constrains Th17 differentiation by inhibiting expression of critical factors in Th17 differentiation, including RORγt, STAT3, STAT4 and NFATc2, and limits Th17-mediated pathology by enhancing production of IL-4 and IL-10.

T cell subsets, involving counter-regulation at the level of transcription factors and cytokines, become more complex. For example, we found that *in vitro* generated Tregs (containing >95% $CD25^{+}FoxP3^{+}$ cells) expressed significant levels of GATA3 and RORγt (Figure S4), indicating that GATA3 is not Th2-specific and RORγt is not Th17-specific. As these cultured Treg cells express FoxP3, T-bet, GATA3 and RORγt, apparently without any tendency to develop into Th1, Th2 or Th17 cells, Treg differentiation seems critically dependent on the balance between these transcription factors and therefore a molecular mechanism should be present in these cells that keep transcription factors in check and prevents Th1, Th2 or Th17 development. Conversely, the presence of RORγt alone is not sufficient to induce commitment to the Th17 lineage. The simultaneous expression of GATA3, T-bet and RORγt in Treg cells are puzzling and in apparent conflict with molecular models in which key transcription factors are essential to stabilize active or silent states of cytokine loci by epigenetic modifications during polarized effector T cell differentiation. Our findings in GATA3 transgenic mice show that GATA3 has the capacity to constrain Th17 differentiation through IL-4 dependent and IL-4 independent pathways, but neither GATA3 expression, nor the consequential production of IL-4, IL-5, IL-13 or IL-10 inhibited the generation of IL-17-producing cells *in vivo*. In this context, these findings are consistent with population-based studies in man [57-59] supporting the hypothesis that Th2-mediated diseases, such as asthma, do not necessarily protect from Th17-mediated autoimmune disorders.

ACKNOWLEDGEMENTS

We would like to thank C. de Haar, K.W. Ling, W.K. Chan, M. van Meurs, G. Dingjan, T. Schonewille and H. Diepstraten (Erasmus MC, Rotterdam) for assistance at various stages of the project and E. Lubberts for valuable discussions.

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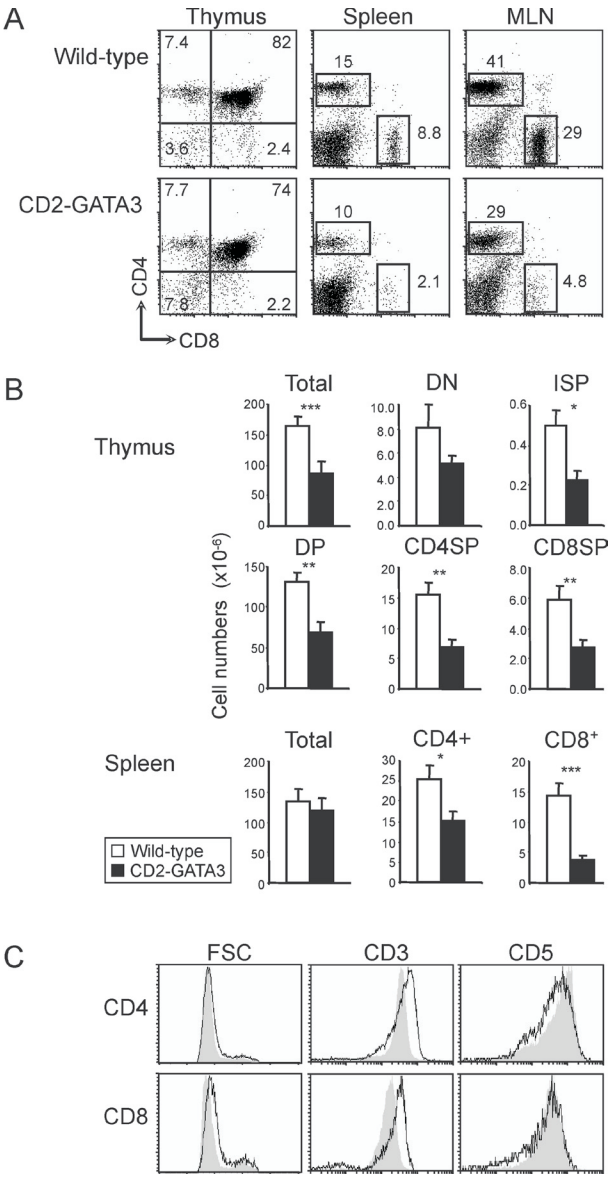


Figure S1. Characterization of CD2-GATA3 Tg mice on the C57BL/6 background. (A) Distribution of T cell populations in the thymus, spleen and mesenteric lymph node (MLN) of wild-type and CD2-GATA3 transgenic mice. Results are displayed as dot plots of CD4/CD8 profiles. Percentages shown represent proportions of cells within a quadrant or gate. (B) Total cell numbers within the indicated cell populations in thymus and spleen. Mean values and SEM are given for eight mice analyzed per group (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *t*-test). (C) Cell size (FSC), surface expression of CD3 and CD5 in splenic CD4 and CD8 T cell populations. Results are displayed as histograms indicating wild-type mice (gray filled histograms) and CD2-GATA3 transgenic mice (black lines). Data shown in A and C are representative for eight mice analyzed per group.

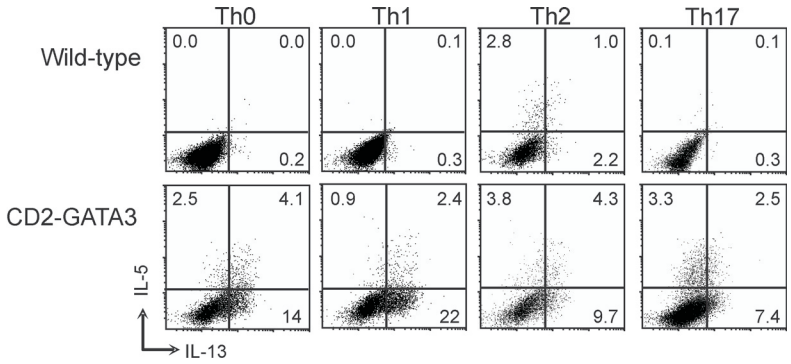
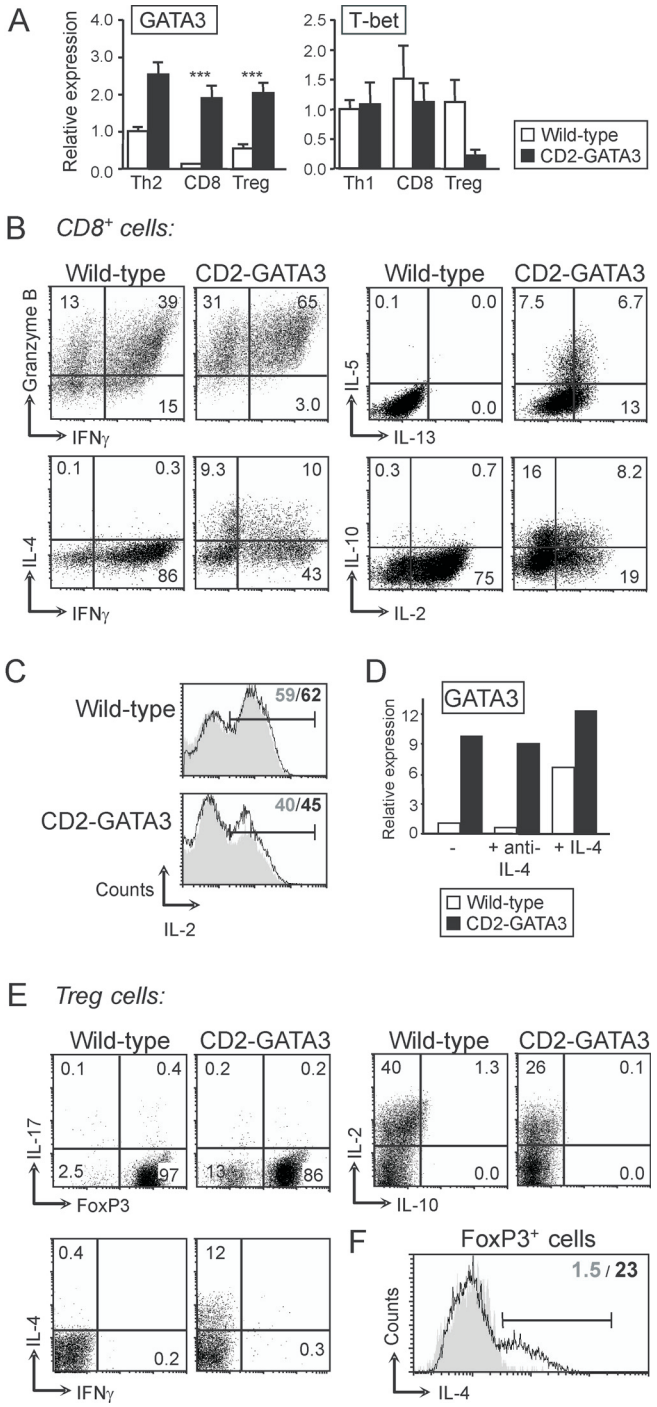


Figure S2. Induction of IL-5, IL-10 and IL13 by GATA3. Flow cytometric analysis for intracellular expression of IL-5 and IL-13 in the indicated gated $CD4^+$ T cell populations. Cells were cultured for seven days. Numbers indicate percentages of total cells within the quadrants.

Figure S3. Enforced GATA3 expression induces Th2 cytokines in CD8 T cells and Treg cells. (A) Quantitative RT-PCR analysis of GATA3 and T-bet expression in the indicated wild-type (white bars) and CD2-GATA3 transgenic (black bars) T effector cell cultures. Expression was normalized as indicated in the legend to Figure 1A. Mean values and SEM are given for four six mice per group (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *t*-test). (B) Flow cytometric analysis of intracellular expression of granzyme B and cytokines in wild-type and CD2-GATA3 transgenic $CD8^+$ T cells, which were cultured for seven days. Numbers indicate the percent of total cells within the quadrants. Data shown are representative for four mice analyzed per group. (C) Flow cytometric analysis of IL-2 expression in wild-type and CD2-GATA3 transgenic $CD8^+$ T cells, stimulated by plate-bound anti-CD3/anti-CD28 and cultured for seven days (gray filled histogram) in comparison with cultures containing anti-IL-4 mAb (black line). Data shown are representative for 3-4 mice per group. (D) Quantitative RT-PCR of GATA3 and ROG expression in wild-type and CD2-GATA3 transgenic CD8 T cells, cultured under the indicated conditions. Expression was normalized to GAPDH and levels in wild-type $CD8^+$ T cells were set to one. Mean values and SEM are given for 2-5 mice analyzed per group. (E) Flow cytometric analysis of intracellular expression of cytokines indicated and the Treg-specific transcription factor FoxP3. Numbers indicate the percentage of total cells within quadrants. (F) Flow cytometric analysis of intracellular IL-4 in wild-type (gray filled histogram) and CD2-GATA3 Tg (black line) $FoxP3^+$ cells. Numbers indicate the percentage of $IL\text{-}4^+$ cells within the $FoxP3^+$ population. Data shown are representative of four mice analyzed per group.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T-bet
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***
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2.0
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1.0
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Th2
CD8
Treg
Th1
CD8
Treg
Wild-type
CD2-GATA3
B
CD8+ cells:
Wild-type
CD2-GATA3
Wild-type
CD2-GATA3
Granzyme B
IFNγ
13
39
15
31
65
3.0
IL-5
IL-13
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7.5
6.7
13
IL-4
IFNγ
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9.3
10
43
IL-10
IL-2
0.3
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16
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+ anti-IL-4
+ IL-4
Wild-type
CD2-GATA3
E
Treg cells:
Wild-type
CD2-GATA3
Wild-type
CD2-GATA3
IL-17
FoxP3
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97
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IL-4

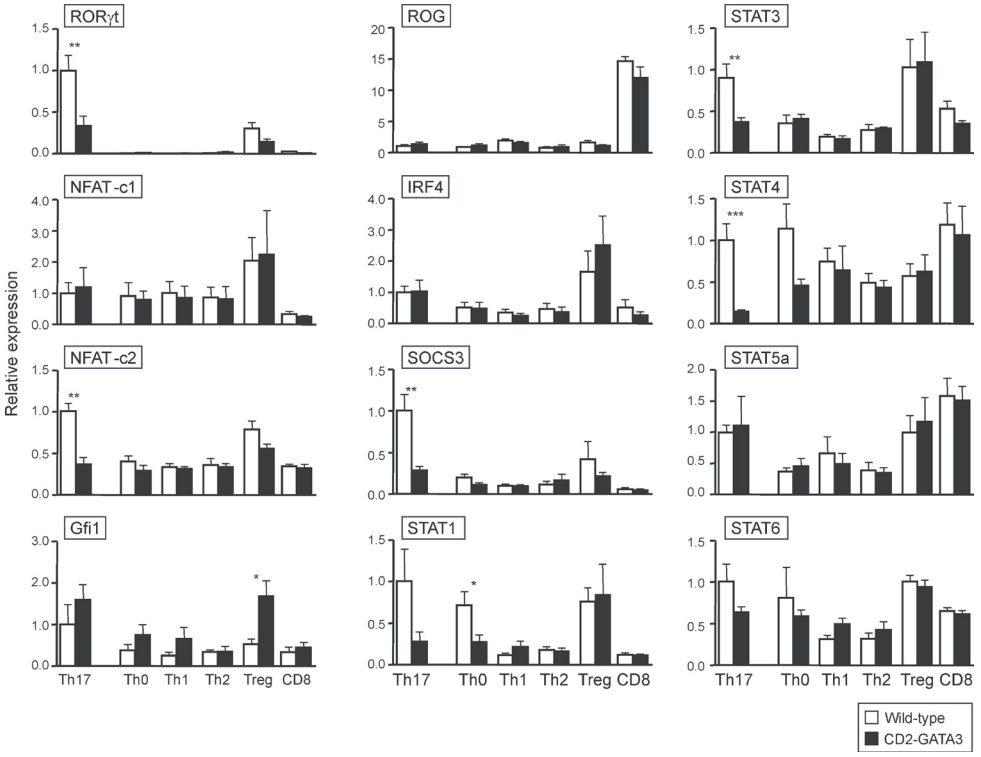

Figure S4. Effect of enforced GATA3 expression on transcription levels of nuclear factors. Quantitative RT-PCR analysis of expression of the indicated nuclear factors in different T effector cell cultures. Expression levels were normalized for GAPDH and expression in Th17 cultures from wild-type mice were set to one. Mean values and SEM are given for 5-11 mice analyzed per group (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *t*-test).

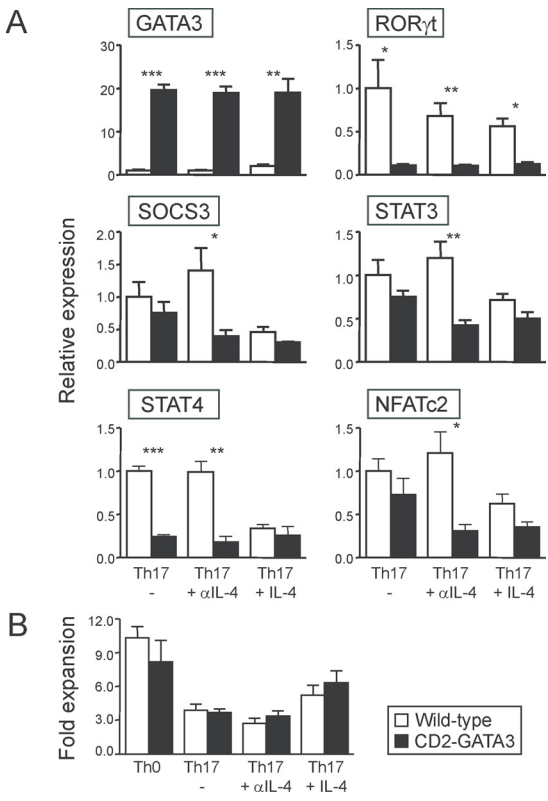

Figure S5. Enforced GATA3 expression affects critical factors in Th17 differentiation in an IL-4-independent manner. (A) Quantitative RT-PCR analysis of expression of the indicated genes in Th17 cultures. Cells were cultured for seven days with or without αIL-4 or IL-4. Expression was normalized for GAPDH and values in Th17 cultures from wild-type mice were set to one. Mean values and SEM are given for eight mice analyzed per group (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *t*-test). (B) Fold expansion of wild-type and CD2-GATA3 transgenic Th0 and Th17 cells with or without αIL-4 or IL-4. Mean values and SEM are given for eight mice analyzed per group.

Table S1. Primers and probes used for quantitative RT-PCR analysis for expression of the indicated genes.

Gene	Forward primer	Reverse primer	Probe
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	TGCATCCTGCACCACCAACTG
GATA3	CATTACCACCTATCCGCCCTATG	CACACACTCCCTGCCTTCTGT	CGAGGCCCAAGGCACGATCCAG
Gfi-1	TCCGAGTTCGAGGACTTTTG	GAGCGGCACAGTGACTTCT	CTTCTCCC
IL-5	ACATTGACCGCCAAAAAGAG	ATCCAGGAACTGCCTCGTC	GAGGAGAG
IL-6	ATCAGGAAATTTGCCTATTGAAA	CCAGGTAGCTATGGTACTCCAGA	TTCCTCTG
IL-21	CCATCAAACCCTGGAAACAA	TCACAGGAAGGGCATTTAGC	AGGAGGAG
IL-22	GTGACGACCAGAACATCCAG	TCCACTCTCTCCAAGCTTTTTC	GGCTGAAG
IRF4	ACCCCATGACAGCACCTTAT	GGGTGGCATCATGTAGTTATGA	CAGCCAG
NFATc1	TCCAAAGTCATTTTCGTGGA	CTTTGCTTCCATCTCCCAGA	GCTCCAGA
NFATc2	CCTGCTGGTACCACCTACTTG	CCGACTGATTGGAGAGTGG	CAGCATCC
ROG	GGCTTCCTCTAGCCCCACT	CCAAGGGCTGAGGGTTTC	CCAGGGCA
RORγt	TTCACCCCACCTCCACTG	TGCAAGGGATCACTTCAATTT	TGCTGTCC
SOCS3	ATTTCGCTTCGGGACTAGC	AACTTGCTGTGGGTGACCAT	CAGCCACC
STAT1	TTGTCAGATCGAACCTTCCTC	TGTCGTTCTACCACGAAGGA	CTTCCAGC
STAT3	CGTGGAGCTGTTCAGAAACTT	AACTGGACACCAGTCTTGATGA	TGGTGGAG
STAT4	CCTTAATTCAGAGCAGCTCAACA	GGTGAGGTGACCATCATTGTAG	TGGCAGAG
STAT5a	TGAAGGCGACCATCATCAG	GACGCAACAGTTGTTCAGGA	CAGCAGGC
STAT6	TCTGAGAGAGGGAGAAGATAGCAG	GGCCCCACAGAGACATGAT	GGCTGCTG
T-bet	TCAACCAGCACCAGACAGAG	AAACATCCTGTAATGGCTTGTG	GGCTGGAG
TNFα	CCACGTCGTAGCAAACCAC	TTTGAGATCCATGCCGTTG	TGGAGGAG

VII

GATA3 protects against severe joint inflammation and bone erosion and reduces Th17 differentiation during experimental arthritis

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Submitted

ABSTRACT

Objective. Rheumatoid arthritis (RA) is associated with the infiltration of T helper (Th) cells in the joint of RA patients. It is unclear, whether IFN-γ producing Th1 cells or the novel IL-17 producing Th17 cells are the pathogenic mediators of joint inflammation in a chronic non-autoimmune arthritis. Therefore, we examined whether the Th2 transcription factor GATA3 can regulate experimental arthritis by modulating Th1 and/or Th17 polarization.

Methods. Arthritis was induced in both wild-type and T cell specific GATA3 transgenic (CD2-GATA3 tg) mice. At 1 and 7 days after the induction of arthritis, knee joints were macroscopically scored and histology was taken. Single-cell suspensions were generated from spleen, lymph node and inflamed knee joints. Cytokine expression of CD4 T cells was determined by flow cytometry and IL-17 expression in the inflamed knee joints was determined by ELISA. Gene expression analysis was performed for Th17-associated factors.

Results. Wild-type mice developed severe joint inflammation, including massive infiltration and bone erosion, which increased in time reaching maximal arthritis scores at day 7. In contrast, only mild joint inflammation was observed in CD2-GATA3 tg mice. This mild effect was further accompanied with systemic and local reduction of IL-17^{+}IFN-γ^{-} and IL-17^{+}IFN-γ^{+}, but not of IL-17^{-}IFN-γ^{+} CD4 T cells and induction of Th2 cytokine expression. Moreover, GATA3 expression resulted in reduced gene expression of the Th17-associated transcription factor RORγt.

Conclusion. These data indicate that enforced GATA3 expression protects against severe joint inflammation and bone erosion accompanied with a reduced Th17, but not Th1 differentiation, during mBSA-induced arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is characterized by autoimmunity, synovial hyperplasia, progressive destruction of cartilage and bone, neo-angiogenesis and infiltration of joint synovium by activated inflammatory leukocytes, such as T helper-1 (Th1) lymphocytes and monocytes. However in the synovium of RA patients, it is difficult to detect the Th1 cytokine IFN-γ, while relatively high expression levels of IL-17A are detected [1]. Since IL-17A is a T cell factor produced by the novel Th17 subset, the question rises whether RA is a Th1 or a Th17 associated disorder. Identification and subsequent modulation of the pathogenic Th subset is a major goal in the treatment of RA. Classically, Th cells were divided into two different subsets namely; Th1 and Th2. Th1 cells are associated with the elimination of intracellular pathogens. In contrast, Th2 cells are characterized by the production of IL-4, IL-5 and IL-13 and are involved in the eradication of parasitic worms and allergic responses. Differentiation of Th1 cells from naïve CD4 T cells is dependent on the IL-12/STAT4 and IFN-γ/STAT1/T-bet signaling pathways. Th2 differentiation is dependent on IL-4 signaling, via the activation of STAT6, resulting in the expression of the transcription factor GATA3.

GATA3 further stabilizes Th2 differentiation, by auto-activating its own expression and by increasing the accessibility of the Th2 cytokine cluster [2, 3].

As Th1 and Th2 cells appear to arise in a mutually exclusive fashion, various mechanisms of counter-regulation exist. It has been described that GATA3 negatively regulates Th1 differentiation by down regulating STAT4 and IL-12Rβ2 [4]. Furthermore, Th1/Th2 differentiation is regulated by an interchromosomal association of Th1 and Th2 loci [5]. On the other hand, T-bet interferes with the binding of GATA3 to its target DNA through a physical interaction with GATA3 [6]. The function of GATA3 as a principal Th1/Th2 switch was shown by conditional gene targeting, whereby the absence of GATA3 expression was sufficient to induce Th1 differentiation in the absence of IL-12 and IFN-γ. Moreover, Th2 cells deficient for GATA3 resulted in reduced Th2 maintenance and responses [7, 8]. Next to the regulatory role during Th1 differentiation, GATA3 acts a negative regulator of regulatory T cells (Treg) by directly inhibiting the expression of the Treg associated factor FoxP3 [9].

GATA3 is further required for embryogenesis, which has been shown by embryonic lethality at day 11 after gestation [10]. Moreover, GATA3 plays essential roles throughout T cell development, including differentiation of the early CD4 and CD8 double negative (DN) T cell stages, TCRβ selection and CD4 positive selection [11-15].

In the past few years a new T helper cell subset has been identified, which produces IL-17 and is therefore referred to as Th17 [16, 17]. IL-17 is a proinflammatory cytokine produced by activated CD4 T cells and has been implicated in a range of autoimmune diseases including, RA, multiple sclerosis (MS) and psoriasis [1, 18, 19]. In mouse models for arthritis, the implication for IL-17 expression has been shown for inflammation and bone destruction [20-24].

Induction of murine Th17 differentiation cells is critically dependent on transforming growth factor-β (TGF-β), IL-6 and the transcription factors RORα, RORγt and IRF4. Additionally, the cytokines IL-21 and IL-23 have been reported to promote IL-17 expression. JAK-STAT signaling, via STAT3 phosphorylation is required to induce IL-17 expression. STAT3 phosphorylation is on its turn negatively regulated by SOCS3 (reviewed in ref: 25).

As mentioned above, various mechanisms through GATA3 exist to regulate Th1 and Th2 differentiation. However, it is still unclear whether GATA3 influences Th17 differentiation and function in chronic inflammatory diseases. In this report, we investigated the effect of GATA3 expression on the function of IL-17 producing cells in experimental arthritis. For this reason, arthritis was induced in CD2-GATA3 transgenic (tg) mice, in which GATA3 expression is controlled by the human CD2 promoter and locus control region, resulting in enforced GATA3 expression throughout T cell development [26]. Enforced GATA3 expression during mBSA-induced arthritis resulted in a striking suppression of severe joint inflammation and bone erosion. Interestingly, this was associated with a remarkable reduction of the fraction of IL-17, but not of IFN-γ producing CD4 T cells isolated from the spleen and draining lymph nodes. Furthermore, an enhanced capacity of Th2 cytokine expression and a reduced expression of Th17 associated genes was found. In addition, to effects the obtained in peripheral CD4 T cells, IL-17 expression in CD4 T cells, isolated from inflamed knee

joints, was even more reduced. Taken together, these data show that enforced GATA3 expression in T cells results in a reduction of severe joint destructive inflammation in mice, which was accompanied with reduced Th17 differentiation, but no reduced Th1 differentiation, systemically as well as locally in the inflamed knee joints.

MATERIALS AND METHODS

Mice

CD2-GATA3 mice [27] were backcrossed on the C57BL/6 background for at least 8 generations and genotyped by PCR and bred in-house. GATA3 primers were: 5'-CAGCTCTGGACTCTTCCCAC-3' and 5'-GTTCACACACTCCCTGCCTT-3'. Mice were kept under specific pathogen free conditions and provided with food and water *ad libitum*. Mice between 8-12 weeks of age were used for experiments. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Flow cytometric analyses

Preparations of single-cell suspensions from spleen and lymph nodes, monoclonal antibody (mAb) incubations and four-color flow cytometry have been previously described [28]. To isolate cells from inflamed tissue, knee joints were dissected, fractionated and incubated with Blendzyme3 (60 µg/ml, Roche Diagnostics, Mannheim, Germany) for 3 hours in Iscove's modified Dulbecco's medium (IMDM) (BioWhittaker, Walkersville, MD), containing 10% heat-inactivated fetal calf serum (FCS) (Sigma, St. Louis, MO), 5 x 10^{-5} M β-mercaptoethanol (Merck, Darmstadt, Germany). After incubation, single cell suspensions were generated. Monoclonal antibodies were purchased from BD Biosciences (San Diego, CA), except biotinylated anti-IL-13 (BAF413, R&D Systems, Minneapolis, MN) and APC-conjugated FoxP3 (FJK-16s, e-Biosciences San Diego, CA). For intracellular detection of cytokines, cells were stimulated with plate-bound anti-CD3 mAb (10 µg/ml in PBS) in the presence of GolgiStop™
(BD Biosciences) for 4h. Cells were harvested, extracellularly stained with anti-CD4 mAb, followed by standard intracellular staining using 2% paraformaldehyde and 0.5% saponin. Samples were acquired on a FACSCalibur™ flow cytometer and analyzed using CELLQuest™ research software (Becton Dickinson, Sunnyvale, CA). Live events were collected based on forward and side scatter.

Purification of effector T cells and *in vitro* T cell stimulation

Effector $CD62L^-$ CD4 T cells were purified from spleens obtained from mBSA immunized mice 7 days after induction of joint inflammation by cell sorting, using a FACSAria cell sorting system and BD FACSDiva software (BD Biosciences). Purity of obtained fractions was typically >98%. $CD62L^-$ CD4 T cells were stimulated for 4h with PMA and Ionomycin (50 ng/ml, 500 ng/ml respectively).

Quantitative PCR analyses

Total RNA of sorted CD62L$^-$ CD4 T cells was extracted using the GeneElute mammalian total RNA miniprep system (Sigma). Isolated RNA was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers. PCR Primers spanning at least one intron-exon junction were designed manually or using ProbeFinder software (Roche Applied Science, Indianapolis, IN) and probes were chosen from the universal probe library (Roche Applied Science) or designed manually and purchased from Eurogentec (Seraing, Belgium). Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). To confirm the specificity of the amplification products, samples were analyzed by standard agarose gel electrophoresis. Threshold levels were set and further analysis was performed using the SDS v1.9 software (Applied Biosystems). The obtained C_t values were normalized to those of glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

Induction of antigen-induced arthritis

Methylated BSA (mBSA, 8 mg/ml) (Sigma) was emulsified in an equal volume of CFA containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco). At day -21, mice were immunized by intra-dermal injection of 100 μl mBSA/CFA emulsion into the base of the tail. On day 0, arthritis was induced by injecting mice intra-articular (i.a.) with 60 μg of mBSA in 0.9% NaCl in 6 μl into both knee joints.

Synovial washouts and cytokine measurements

To determine the expression levels of several cytokines in synovial wash-outs, patellae with adjacent synovium was isolated from knee joints as described earlier [29]. In brief, patellae with adjacent synovium was incubated in RPMI 1640, supplemented with 0.1% BSA, gentamicin (50 μg/ml), and L-glutamine (2 mM) (200 μL/patellae) for 1h at room temperature. IL-17 cytokine level was measured in the obtained supernatant by using the Duoset ELISA development kit (R&D Systems) according to the manufacture's instructions.

Histological assessment

Mice were killed 1 and 7 days after arthritis induction and the rear limbs were removed and processed for hematoxylin and eosin (H&E) staining. Sections were assessed for joint inflammation (infiltrate and exudate) and bone erosion [30]. The severity of inflammation was scored on a scale from 0-3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity and 3 = maximal cellularity) and the severity of bone erosion was also scored from 0-3 (0 = no bone erosion, 1 = mild bone erosion, 2 = moderate bone erosion and 3 = maximal bone erosion).

Statistical analysis

Differences between experimental groups were tested with the Mann Whitney U test or stated otherwise. *P* values less than 0.05 were considered significant.

RESULTS

T cell specific overexpression of GATA3 protects against development of severe joint inflammation and bone erosion

To examine whether T cell specific GATA3 expression affects joint inflammation during arthritis, we induced antigen-induced arthritis (AIA) in both CD2-GATA3 tg and wild-type C57BL/6 mice. For this reason, mice were immunized with methylated bovine serum albumin (mBSA) in complete Freund adjuvant (CFA) and 21 days later mBSA was injected intra-articular (i.a) in the knee joints [30].

At day 1 after arthritis induction, similar mild arthritis scores (~0.5) were observed in wild-type and CD2-GATA3 tg mice. In wild-type mice this mild joint inflammation progressed to severe joint inflammation, which gradually increased to maximum arthritis scores 7 days after arthritis induction. In contrast, the mild joint inflammation observed at day 1 in CD2-GATA3 tg mice did not increase in time and only mild arthritis scores (<0.5) were detected at day 7 (Figure 1A).

This severe joint inflammation in wild-type mice was accompanied with large and massive infiltrations of inflammatory cells, which was significantly lower in CD2-GATA3 tg mice. Moreover, bone erosion was present in wild-type mice, but strongly reduced in CD2-GATA3 tg mice (Figures 1B and C).

Taken together, T cell specific enforced expression of GATA3 protects against the development of severe joint inflammation and bone erosion in mBSA-induced arthritis.

Enforced GATA3 expression inhibits the differentiation of IL-17$^+$ and IL-17$^+$IFN-γ^+, but not of IFN-γ^+ CD4 T cells

Induction of arthritis with mBSA, is driven by CD4 T cells [31]. Both IFN-γ and IL-17 producing CD4 T cells have been found in mBSA arthritis [31-33]. However it is unclear, whether IFN-γ producing Th1 or IL-17 producing Th17 are the pathogenic mediators of joint inflammation. Because enforced GATA3 expression protected against severe joint inflammation and bone erosion, we examined the influence of GATA3 on Th1 and Th17 cytokine expression in this experimental arthritis model.

At 1 day after arthritis induction, similar fractions of ~1.5% of wild-type and ~1.2% of CD2-GATA3 tg splenic CD4 T cells produced IL-17 (Figure 2A and B). In naïve wild-type and CD2-GATA3 tg mice less than 0.4% of splenic CD4 T cells express IL-17 (data not shown). At day 7 after arthritis induction, a large increase in the fraction of IL-17$^+$ splenic CD4 T cells (~3.1%) was found in wild-type mice, while this increase was absent in CD2-GATA3 tg mice (Figure 2A and B). Similarly, a fraction of IL-17$^+$ cells (~2.5%) was present within the CD4 T cell population of wild-type draining lymph nodes, while only a minor fraction of IL-17$^+$ cells (~0.3%) was observed in CD2-GATA3 tg mice (Figure 2A).

The population of IL-17 producing CD4 T cells can be further divided in IL-17$^+$ IFN-γ^- and IL-17$^+$IFN-γ^+ cells. Low, but substantial fractions of ~0.4% at day 1 and ~0.6% at day 7 of these IL-17$^+$IFN-γ^+ cells were identified within the CD4 T cell population of the spleen and draining lymph nodes of wild-type mice. In contrast, these double positive cells were hardly detectable (≤ 0.2%) in CD2-GATA3 tg mice

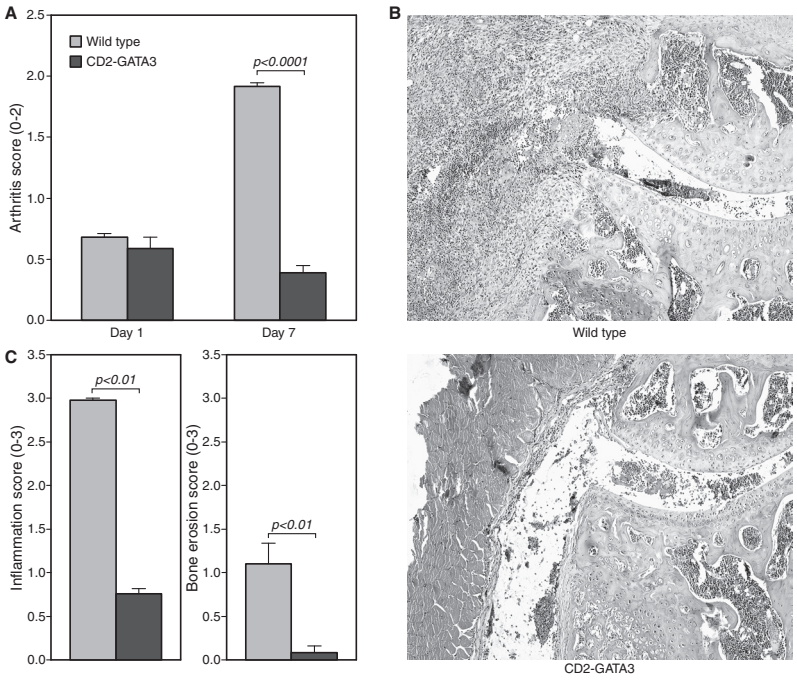

Figure 1. GATA3 protects against mBSA induced joint inflammation. (A) Macroscopical scores of wild-type and CD2-GATA3 tg mice at days 1 and 7 after intra-articularly mBSA injection in the knee joint. Mean values and SEM are given for 6 mice analyzed per group at day 1 and 11 mice per group at day 7. Data are obtained from at least 2 separate experiments. (B) Histological (H&E) staining of knee joint sections obtained from wild-type and GATA3 mice 7 days after mBSA injection. Data are representatives of 6 histological sections per group (original magnification 100x). (C) Inflammation and bone erosion scores of wild-type and CD2-GATA3 tg mice, 7 days after the induction of arthritis. Data are obtained from 6 histological sections per group. (See Appendix page 220 for a full-color representation of this figure).

(Figure 2A and B). Enforced GATA3 expression had no suppressive effect on the total fraction of IFN-γ producing CD4 T cells. Even a slight increase of total IFN-γ production was found on both days 1 and 7 after arthritis induction in CD2-GATA3 tg mice (Figure 2B).

Next to the CD4 T cells, we analyzed the effect of GATA3 expression on regulatory T cells (Treg) and TCR$\gamma\delta$ expressing T cells. No effects were found in the population of FoxP3 expressing CD4 T cells and similar low fractions of IL-17 producing FoxP3 cells were observed (Figure 3A and B). Among TCR$\gamma\delta$ T cells, comparable large fractions (~24-27%) of IL-17 producing cells were found in both wild-type and CD2-GATA3 tg mice (Figure 3C and D).

Taken together, these data indicate that the suppression of joint inflammation in CD2-GATA3 tg mice was associated with a suppressed expansion of IL-17$^+$IFN-γ^- and IL-17$^+$IFN-γ^+ producing CD4 T cells, but not of IL-17$^-$IFN-γ^+ CD4 T cells.

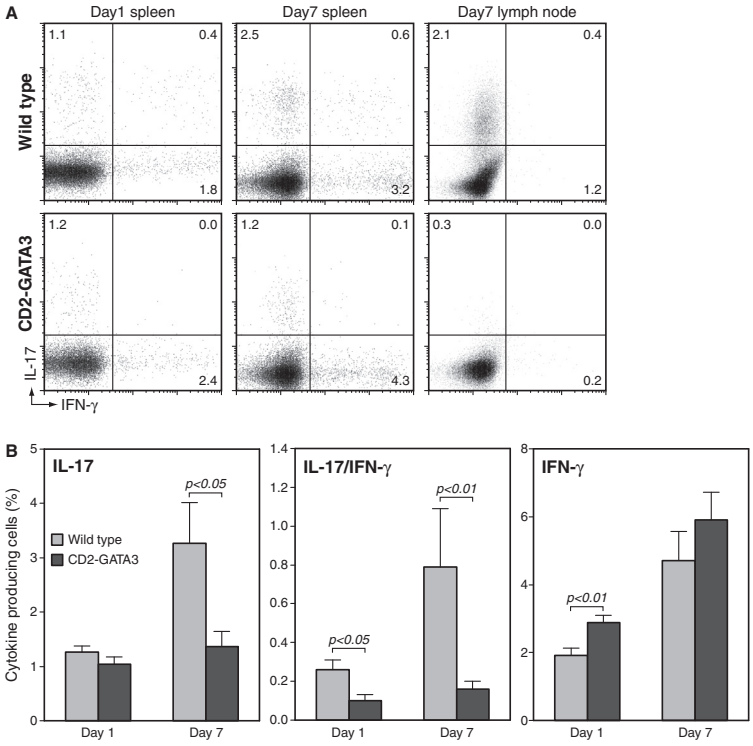

Figure 2. Enforced GATA3 expression results in reduced IL-17 expression, but not of IFN-γ expression, by CD4 T cells. (A) Flow cytometric analysis for intracellular IL-17 and IFN-γ expression in CD4 T cells obtained form spleen and draining lymph nodes at day 1 (spleen only) and day 7 after arthritis induction. Numbers indicate the percentage of cells within a quadrant. Dates are representatives of at least 2 separate experiments wherein 6 mice were analyzed per group at day 1 and 11 mice per group at day 7. (B) Quantification of flow cytometric analysis of the IL-17$^+$, IL17$^+$IFN-γ$^+$ and IFN-γ$^+$ CD4 T cell subsets at day 1 and 7 after arthritis induction. Bars represent the percentage of cytokine producing splenic CD4 T cells from wild-type (white bars) and CD2-GATA3 tg mice (black bars). Mean values and SEM are given for 6 mice analyzed per group at day 1 and 11 mice per group at day 7.

Enforced GATA3 expression results in increased Th2 and reduced IL-2 expression

Enforced expression of GATA3 results in increased expression of Th2 associated cytokines, like IL-4, IL-5, IL-13 and IL-10 in activated peripheral CD4 T cells [26]. To examine whether enforced GATA3 expression was sufficient to induce these cytokines in mBSA-induced arthritis, we examined the expression of IL-4, IL-5 and IL-13. Seven days after arthritis induction, limited percentages of IL-4 producing CD4 T cells were found in the spleens of wild-type mice (~1.3%), while in CD2-GATA3 tg mice a large fraction (~10.3%) of CD4 T cells produced IL-4 (Figures 4A and B). From these cells, which expressed IL-4, about ~26% (2.7% out of 10.3%)

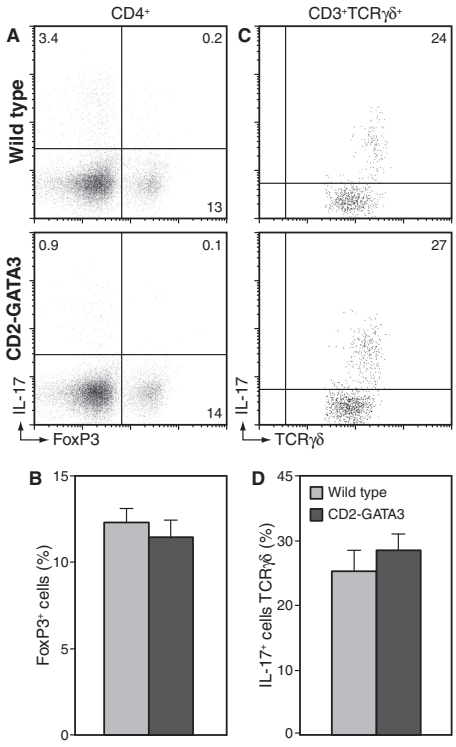

Figure 3. Suppressed joint inflammation in mBSA immunized CD2-GATA3 mice was not associated with an increased Treg cell population and altered IL-17 expression by Treg and TCRγδ cells. (A) Flow cytometric analysis for intracellular expression of FoxP3 and IL-17 in splenic CD4 T cells. (B) Quantification of FoxP3 expression. (C) Flow cytometric analysis for intracellular IL-17 expression of $CD3^+$ and $CD4^-$ gated TCRγδ T cells. (D) Quantification of intracellular IL-17 expression by TCRγδ T cells. Splenic cells were gated for CD4 and obtained 7 days after arthritis induction. Numbers indicate the percentage of cells within a quadrant. Data are representatives of at least 2 separate experiments wherein 6 mice were analyzed per group.

co-expressed IFN-γ. In addition to enhanced IL-4 expression, the fractions of IL-5 and IL-13 producing cells were larger in CD2-GATA3 tg mice (~1.4 and ~2.6% respectively), compared to wild-type mice (~0.2 and 0.6% respectively) (Figures 4A and B).

Recently, a few reports described a role for IL-10 in restraining Th17 mediated pathology [34-36]. For this reason, we were interested whether enforced GATA3 expression in the context of mBSA-induced arthritis, resulted in increased populations of IL-10 producing splenic CD4 T cells. However, only small populations (~0.4%) of $IL\text{-}10^+$ cells were identified in CD2-GATA3 tg mice, which were in the same range of wild-type mice (~0.2%). IL-2 has been shown to inhibit Th17 differentiation [37]. However, in CD4 T cells of CD2-GATA3 Tg mice, we found reduced expression of IL-2, compared to wild-type CD4 T cells (Figure 4A and B).

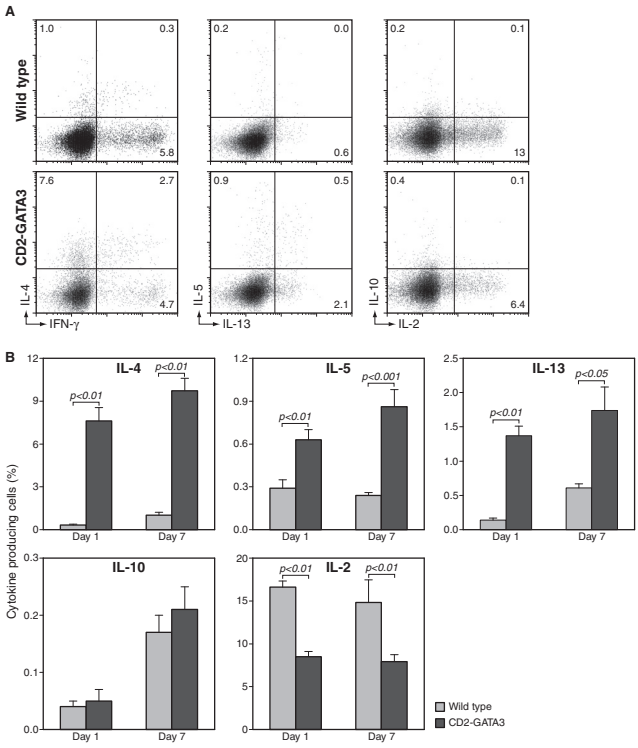

Figure 4. Enforced GATA3 expression induces a systemic expression of Th2 cytokines and limits the expression of IL-2 during mBSA-induced arthritis. (A) Flow cytometric analysis for intracellular cytokine expression in gated splenic CD4 T cells from wild-type and CD2-GATA3 tg mice at day 7 after the induction of mBSA-induced arthritis. Numbers indicate the percentage of cells within the quadrants. Data are representatives of at least 2 separate experiments wherein 6 mice were analyzed per group at day 1 and 11 mice per group at day 7. (B) Quantification of flow cytometric analysis for intracellular expression of the indicated cytokines at day 1 and 7 after arthritis induction. Bars represent the percentage of splenic CD4 T cells producing the indicated cytokine from wild-type (white bars) and CD2-GATA3 tg mice (black bars). Mean values and SEM are given for 6 mice analyzed per group at day 1 and 11 mice per group at day 7.

These findings indicate that during the induction of mBSA-induced arthritis, enforced GATA3 expression is sufficient to induce the expression of Th2 associated cytokines and to reduce the expression of IL-2.

GATA3 expression has positive and negative effects on Th17 differentiation during arthritis

To find out, whether the suppression of the IL-17$^+$ T cell population in CD2-GATA3 tg mice was associated with a decreased expression of genes involved in Th differentiation, quantitative RT-PCR analysis were performed. For this analysis, we

used FACS sorted effector CD62L$^-$ CD4 T cells obtained from spleens of both wild-type and CD2-GATA3 tg mice, 7 days after arthritis induction.

First, expression of GATA3 was increased ~10 fold, in CD2-GATA3 tg mice. Secondly, we found a decreased expression level of RORγt, but not of RORα. Both these factors were shown to be crucial for Th17 differentiation [38, 39]. Thirdly, we found increased expression of the Th1 associated transcription factor T-bet, which might be responsible for the slight increase in IFN-γ (Figure 2B and 5). Besides these transcription factors, JAK-STAT signaling is crucial to integrate cytokine signals to Th differentiation programs. Likewise, STAT3 signaling was recently shown to be required for Th17 differentiation and IL-17 expression [40, 41]. Although we observed increased expression of STAT3 in CD2-GATA3 tg effector CD4 T cells, we also found increased expression of SOCS3, which is a negative regulator of STAT3 activation [41]. When compared with wild-type mice, higher levels of STAT1 (although not statistically different) and STAT4, both of which are essential for Th1 differentiation, were found in effector CD4 cells of CD2-GATA3 tg mice (Figure 5). Despite reduced proportions

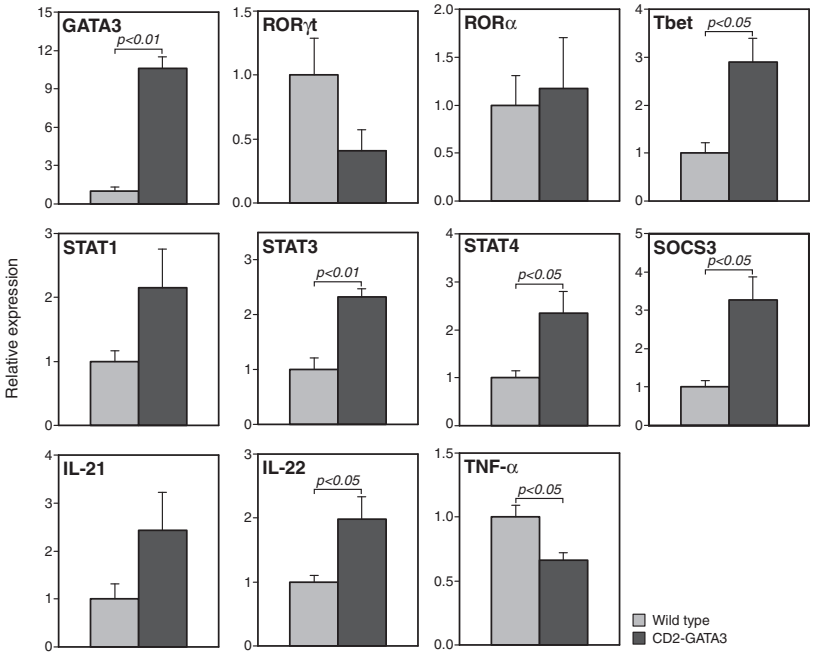

Figure 5. Enforced GATA3 expression during mBSA-induced arthritis affects factors involved in Th17 differentiation. Quantitative RT-PCR analysis of expression of the indicated genes in sorted CD62L$^-$ CD4 T cells from wild-type (white bars) and CD2-GATA3 transgenic mice (black bars). Cells were obtained 7 days after mBSA-induced arthritis. Expression levels were normalized for GAPDH and values of sorted memory T cells from wild-type mice were set to one. Mean values and SEM are given for 4 mice analyzed per group. For statistical analysis a Student's t-test was performed.

of IL-17 producing CD4 cells (Figure 2), we found abundant expression of IL-21 and IL-22. Expression of TNF-α was reduced (~60% of wild-type levels).

Altogether, these data suggest that enforced expression of GATA3 negatively affects Th17 differentiation via reduced RORγt expression.

GATA3 expression results in reduced IL-17 and enhanced Th2 cytokine expression by joint infiltrating CD4 T cells

The suppression of joint inflammation in CD2-GATA3 tg mice was associated with a systemic decrease in the population of IL-17$^+$ CD4 T cells. Next to this, we examined the capacity of joint infiltrating CD4 T cells to express IL-17 locally at the site of inflammation at day 7 after the induction of arthritis. In wild-type mice, about ~14% of the CD4 T cells produced IL-17, while a significant reduction was observed in the IL-17 producing fraction (~3.9%) in CD2-GATA3 tg mice (Figure 6A and B).

In addition, when IL-17 cytokine expression was measured by ELISA in synovial washouts, a significant reduction of ~3.6 fold, was observed in CD2-GATA3 tg mice, compared to wild-type mice (Figure 6C).

The amount of IL-17 expression, as determined by ELISA, could partly be explained by a reduction of ~1.6 fold of joint infiltrating CD4 T cells in CD2-GATA3 tg mice (Figure 6D). Of note, this reduction was in line with a ~1.6 fold reduction in the total numbers of splenic CD4 T cells in untreated CD2-GATA3 tg mice (Figure 6E). Therefore, this strongly suggests that enforced GATA3 expression does not influence homing of CD4 T cells.

Furthermore, we examined the expression of Th2 associated cytokines by joint infiltrating T cells. A striking fraction of ~22% of the CD2-GATA3 tg CD4 T cells produced IL-4, while only a fraction of ~0.5% of wild-type CD4 T cells produced IL-4 (Figure 6F and G). In addition, increased production of IL-13 and IL-5 was detected in CD2-GATA3 tg joint infiltrating CD4 T cells. IL-10 production was hardly detected in both CD2-GATA3 tg and wild-type mice (Figure 6F).

When taken together, these data show that reduced joint inflammation in mBSA immunized CD2-GATA3 tg mice was associated with reduced IL-17 and enhanced Th2 cytokine expression by joint infiltrating CD4 T cells.

DISCUSSION

The transcription factor GATA3 is a master regulator of Th2 differentiation and Th2 cytokine expression. A lot of knowledge has been gained on the role of GATA3 in the regulation of Th1 and Th2 differentiation. However the effect of GATA3 expression on Th17 differentiation and function during arthritis is unclear.

In this report, we describe that enforced T cell specific expression of GATA3 in an experimental arthritis model suppressed severe joint inflammation and bone erosion, which was associated with reduced Th17 differentiation. Wild-type mice developed severe arthritis, whereby joint inflammation was associated with bone erosions and massive infiltration of inflammatory cells, while only mild joint inflammation was

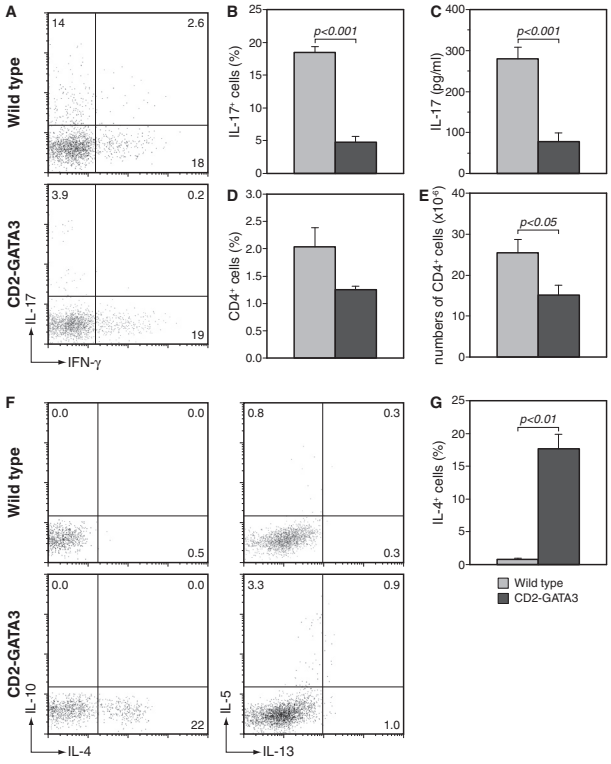

Figure 6. Enforced GATA3 expression results in reduced IL-17 expression and enhanced Th2 cytokine expression by joint infiltrating CD4 T cells. (A) Flow cytometric analysis for intracellular IL-17 and IFN-γ expression in joint infiltrating CD4 T cells isolated 7 days after induction of arthritis. Numbers indicate the percentage of cells within a quadrant. Data are representatives of 3 mice analyzed per group. (B) Quantification of flow cytometric analysis for intracellular IL-17 in joint infiltrating CD4 T cells. Percentage of IL-17 producing CD4 T cells from wild-type (white bars) and CD2-GATA3 tg mice (black bars). Mean values and SEM for 3 mice per group. (C) ELISA analysis of IL-17 expression in synovial washouts from wild-type (white bars) and CD2-GATA3 tg mice (black bars). Mean values and SEM for 6 synovial washouts per group. (D) Fraction of CD4 T cells within the total population of joint infiltrating cells in wild-type (white bars) and CD2-GATA3 tg mice (black bars). Mean values and SEM for 3 mice per group. (E) Total splenic CD4 T cell numbers of untreated wild-type and CD2-GATA3 tg mice. Mean values and SEM for 8 mice per group. (F) Flow cytometric analysis for intracellular IL-4, IL-5, IL-13 and IL-10 expression. Data are representatives of 3 mice per group. (G) Quantification of percentage of IL-4 expressing CD4 joint infiltrating T cells in wild-type (white bars) and CD2-GATA3 tg mice (black bars). Mean values and SEM for 3 mice per group. For statistical analysis a Student's t-test was performed.

observed in CD2-GATA3 tg mice. This suppressed joint inflammation was associated with reduced IL-17 expression by CD4 T cells obtained from the spleen, draining lymph nodes and synovium.

Surprisingly, enforced GATA3 expression had no suppressive effect, but even moderately enhanced IFN-γ expression by CD4 T cells. This is also reflected by

enhanced gene expression of Th1 associated factors like, T-bet, STAT1 and STAT4 in sorted splenic effector T cells. However, the co-expression of a large fraction of CD4 T cells, which co-expressed both IL-4 and IFN-γ at a single cell level have to be taken into account, which makes it hard to judge whether these cells are true Th1 cells (Figure 4A). In addition, involvement of the strong Th1 skewing potential of complete Freund adjuvant, may explain the incapability of GATA3 to suppress IFN-γ, which is indeed the case when CD2-GATA3 tg CD4 T cells are cultured *in vitro* [26]. However, the fact that expression of IFN-γ is elevated in CD2-GATA3 tg mice, while IL-17 expression is suppressed, indicate that the induction of arthritis in mBSA immunized mice, is mediated by Th17 cells, rather than by Th1 cells. A similar observation for a critical role for IL-17 producing CD4 T cells, was made for the autoimmune collagen induced arthritis model [20].

The role of IL-17$^+$IFN-γ$^+$ double positive cells in the pathology of arthritis is still unclear. These cells have been found in collagen induced arthritis [20] and Lubberts *et al.*, unpublished observations, as well as in early RA patients (Colin *et al.*, unpublished observations). In the present study, we could hardly detect expression of both IL-17 and IFN-γ in single CD4 T cells of CD2-GATA3 tg mice, while substantial fractions of these double positive cells were found in wild-type mice. This might indicate a pathogenic potential of IL-17$^+$IFN-γ$^+$ CD4 T cells in arthritis.

The large fraction of about 17% of wild-type joint infiltrating CD4 T cells that express IL-17, indicate the local function of IL-17 expression in joint inflammation. Especially in these joint infiltrating CD4 T cells, enforced GATA3 expression significantly suppressed IL-17 expression. Moreover, enforced GATA3 expression greatly enhanced the expression of IL-4 in these cells. It has been described that IL-4 has a suppressive potential on destructive arthritis and that IL-4 can inhibit the differentiation of Th17 cells [42]. However, treatment of mBSA immunized mice with high doses of ~1.2 mg of neutralizing anti-IL-4 mAb per mouse did not increase joint inflammation or IL-17 production in CD2-GATA3 tg mice (data not shown). Although, we cannot exclude that not all IL-4 is neutralized by this antibody treatment, these data suggest that the protection of severe arthritis and Th17 expansion may not be fully dependent on IL-4.

The reduced IL-17 expression of CD2-GATA3 tg CD4 T cells might as well be a result of an effect on the expression of Th17 associated factors. Gene expression analysis indicated a suppressed gene expression of RORγt in sorted effector CD4 T cells, while expression of STAT3 was even enhanced. However, gene expression of SOCS3 a negative regulator of STAT3 phosphorylation, was enhanced as well. In addition, enforced GATA3 expression did result in reduced gene expression of TNF-α, but not of IL-21 and IL-22.

It has been described that GATA3 is negatively regulated by TGF-β, which is required for Th17 and Treg differentiation [43]. This negative regulation in Treg cells is possibly required to inhibit GATA3 expression in its capacity to directly inhibit FoxP3 expression [9]. A speculative hypothesis could be that a similar mechanism is present in Th17 cells, whereby GATA3 is capable to directly or indirectly inhibit the differentiation of Th17 cells by repressing the expression of RORγt. Future experiments are required to unravel direct or indirect effects of GATA3 on RORγt expression.

Enforced GATA3 expression was not sufficient to provide a complete suppression of joint inflammation and to provide a complete blockade of IL-17 expression. Especially at the 1 day after arthritis induction, no differences were observed in terms of joint inflammation and IL-17 expression. This might suggest that GATA3 is not capable to inhibit the induction of Th17 cells, but that GATA3 is more likely to be involved in the regulation of the survival or pathogenic function of Th17 cells.

Taken together this report describes the suppressive capacity of enforced GATA3 expression on severe joint inflammation and bone erosion during arthritis, which is associated with inhibited Th17 differentiation. These data indicate that selective modulation of specific transcription factors (GATA3 up, RORγt down) might lead to new therapeutic applications, which may improve current therapy and even reach the goal to prevent the development of chronic inflammatory diseases, such as RA.

ACKNOWLEDGEMENTS

We would like to thank E. de Haas, T. Schonewille and the people from the EDC Animal Facility (Erasmus MC, Rotterdam) for and the technical assistance at various stages of the project. Grant support: This work was partly supported by the Dutch Cancer Society and the Dutch Arthritis Association

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VIII

General discussion

GATA factors: key regulators in complex networks

Parts of this chapter have been published as a review in Stem Cell Research Developments

This thesis describes research on the molecular mechanisms underlying the role of GATA transcription factors in the T cell lineage. Since GATA factors are crucial regulators of proliferation and differentiation, it is essential that the expression of GATA factors is tightly regulated. This is underscored by various findings implicating GATA family members in hematopoietic disorders, such as leukemia. By using gene targeting and transgenic approaches in mice, the mechanisms underlying the key functions of GATA factors in haematopoiesis, T cell development and Th cells were investigated. Detailed knowledge on the *in vivo* function of GATA transcription factors will not only have implications in the context of T cell leukemia, but also in stem cell transplantation, effector T cell mediated diseases and in oncology in general, given the involvement of GATA3 in breast cancer.

Function of GATA2 in embryonic hematopoietic stem cells

GATA2 is essential for embryonic development and early hematopoiesis. *GATA2*$^{-/-}$ mutants die at embryonic stage E10.5 with severe anemia. E9.5 GATA2$^{-/-}$ embryos are pale and have lower numbers of circulating blood cells [1]. *In vitro*, clonogenic progenitor colonies obtained from *GATA2*$^{-/-}$ para-aortic splanopleura, yolk sac (YS) and embryonic stem (ES) cells are dramatically reduced, but can be partially rescued by crossing into the *p53*$^{-/-}$ background [2]. Consistently, when *GATA2*$^{-/-}$ ES cells were introduced into wild-type blastocysts, these cells contributed at a low level to the embryonic circulation in the chimeras, but not to the fetal liver hematopoietic compartment [1]. Therefore, it can be concluded that GATA2 plays a minor or redundant role in primitive hematopoiesis, whereas the development of the definitive hematopoietic system is highly dependent on GATA2. Hematopoietic progenitor cells arise in *GATA2*$^{-/-}$ embryos and can be derived from *GATA2*$^{-/-}$ ES cells, but they fail to proliferate or survive [2]. Thus, GATA2 function is dispensable for the generation of hematopoietic progenitors, but is crucial for their maintenance and expansion [1].

Since *GATA2*$^{-/-}$ mutants die before the first hematopoietic stem cells (HSCs) are detectable in the embryo at E10.5, the effect of *GATA2* deletion in HSC production and function in the embryo cannot be analyzed *in vivo.* Heterozygous *GATA2*$^{+/-}$ mutant mice, which express reduced levels of GATA2 in hematopoietic cells, survive until adulthood without gross abnormalities. Studies on *GATA2*$^{+/-}$ mutant embryos have demonstrated that they are haploinsufficient and have revealed differential responses of YS and AGM to GATA2 gene dosage, thereby affecting the production, expansion and maintenance of HSCs in the developing embryo (**Chapter II**). *GATA2*$^{+/-}$ mutants have reduced numbers of CFU-S (colony forming units - spleen), both in the YS and in the aorta-gonads-mesonephros (AGM) region. While this defect could be intrinsic to the expansion or survival of these erythro-myeloid progenitors, it could alternatively be due to the reduced HSCs production in both YS and AGM regions of the *GATA2*$^{+/-}$ mutants. Although HSCs are present in these embryonic sites in the *GATA2*$^{+/-}$ mutants, the detected HSC activities are reduced in both tissues. As the embryo develops, expansion of HSC activities in the *GATA2*$^{+/-}$ YS appears to be normal, both *in vivo* and *ex vivo*, while the *GATA2*$^{+/-}$ AGM region completely fails to maintain and expand the residual HSC activity. Therefore, while GATA2 gene

dosage affects HSC production of both YS and AGM region, there is an intrinsic difference between YS and AGM region in the response to GATA2 levels in terms of HSC proliferation and survival. We conclude that HSC expansion and maintenance in the AGM region, but not in the YS, is dependent on GATA2 gene dosage (**Chapter II**).

While *GATA2*$^{+/-}$ bone marrow cells express lower levels of GATA2 mRNA, the hematological profile of *GATA2*$^{+/-}$ mice, and the efficiency of engraftment of *GATA2*$^{+/-}$ bone marrow cells to the recipient's hematopoietic system, is comparable to wild-type bone marrow cells. Steady state hematopoiesis is normal in *GATA2*$^{+/-}$ mice, but phenotypically defined HSCs are under-represented in *GATA2*$^{+/-}$ mice. *GATA2*$^{+/-}$ HSC defects are only uncovered in a competitive transplantation scenario. When equal numbers of wild-type and *GATA2*$^{+/-}$ cells are transplanted into lethally irradiated recipients, a lower contribution of *GATA2*$^{+/-}$ cells to the hematopoietic system is observed. This is not due to homing defects in the *GATA2*$^{+/-}$ HSCs, since a similar study, using sub-lethally irradiated *GATA2*$^{+/-}$ recipients, the same low dose of wild-type bone marrow cells is able to out-compete the residual *GATA2*$^{+/-}$ HSC. Reciprocal transplantation using *GATA2*$^{+/-}$ donor cells and wild-type recipients showed that a higher dose (about 10-fold) of donor cells is required to out-compete the endogenous HSCs. These data clearly show that proliferation of *GATA2*$^{+/-}$ HSCs is less deficient, when compared to wild-type HSCs (**Chapter II**).

Interestingly, flow cytometric analysis showed that *GATA2*$^{+/-}$ bone marrow HSCs are phenotypically more quiescent, which explains the proliferation defects in competitive transplantation experiments, this predicts a delayed response to proliferation stress. When *GATA2*$^{+/-}$ mice were challenged by the cytotoxic drug 5-FU, which specifically targets cycling cells, regeneration of the hematopoietic system in the *GATA2*$^{+/-}$ mutants was found to be delayed. The recoveries of all progenitors studied in the *GATA2*$^{+/-}$ mutants indeed were decreased at various time points after 5-FU treatment, but *GATA2*$^{+/-}$ bone marrow cells were able to fully recover when given sufficient time (**Chapter II**). In conclusion, the GATA2 gene dosage affects HSC proliferation during regeneration of the lympho/hematopoietic system, most likely by regulating HSC cell cycle entry.

Identification of GATA3 targets at the double positive T cell stage

Like GATA2 deficiency, deletion of GATA3 expression results in early embryonic death [3], making it impossible to investigate the role of GATA3 during T cell development *in vivo*. However, complex GATA3 gene targeting and T cell specific conditional GATA3 gene deletion approaches have proven the essential role of GATA3 in early T cell commitment [4-6], TCRβ selection [7] and survival or development of CD4 single positive (SP) [7] thymocytes.

To investigate the function of GATA3 during T cell differentiation in the thymus in greater detail, T cell specific CD2-GATA3 transgenic mice were generated. Enforced GATA3 expression in these mice resulted in an inhibition of CD8 T cell maturation, double positive (DP) cell size increase and in the induction of DP thymic lymphoma [8]. To further examine the role of GATA3 in CD4/CD8 lineage commitment, we crossed

the CD2-GATA3 transgenic mice with mice in which the *GATA3* gene is conditionally deleted in the T cell lineage using the CD4-Cre lox system. In this complex cross any differential expression of GATA3 among DP cells taking the CD4 or CD8 pathway was eliminated, because endogenous GATA3 was entirely substituted by transgenic GATA3, which is expressed at equal levels in developing CD4 and CD8 cells. These mice both produced CD4 and CD8. Therefore we conclude that GATA3 expression levels are not decisive for determination of CD4/CD8 lineage choice (**Chapter V**).

Although, *in vivo* studies provided more knowledge about the role of GATA3 during T cell development, GATA3 targets that can explain the GATA3 function during T cell development are largely unknown. For this reason, we analyzed the effects of enforced GATA3 expression in a gene expression profiling study, comparing wild-type and CD2-GATA3 transgenic mice. Although the CD2-GATA3 transgene is already expressed at double negative (DN) T cell stage, the DP T cell stage was chosen as the prime stage to identify possible GATA3 targets. At DP T cell stage TCRα gene segments are recombined by the V(D)J recombination machinery. Successful rearrangement results in the expression of a TCR on the cell surface. At this point the activity of the V(D)J recombination machinery is reduced and GATA3 expression is up-regulated and remains high during CD4 positive selection and declines during CD8 positive selection in the thymus [5]. Importantly, expression of GATA3 is specifically induced at the DP stage upon TCR signaling in the context of major histocompatibility complex (MHC) class II [9], whereas TCR signaling in the context of MHC class I is not associated with GATA3 expression.

To identify GATA3 targets, gene expression profiles were generated from sorted wild-type and CD2-GATA3 transgenic DP cells. To specifically identify targets of GATA3 in the context of CD4 positive selection, gene expression profiles were analyzed of wild-type and CD2-GATA3 transgenic DP cells harboring the MHC class II restricted DO11.10 TCR transgene (**Chapter III**). We found that as a result of enforced GATA3 expression, recombination activating gene 1 (Rag1), Rag2 and terminal deoxynucleoti dyltransferase (TdT) and various genes involved in signal transduction were reduced. Upon successful V(D)J recombination at the TCRα locus leading to positive selection, the recombination machinery has to be down-regulated to prevent ongoing gene rearrangement. During positive selection towards the CD4 lineage TCR expression is up-regulated. The concomitant GATA3-mediated down-regulation of the expression of various signaling molecules may be required to limit signal strength in the process of positive selection. In this context, GATA3 binding sites have been identified in the promoter region murine Rag2 [10] and in human Rag1 (Taghon *et al*., unpublished observations). Mutation of GATA3 binding sites in the murine Rag2 promoter resulted in the reduction of promoter activity in T cells, indicating that GATA3 has the capacity to act as a critical direct positive regulator of the V(D)J recombination machinery (Figure 1A). GATA3 may additionally function as an indirect negative regulator, e.g. by controlling expression of GADD45 or Foxo1, which have recently been shown to be involved in regulating Rag expression in the B cell lineage [11].

The process of positive selection at the DP stage is associated with increased rates of proliferation and survival. Enforced GATA3 expression resulted in differential

expression of crucial regulators of these processes, like c-Myc, Cyclin D2, Mad4 and the Cop9 signalosome complex subunit 2. In line with this, in the presence of the GATA3 transgene, genes encoding factors involved in cellular metabolism were increased, such as Vldl receptor, thrombospondin-2, alpha-actinin-2, collagen alpha 2(I) and myosin IF. When DP T cells are activated via TCR signalling in the context of MHC class II, GATA3 expression is induced [9]. Regulators of TCR signalling, such as cytotoxic T lymphocyte antigen 4 (Ctla4) [12] were found to be affected by enforced GATA3 expression at the DP T cell stage and may be involved in a negative feedback-mechanism regulating TCR signalling (Figure 1A). As an effect of enforced GATA3 expression, Notch1 expression was slightly reduced. Interestingly, we observed up-regulated expression of the peptidyl-prolyl-isomerase Pin1, a negative regulator of intracellular Notch1 levels [13]. Up-regulated gene expression levels of B cell translocation gene 1 (Btg1) and CCR4-associated factor 1 (Caf1) were identified as a result of GATA3 expression. Both Btg1 as Caf1 have been shown to be involved in the negative regulation estrogen receptor α (ERα) expression [14]. In breast cancer, GATA3 has been shown to directly regulate the expression of ER expression and ERα directly regulates the expression of GATA3, indicating that these two factors are involved in a positive cross-regulatory loop [15]. Taken together, we conclude that GATA3 might regulate ERα in two independent pathways: indirectly via the down-regulation of Btg1 and Caf1 and directly via induction of ERα gene expression (Figure 1B).

Together, gene expression profiling revealed new putative GATA3 target genes at the DP T cell stage. These targets may explain the effects of enforced GATA3 expression on proliferation and survival associated with the process of positive selection, especially towards the CD4 T cell lineage. However, the identified targets cannot obviously be linked to induction of positive selection towards the CD4 lineage, which is consistent with the notion that GATA3 expression is insufficient to induce positive selection and commitment to the CD4 cell lineage [7, 8].

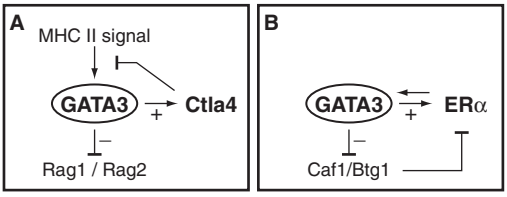

Figure 1. GATA3 act as a negative regulator of V(D)J recombination and positively regulates the expression of ERα. (A) GATA3 is induced upon TCR signaling in the context of an MHC class II signal, leading to a negative regulation of RAG1 and RAG2, which are critical factors involved in V(D)J recombination. TCR signaling on its turn might be regulated by GATA3 in a negative feedback-mechanism, by inducing the expression of Ctla4. (B) GATA3 acts as positive regulator of ERα by two separate pathways: directly by inducing the transcription of ERα and indirectly by reducing the expression of Caf1 and Btg1, which are negative regulators of ERα.

Interactions of GATA3, c-Myc and Notch1 during T cell development and lymphomagenesis

The induction of lymphomas in CD2-GATA3 transgenic mice appeared to be independent of the processes of positive and negative selection in the thymus. However, particularly during positive selection towards the CD4 lineage, enforced GATA3 expression resulted in increased DP cell size and dramatically induced the rate of lymphoma induction (**Chapter IV**).

The identification of GATA3 targets in non-malignant and malignant DP cells revealed the oncogene and lymphocyte cell size regulator, c-Myc, as an important GATA3 target for the induction of DP lymphomas in CD2-GATA3 transgenic mice (**Chapter III and IV**). Additional evidence for the interaction of GATA3 and c-Myc during T cell development came from the finding that *GATA3*$^{-/-}$ TCRβ-expressing DN3 cells cannot increase their cell size during β-selection [7, 8] (**Chapter I**). Enforced GATA3 expression in *Rag2*$^{-/-}$ mice resulted in up-regulated expression levels of c-Myc at the DN stage (van Hamburg *et al*., unpublished). The interaction of GATA3 and c-Myc appeared to be context-dependent, as c-Myc expression levels were unaffected by the presence of the CD2-GATA3 transgene in *in vitro* cultured Th2 cells (van Hamburg *et al*., unpublished).

The finding of up-regulated expression of Mad4, a repressor of c-Myc [16], suggest sthat GATA3 is both involved in a positive regulation of c-Myc expression and in a negative feedback mechanism regulating the expression of c-Myc, via Mad4 (Figure 2A).

Taken together, these findings make it attractive to hypothesize that c-Myc is an important downstream target of GATA3 at the DN and DP stage of T cell development, whereby GATA3 is induced after pre-TCR and TCR signaling (Figure 2A). This hypothesis is further supported by striking functional parallels between GATA3 and c-Myc during T cell development (**Chapter IV**).

Intriguingly, it was recently reported that Notch1, which signaling pathway plays a critical role in T cell development and in the pathogenesis of human T cell lymphoblastic leukemia [17-20], has the capacity to directly regulate c-Myc gene expression. It appears that Notch1 and c-Myc govern two directly interconnected transcriptional programs containing common target genes that together promote leukemic cell growth [21, 22]. As we did not find evidence for activation of Notch1 signaling in non-transformed CD2-GATA3 transgenic DP cells, we conclude that up-regulated c-Myc expression in these cells is independent of Notch1. In contrast, we found that enforced GATA3 expression was associated with modestly reduced Notch1 expression. The slight reduction of Notch1 gene expression by enforced GATA3 expression might indicate a negative feedback-mechanism, whereby GATA3 regulates the expression of Notch1. In this context, we found that enforced GATA3 expression resulted in up-regulation of Pin1 expression. Pin1 is an important regulator of cell proliferation and differentiation in the thymus, and acts as a negative regulator of intracellular Notch1 levels [13] (Figure 2B). GATA3 has been recently identified as a direct target of Notch1 during Th2 differentiation [23-25] and it is very well possible that Notch1 acts upstream of GATA3 in DP cells as well (Figure 2B).

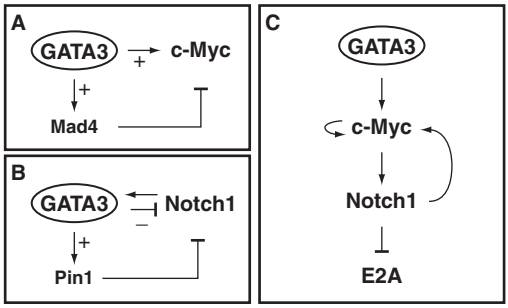

Figure 2. Interactions involving GATA3, c-Myc and Notch1 during CD4 positive selection in the thymus and lymphomagenesis. (A) GATA3 acts as positive regulator of c-Myc at the DP stage of T cell development in the thymus. At the same time, GATA3 might be involved in the negative regulation of c-Myc expression, via the induction of Mad4m a negative regulator of c-Myc. (B) Upon TCR signaling in the context of an MHC class II signal, GATA3 may serve as a negative regulator of Notch1 signaling at the level of Notch1 gene expression and via the induction of Pin1 expression, which acts as an inhibitor of intracellular Notch1 protein levels. (C) GATA3, c-Myc and Notch1 cooperate in the induction of thymic lymphoma. Secondary events, like a trisomy of chromosome 15 harboring the c-Myc gene, activating Notch1 mutations and reduction of E2A gene expression are involved in malignant transformation of CD-GATA3 transgenic thymocytes.

More evidence for a link with GATA3 and Notch includes the finding that the impairment of T lymphopoiesis in *GATA3*$^{-/-}$ hematopoietic progenitor cells was rescued only by introduction of both GATA3 and the intracellular region of Notch1, but not by either alone [26]. In addition, activation of the Notch1 pathway controls lineage commitment of early thymic precursors by altering the levels between GATA3 and Spi-B, which is an Ets family member transcription factor controlling plasmacytoid dendritic cell development [27]. Moreover, GATA2 is regulated by Notch1 in the generation of HSCs in the developing embryo [28-30].

In line with this we found that enforced GATA3 expression in *Rag2*$^{-/-}$ mice resulted in decreased Notch1 expression at the DN stage (van Hamburg *et al.*, unpublished). A function of GATA3 as a negative regulator of Notch1 signalling would be supported by transcriptome analysis of laser-dissected *GATA3*$^{-/-}$ hair follicles, which revealed overrepresentation of the Notch signaling pathway [31]. Interestingly, the phenotype of Notch1 transgenic mice demonstrated that Notch1 activity inhibits the DP to SP transition by abrogating TCR signaling [32], indicating that termination of Notch1 signaling is essential for the generation of SP cells. Our microarray data support an important role for GATA3 in the down-regulation of Notch1 signaling. GATA3-deficiency inhibits the DN3 to DN4 transition and both GATA3-deficiency and expression of activated Notch inhibit the DP to SP transition. From these findings, we propose that GATA3 is essential for termination of Notch signaling in developing DN and CD4 SP thymocytes, leading to reduced activity of the V(D)J recombination machinery and increased rates of survival and proliferation.

Taken together, the following picture emerges with regard to the primary oncogenic potential of GATA3 might be the induction of c-Myc expression, resulting in a pre-malignant stage. Secondary events, including trisomy 15 and activation of Notch signaling cooperate to establish malignant transformation (Figure 2C). Activated Notch1 signaling in CD2-GATA3 transgenic lymphoma could be responsible for down-regulating E2A, resulting in an increased survival and proliferation [33, 34] (Figure 2C). Interestingly, the relatively low GATA3 expression observed in the resulting lymphomas masks the primary role of GATA3 overexpression in lymphomagenesis in our CD2-GATA3 lymphoma model. This may have implications for human cancers, such as T-ALL and breast cancer, in which GATA3 may also cooperate in transformation with c-Myc and Notch1.

GATA3 controls CD5 and TCR expression in the CD4 lineage

In *GATA3*$^{-/-}$ DP cells, the proximal components of TCR signaling for positive selection, such as phosphorylation of LCK and ZAP70 appear to be unaffected [7]. However, in the absence of GATA3, TCR signaling does not induce cell size increase or CD69 and TCR up-regulation. Conversely, in the CD2-GATA3 transgenic mice we identified a small increase in DP cell size and TCR$\alpha\beta$/CD3 expression levels in CD69^{+} DP cells, suggesting that enforced GATA3 expression may influence the kinetics of positive selection [8]. Enforced expression of GATA3 is associated with decreased levels of CD5 at the DP stage, while in the absence of GATA3, CD5 levels are slightly elevated, when compared to wild-type mice. CD5 expression is first induced at the early DN stage, up-regulated by pre-TCR signaling and increases progressively as T cells develop from the DN to the DP stage in a signal dose-dependent manner [35]. CD5 is a negative regulator of TCR signaling and thus participates in the fine-tuning of the TCR repertoire [36]. Taken together, GATA3 has the ability to down-regulate CD5 and to up-regulate TCR expression in developing CD4 T cell lineage cells (**Chapter V**).

As GATA3 expression is induced by TCR signaling, these findings implicate GATA3 as a key regulator in a positive feedback loop (Figure 3). Induction of GATA3 by TCR signaling will increase TCR expression and thereby enhance the induction of GATA3. Moreover, GATA3 has the ability to increase TCR signal strength in an independent parallel pathway by reducing CD5, leading to an efficient mode of signal amplification in this positive regulatory loop. As a result, during development of DP to CD4 SP cells the expression levels of the TCR and GATA3 will increase (Figure 3).

It has been shown that basic helix-loop-helix (bHLH) transcription factor E2A is a negative regulator of CD5 expression during T cell development [37]. Although E2A gene transcription was not increased as a result of enforced GATA3 expression in DP T cells (**Chapter III and IV**), we found increased levels of E2A protein expression (**Chapter V**). It is possible that GATA3 acts via E2A protein stabilization. In this context it is interesting that GATA3 expression results in reduced expression of the transcription factor ID3 (**Chapter III**), which is an important regulator for thymocyte selection, via the regulation of E2A [38]. This might indicate a role for GATA3 in down-regulating CD5 via ID3 during thymocyte selection.

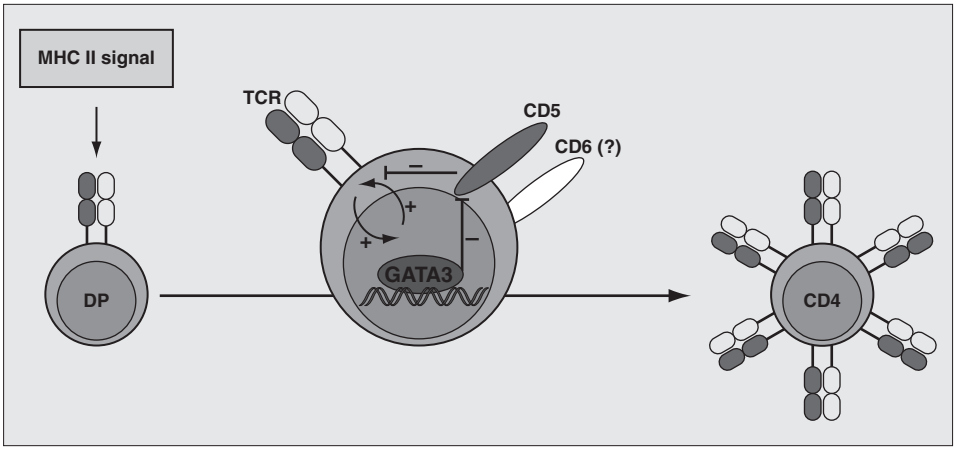

Figure 3. Schematic overview of the control of CD5 and TCR expression by GATA3 during CD4 selection. Upon TCR signaling in the context of an MHC class II signal, GATA3 expression is induced. On its turn GATA3 establishes a positive feedback loop that increases TCR surface expression in developing CD4 lineage cells. In this context, GATA3 acts as a negative regulator of CD5 and CD6 expression, resulting in increased TCR signal strength. (See Appendix page 221 for a full-color representation of this figure).

Enforced GATA3 expression was associated with an increased rate of apoptosis in DP T cells. As CD5 acts as a negative regulator of TCR-mediated signal transduction [36, 39], reduction of CD5 expression levels in CD2-GATA3 transgenic DP cells is expected to increase TCR signal strength, which will result in negative selection and thus deletion. However, it cannot be excluded that the impaired thymocyte expansion and the partial developmental arrest found in thymocytes with premature TCR$\alpha\beta$ expression and signaling [40] or increased c-Myc levels, may also contribute to the observed reduction in the DP cell population in CD2-GATA3:DO11.10 double transgenic mice (**Chapter V**). The CD5 gene is located in the vicinity of CD6 gene on chromosome 19 (Figure 4A). CD5 and CD6 are closely related lymphocyte surface receptors of the scavenger receptor cysteine-rich superfamily. They are both expressed on thymocytes, mature T cells, and B1a cells and the expression of CD5 and CD6 in T cells limits TCR responsiveness [35, 36, 41-43]. However, in mature T cells the engagement by the physiological ligand of CD6 (CD166) leads to co-stimulation, by activation of Slp76 [42]. Out of our gene expression profiling study, we found that the expression of CD5 was strongly correlated with CD6 and that both CD5 and CD6 were inversely correlated with the expression of GATA3 (Figure 4B). This may indicate that in DP thymocytes GATA3 acts as a negative regulator for both CD5 and CD6, resulting in induced TCR responsiveness, which influences the process of DP selection (see Figure 3).

It may be that GATA3 directly regulates TCRα and -β transcription, as binding sites have been identified in these loci [44, 45]. As GATA3 expression is not induced in the CD8 cell lineage [9], different nuclear factors should be responsible for the regulation of the expression level of CD5 and TCR molecules in the CD8 lineage .

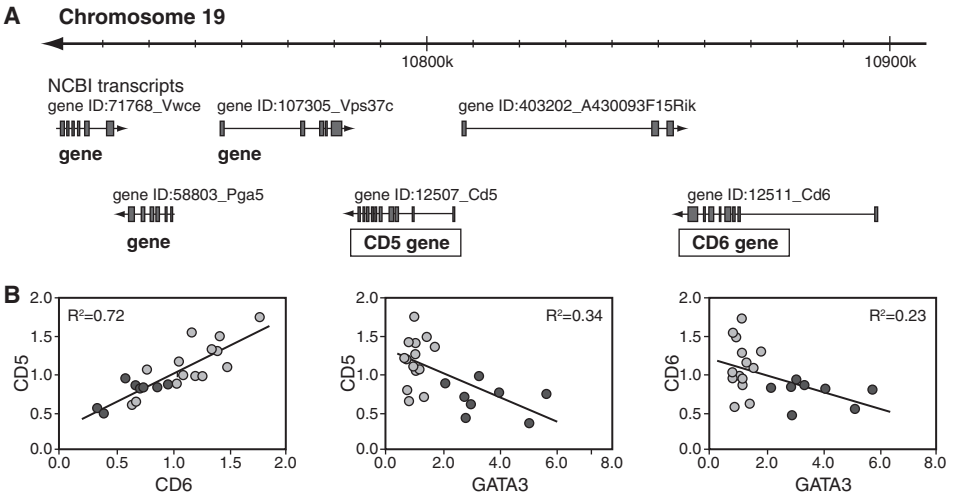

Figure 4. GATA3 acts as a negative regulator of both CD5 and CD6. (A) CD5 and CD6 are closely related lymphocyte surface receptors, involved in the negative regulation of TCR signaling. The gene encoding CD5 is located in the neighborhood of CD6 on chromosome 19 (source: www.emsembl.org). (B) Correlating gene expression levels of CD5 and CD6 were found at the DP stage. Both CD5 and CD6 expression were inversely correlated with GATA3 gene expression levels, indicating that both CD5 and CD6 are reduced by GATA3, resulting in increased TCR receptor strength. Gray dots represent the relative gene expression levels of the indicated genes in wild-type and DO11.10 transgenic sorted DP cells and black dots represent the relative gene expression levels of CD2-GATA3 and CD-GATA3:DO11.10 transgenic sorted DP cells.

GATA3 and T helper cell differentiation

The transcription factor GATA3 acts as a master regulator of Th2 differentiation and Th2 cytokine expression (see **Chapter I**, Figure 5). The molecular mechanisms of counter-regulation, by which GATA3 affects the differentiation of naïve CD4 T cells towards various effector T cell lineages, were investigated by *in vitro* culture studies (**Chapter VI**). These experiments confirmed observations by various groups, that GATA3 supports Th2 development and inhibits Th1 development. In addition, the finding that GATA3 expression is sufficient to induce Th2 cytokine expression, even under Th1 culture condition when the cells express the T-box transcription factor (T-bet), indicate that T-bet is apparently unable to counteract GATA3. Nevertheless T-bet is able to support Th1 differentiation in the presence of GATA3.

We were interested in the effects of GATA3 on differentiation of the novel Interleukin-17 (IL-17) producing Th17 subset. Enforced GATA3 expression allowed for the expression of IL-17 and unique IL-4/IL-17 and IL-13/IL-17 co-expressing cells were observed. In addition, expression of IL-10, recently shown to restrain Th17 mediated pathology [46-48], was slightly enhanced. Furthermore, GATA3 had the capacity to inhibit the expression of IL-2, a negative regulator of IL-17 differentiation [49]. The reduced IL-2 production, even in Th1 or Th17 cultures in the presence of anti-IL-4

antibodies, indicates that GATA3 directly regulates IL-2 through an IL-4-independent mechanism (**Chapter VI**).

Gene expression analysis indicates that enforced GATA3 expression is associated with the reduced expression of Th17 associated transcription factors retinoid related orphan receptor-γt (RORγt) and signal transducer and activator of transcription 3 (STAT3) [50, 51]. Beside these inhibitory effects, GATA3 expression stimulated Th17 effects as well. The transcription factor STAT1, known to suppress Th17 differentiation via IL-27 signaling [52] and of suppressor of cytokine signaling 3 (SOCS3), which acts as a negative regulator of STAT3 phosphorylation [51] were both down-regulated as a result of GATA3 expression. Interferon regulatory factor 4 (IRF4), another factor which is essential for Th17 differentiation [53], was not affected by enforced GATA3 expression. Expression of the nuclear factor of activated T cells-c2 (NFATc2) was reduced as a result of GATA3 expression (**Chapter VI**). So far, NFATc2 has not been implicated in Th17 differentiation. However, the interesting finding that NFATc2 has the potential to stimulate IL-17 and IL-21 and the finding that NFATc2 is induced by IL-6 [54-56], points towards a role of NFATc2 in early Th17 differentiation. It was shown that NFATc2 can interact with IRF4 on the protein level, in the modulation of IL-4 expression [57]. A speculative hypothesis would be that the NFATc2 could serve as the missing IRF4 interacting factor in Th17 differentiation.

All together GATA3 has various stimulatory and inhibitory effects on Th17 differentiation; enforced GATA3 expression still allows the generation of IL-17 producing cells *in vitro*.

GATA3 and Th17 mediated pathology *in vivo*

The **Chapters VI and VII** describe the suppressive capacity of enforced GATA3 expression on paralysis during experimental autoimmune encephalomyelitis (EAE) and severe joint inflammation during antigen induced arthritis (AIA), which is associated with reduced Th17 mediated pathology. In gut-draining tissue and in lymph nodes from CD2-GATA3 transgenic mice, normal numbers of IL-17 producing cells were found. Because of the observed effects of GATA3 on the expression of Th17 associated genes, we investigated whether GATA3 affected Th17 mediated-pathology *in vivo* (**Chapter VI**).

It appeared that CD2-GATA3 transgenic mice were not susceptible to the induction of EAE. However, no differences in the expression of IL-17 by peripheral CD4 T cells between CD2-GATA3 transgenic and wild-type mice were observed. On the other hand, increased IL-4 and IL-10 cytokine expression was observed in CD2-GATA3 transgenic peripheral CD4 T cells. Gene expression of the Th17-associated transcription factors RORγt and STAT3 were reduced in splenic effector CD4 T cells (**Chapter VI**). These findings suggest that GATA3 mainly acts as an inhibitor of Th17-mediated pathology and not by IL-17 expression alone, possibly by driving IL-4 and IL-10 production, or by direct inhibition of Th17 associated factors. In this context, we found that in the presence of neutralizing antibodies to IL-10, EAE can be induced in CD2-GATA3 transgenic mice, albeit with an incidence and severity that is lower than in wild-type mice (van Hamburg *et al*., unpublished).

The finding that in CD2-GATA3 transgenic mice immunization with myelin oligodendrocyte glycoprotein (MOG) results in IL-17 production by CD4 T cells, from the spleen and draining lymph nodes, while these mice nevertheless do not develop EAE, indicate that IL-17 expression alone is not sufficient to induce this disease. Controversy remains about the role of Th17 cells as the central players of pathogenic mediators in autoimmune diseases (reviewed by Steinman *et al*., 58). It has been argued that EAE may also have a Th1 component, but our EAE experiments did not provide evidence for Th1 mediated pathology, as we found low percentages of IFNγ expressing CD4 cells in draining lymph nodes and spleen that were similar in wild-type and CD2-GATA3 transgenic mice.

Besides the EAE model we used the experimental mouse model for AIA to investigate the effect of GATA3 expression on Th17 mediated pathology. Compared to wild-type mice, CD2-GATA3 transgenic mice were protected against severe joint inflammation and bone erosion in the AIA model. In contrast to our findings in EAE, the induction of AIA in CD2-GATA3 transgenic mice resulted in reduced numbers of IL-17 expressing cells in the spleen, lymph nodes and especially at the site of inflammation in the joints. The reduction of IL-17 expression was further associated with increased IL-4, IL-5 and IL-13 and reduced IL-2 cytokine expression. At the transcriptional level we found reduced gene expression of RORγt in effector CD4 T cells as a result of enforced GATA3 expression (**Chapter VII**).

It has been described that IL-4 has a suppressive potential on destructive arthritis and that IL-4 can inhibit differentiation of Th17 cells [59]. However, administration of neutralizing IL-4 antibodies to CD2-GATA3 transgenic mice during the induction of AIA did not increase joint inflammation or IL-17 production (van Hamburg *et al*., unpublished). Although we cannot exclude that not all IL-4 is neutralized, these data suggest that the protection of severe arthritis and Th17 expansion by the CD2-GATA3 transgene may not be fully dependent on IL-4 (**Chapter VII**). While CD2-GATA3 transgenic mice were not susceptible to induction of EAE and AIA, lack of AIA induction in CD2-GATA3 transgenic mice was associated with a reduction of Th17 differentiation. The mechanisms underlying this discrepancy are unclear. Some differences were observed in the effects of enforced GATA3 expression, when comparing the EAE and AIA models. It appeared that in the EAE model, IL-10 expression, which has been shown to restrain Th17 mediated pathology [46-48], was induced as a result of enforced GATA3 expression, while hardly any IL-10 expression was observed in the AIA model. Another point could be the different kinetics of Th17 differentiation in EAE and AIA. In the AIA model mice were immunized with antigen and at 21 days a second injection of antigen was provided, leading to a strong T cell mediated response [60]. EAE was induced by immunization with antigen and after ~10-12 days, clinical disease symptoms were observed. It also needs to be taken into account that AIA is mainly T cell mediated, while EAE requires both humoral and cellular immunity [60, 61]. Another explanation for the protection against the induction of EAE in CD2-GATA3 transgenic mice could be an impaired central nervous system (CNS) invading capacity of Th17 cells. This might be unlikely, because during AIA induction, CD2-GATA3 transgenic CD4 T cells did not manifest any defects in homing

towards the inflamed joints. Future experiments should point out whether IL-17 producing T cells are present in the CNS of CD2-GATA3 transgenic mice upon MOG immunization (**Chapter VI and VII**).

The lack of any effect of providing neutralizing IL-4 antibodies during AIA induction suggests that the action of GATA3 is not fully dependent on IL-4 and that there are IL-4 independent mechanisms as well. A putative target for GATA3, in constraining Th17 differentiation and pathology, would be RORγt. In both EAE and AIA *in vivo* models and in *in vitro* Th17 cultures reduced RORγt expression levels were found as a result of GATA3 expression (**Chapter VI and VII**). Micro array analysis indicated that RORγt was slightly reduced in DP thymocytes and lymphomas of CD2-GATA3 transgenic mice (**Chapter III and IV**). Sequence analysis revealed that the ~500 base pair up-stream region of RORγt harbours GATA3 binding sites. Future experiments, such as chromatin immunoprecipitation and transfection assays, should provide information whether RORγt is a direct or indirect target of GATA3.

Concluding remarks

In this thesis we studied the control of the T cell developmental program by GATA family transcription factors. GATA2 is essential for embryonic HSC production and adult HSC function. It also functions to limit the differentiation of thymocytes into non-T lineages. In contrast, GATA3 is implicated in nearly all major fate decisions during T cell development, starting from T cell specification, β-selection, positive selection and even up to the generation of mature effector Th2 cells. GATA factors are crucial regulators of the balance between cellular proliferation and differentiation. Interestingly, GATA3-mediated regulation of cell fate most likely involves the induction of proliferation at the stages of T cell commitment, β-selection or Th2 differentiation, but not during positive selection of CD4 SP cells in the thymus. Thus, the mechanism by which GATA3 regulates cell fate decisions during T cell differentiation may differ between the individual developmental stages. From our studies it became obvious that GATA3 functions in complex gene networks, whereby positive and negative feedback mechanisms and multiple levels of regulation exist.

The various transgenic and (conditional) knock-out mouse models for GATA2 and GATA3 have evolved as valuable tools to gain insight into the *in vivo* functions of these factors, in particular, since the functions of the GATA factors are context-dependent, with clear differences e.g. between fetal and adult hematopoiesis, or between T cell differentiation in the thymus and the activation of peripheral T cells. Moreover, expression levels of the GATA factors are decisive, as is obvious from the phenotypic consequences of reduced or enhanced expression of GATA2 or GATA3. The essential role of GATA factors in the regulation of cell division and differentiation is also underscored by their involvement in the pathogenesis of specific leukemias.

Knowledge on the molecular mechanisms underlying the control of T cell development by GATA factors may facilitate improvement of clinical strategies for the generation of T cells from HSCs, e.g. after of bone marrow or stem cell transplantation. The identification of putative GATA3 targets might provide new targets for future molecular drugs and tumor specific therapies that can replace

current intensive treatments against human cancers, such as T-ALL or breast cancer. Since overexpression of GATA3 is protective for EAE and arthritis, modulation of specific transcription factors, such as GATA3 and RORγt, may lead to new therapeutic applications in the treatment or prevention of autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA).

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SUMMARY

Within the immune system, T lymphocytes play a central role in the defense against pathogens and are mainly generated in the thymus. Each individual T lymphocyte or T cell has its own unique T cell receptor (TCR), which recognizes a specific foreign substance, called antigen. Once a TCR recognizes an antigen, the T cell gets activated, starts to proliferate in a clonal fashion and differentiates towards different types of effector T cells. Besides immediate defense, lifelong protective immunity against the same antigen is assured by the generation of memory T cells.

T cells are derived from hematopoietic stem cells (HSCs) in the adult bone marrow, which differentiate into early T cell progenitors that migrate to the thymus. Within the thymus complex differentiation and selection processes take place to generate T cells that express a functional TCR, which recognizes foreign antigen, but no self antigen. Defects in T cell development and function can result in increased susceptibility to infections and even development of leukemias, allergies and autoimmune diseases. On the other hand, T lymphocytes can be manipulated to eradicate tumor and control graft rejection after organ transplantation. Therefore, in addition to biological interest, knowledge on the molecular mechanisms underlying T cell biology is important for understanding the etiology of a wide variety of diseases and potentially improves current therapies.

Essential proteins in the development of T cells from HSCs towards mature effector T cells are GATA transcription factors, which are crucial regulators of cellular proliferation and differentiation. GATA transcription factors bind to DNA at a consensus DNA sequence (A/T)GATA(A/G), through conserved zinc-finger domains. The mammalian GATA family consists of 6 members, from which GATA1, 2 and 3 are expressed within the hematopoietic system. The expression of GATA proteins is tightly regulated and aberrant expression has been implicated in human diseases, such as specific leukemias.

This thesis describes the research performed to investigate the role of GATA transcription factors in the T cell lineage. For this reason, GATA gene targeting and transgenic approaches were used in mice. One of the GATA factors shown to be essential for early hematopoiesis is GATA2. The fact that *GATA2* deficient murine embryos die before the first HSCs are generated makes it difficult to investigate the effect of GATA2 deficiency on the generation and function of HSCs *in vivo*. Therefore, the effect of reduced GATA2 gene expression on the generation and expansion of HSCs was examined in heterozygous *GATA2*$^{+/-}$ mice (**Chapter II**). From this investigation, it was found that reduced GATA2 expression played a role during the generation of HSCs in both the developing embryo as in adult mice. In *GATA2*$^{+/-}$ mice, the expansion and maintenance of HSCs was affected in the aorta, gonads and mesonephros region, but not in the yolk sac of the developing embryo. In adult mice, GATA2 gene dosage affected the proliferation of HSCs in the bone marrow. This resulted in a delayed regeneration of the hematopoietic system in *GATA2*$^{+/-}$ mice, after treatment with the cytotoxic drug 5-FU.

Another GATA factor important for hematopoietic development is GATA3. Like *GATA2* deficient mice, *GATA3* deficient mice die during early embryonic

development. To investigate the role of GATA3 during T cell development, the effects of T cell specific deletion of GATA3 or enforced transgenic GATA3 expression on T cell development were examined. In previous studies it has been shown that enforced GATA3 expression resulted in the induction of thymic lymphoma, impaired CD8 differentiation and inhibited T helper-1 (Th1) differentiation. In contrast, to Th2 cells, targets of GATA3 in the thymus that can explain these effects are unknown. As the expression of GATA3 is induced at the CD4/CD8 double positive (DP) stage, where it is essential for the generation of CD4 cells, we generated gene expression profiles from DP thymocytes, obtained from CD2-GATA3 transgenic and wild-type mice (**Chapter III**). Comparisons of these gene expression profiles revealed new putative GATA3 target genes involved in cell size regulation, lymphomagenesis and CD4 selection, including genes involved in the V(D)J-recombination machinery, like Rag1, Rag2 and TdT and of various signal transduction proteins. Furthermore, GATA3 was found to regulate key genes involved in cellular proliferation and survival, including Mad4, c-Myc, Cop9, Caf1 and Btg1. Moreover, GATA3 induces the intracellular Notch inhibitor Pin1 and downregulates Notch1 transcription. As both GATA3-deficiency and expression of activated Notch inhibit the DP to SP transition, we propose that GATA3 is essential for termination of Notch signaling in developing CD4 SP thymocytes.

In GATA3 induced lymphomas, high gene expression levels were found of the oncogene and cell size regulator c-Myc, which might explain the observed cell size increase in pre-malignant CD2-GATA3 transgenic DP cells (**Chapter IV**). Furthermore, secondary events, such as trisomy of chromosome 15 harboring the gene encoding c-Myc, activating Notch1 mutations and reduced E2A levels were found in the lymphomas. Therefore we conclude that GATA3 cooperates with c-Myc and Notch1 in the malignant transformation of DP thymoctytes. These findings may have implications for human cancers in which GATA3 is expressed, such as T cell acute lymphoblastic leukemia (T-ALL) and breast cancer.

Another identified GATA3 target was CD5, which functions as a negative regulator of TCR signaling (**Chapter V**). T cell specific deletion of GATA3 resulted in increased cell surface expression of CD5. In contrast, enforced GATA3 expression during CD4 selection resulted in decreased expression of CD5, while TCR surface expression was enhanced. It has been shown that GATA3 expression is induced upon TCR signaling, specifically in the context of MHC class II, during the process of positive selection towards the CD4 lineage. From these findings, we concluded that GATA3 establishes a positive feedback loop that increases TCR surface expression during CD4 selection.

Upon antigenic stimulation naive CD4 SP T cells differentiate into effector T cells, which are classically divided into two functionally distinct subsets, termed T helper-1 (Th1) and T helper-2 (Th2). Th1 cells, which produce IFNγ, are associated with the elimination of intracellular pathogens. Th2 cells, which produce IL-4, Il-5 and IL-13, are critically important for the eradication of parasitic worms, but are also implicated in allergic responses. More recently, new lineages of effector CD4 T cells have been identified, including $CD25^+$ regulatory T (Treg) cells and a helper T cell subset producing IL-17 and for this reason termed Th17. Treg cells are involved in

the suppression of effector T cell function and are essential for tolerance against self-antigens. Th17 cells are implicated in autoimmune diseases, like multiple sclerosis (MS) and rheumatoid arthritis (RA).

Differentiation towards individual T cell subsets and their maintenance is critically dependent on specific transcription factors, such as T-bet for Th1, GATA3 for Th2 and FoxP3 for Treg cells. The transcription factor RORγt was identified as the regulator of Th17 cells. GATA3 has been shown to directly inhibit the expression of T-bet and FoxP3. The effects of GATA3 on Th17 cells, are unknown. When naïve CD2-GATA3 transgenic CD4 cells were cultured under Th17 polarizing conditions we found that enforced GATA3 expression allowed for the differentiation of IL-17 producing CD4 cells (**Chapter VI**). Although IL-17 expression was not affected *in vivo*, GATA3 transgenic mice were not susceptible for the induction of experimental autoimmune encephalomyelitis (EAE), a mouse model for MS. The induced expression of cytokines, such as IL-10 (which has been shown to inhibit Th17 effector function) and the reduction of the expression of Th17 associated factors, such as RORγt, might explain the effects of GATA3 on Th17 mediated pathology.

Next to EAE, the effects of enforced GATA3 expression were investigated in antigen induced arthritis, a mouse model for rheumatoid arthritis (RA) (**Chapter VII**). During the induction of arthritis, enforced expression of GATA3 was protective against joint inflammation and bone erosion. This protection was associated with reduced expression of IL-17, but not of IFNγ, by both peripheral and joint infiltrating CD4 cells. Furthermore Th2 cytokines, such as IL-4 were induced. Although, it has been shown that IL-4 is protective against arthritis. Treatment with neutralizing IL-4 antibodies was not sufficient to induce arthritis in CD2-GATA3 transgenic mice.

The research described in this thesis provides more knowledge underlying the molecular mechanisms involved in the function of GATA factors in the T cell lineage. This might have implications for improving clinical strategies to generate T cells from HSCs, particular in the context of bone marrow or stem cell transplantation. Moreover, the identification of putative GATA3 targets provides new targets for future molecular drugs and tumor specific therapies that can replace current intensive treatments against human cancers, such as T-ALL or breast cancer. Since overexpression of GATA3, is protective for EAE and arthritis, modulation of specific transcription factors, such as GATA3 and RORγt, might lead to new therapeutic applications in the treatment or prevention of autoimmune diseases, such as MS and RA.

SAMENVATTING

T-lymfocyten spelen een centrale rol in de verdediging tegen ziektekiemen en worden hoofdzakelijk gevormd in de thymus. Elke individuele T-lymfocyt of T-cel heeft een unieke T-cel receptor (TCR), die specifiek gericht is tegen een lichaamsvreemde stof, ook wel antigeen genoemd. Wanneer een TCR een antigeen herkent, dan zal de T-cel geactiveerd raken, gaan delen en vervolgens differentiëren tot een effector T-cel. Door de aanmaak van geheugen T-cellen wordt er gezorgd dat er naast een directe verdediging tegen ziektekiemen, een levenslange immuniteit opgebouwd kan worden tegen een specifiek antigeen.

T-cellen ontstaan uit hematopoëtische stamcellen (HSC) in het beenmerg. Deze cellen differentiëren tot vroege voorloper T-cellen die naar de thymus migreren. In de thymus vinden vervolgens complexe differentiatie- en selectieprocessen plaats om T-cellen te genereren die een functionele TCR tot expressie brengen. Deze TCR dient wel een lichaamsvreemde, maar geen lichaamseigen antigenen te herkennen. Fouten of gebreken in de ontwikkeling of de functie van T-cellen kunnen resulteren in een verhoogde kans op infecties en zelfs in het ontstaan van leukemie, allergie of auto-immuniteit. Aan de andere kant kunnen T-cellen gemanipuleerd worden om afstoting na orgaantransplantatie tegen te gaan en om tumoren te herkennen en te verwijderen. Om deze redenen is het belangrijk om een beter inzicht te verkrijgen in de moleculaire mechanismen die ten grondslag liggen aan de ontwikkeling van T-cellen uit HSC naar rijpe effector T-cellen. Hierdoor kan het ontstaan van verschillende ziekten beter begrepen worden en kunnen bestaande therapieën verbeterd worden.

Essentiële eiwitten in de ontwikkeling van T-cellen uit HSC naar effector T-cellen zijn GATA transcriptie factoren, die een cruciale rol vervullen in de regulatie van celdeling en differentiatie. GATA transcriptie factoren binden aan de DNA sequentie (A/T)GATA(A/G), door middel van evolutionair geconserveerde zink-vinger domeinen. Bij de mens en de muis zijn er 6 verschillende GATA factoren bekend, waarvan GATA1, -2 en -3 tijdens de aanmaak van bloedcellen, ook wel hematopoëse genoemd, tot expressie komen. De expressie van GATA factoren is nauwkeurig gereguleerd en afwijkende expressie is in relatie gebracht met humane ziekten, zoals bepaalde leukemieën.

Dit proefschrift beschrijft het uitgevoerde onderzoek naar de rol van GATA transcriptie factoren in de T-cel en hun ontwikkeling. Voor dit onderzoek zijn in muizen specifieke GATA genen uitgeschakeld of GATA factoren versterkt tot expressie gebracht. GATA2 is een van de GATA factoren, waarvan bekend is dat het een essentiële rol speelt tijdens de vroege hematopoëse. *GATA2* deficiëntie bij muizen is lethaal tijdens de vroege embryonale ontwikkeling, vóór het moment dat de eerste HSC gevormd zijn. Dit bemoeilijkt het onderzoek naar het effect van GATA2 in de ontwikkeling en de functie van HSC *in vivo*. Om deze reden is het effect van verlaagde GATA2 genexpressie onderzocht op de ontwikkeling en expansie van HSC, tijdens zowel de embryonale ontwikkeling als in volwassen heterozygote *GATA2*$^{+/-}$ muizen (**Hoofdstuk II**). De expansie en handhaving van HSC bleek in *GATA2*$^{+/-}$ muizen negatief beïnvloed te zijn in de aorta-gonads-mesonephros (AGM) regio, maar niet

in dooierzak van het ontwikkellende embryo. Verlaagde genexpressie van GATA2 had een negatieve invloed op de celdeling van HSC in het beenmerg van volwassen muizen. Dit resulteerde in een vertraagde regeneratie van het hematopoëtisch systeem in *GATA2*$^{+/-}$ muizen, na behandeling met het cytotoxisch middel 5-FU.

Een andere GATA factor die een belangrijke rol vervult tijdens de hematopoëse is GATA3. Net zoals bij *GATA2* deficiënte muizen, is *GATA3* deficiëntie bij muizen lethaal tijdens de vroege embryonale ontwikkeling. Om de rol van GATA3 gedurende de T-cel ontwikkeling nader te onderzoeken is er gebruik gemaakt van muizen waarin het GATA3 gen specifiek tijdens de T-cel ontwikkeling is uitgeschakeld of waarin GATA3 expressie versterkt is. In het verleden is aangetoond dat een versterkte expressie van GATA3 resulteert in de inductie van leukemie, een verminderde differentiatie van CD8 enkelpositieve (SP) T-cellen en een geremde T-helper-1 (Th1) differentiatie. Met uitzondering van de Th differentiatie, zijn er geen targets van GATA3 bekend die deze effecten kunnen verklaren. Om deze targets op te sporen hebben we het effect van versterkte expressie van GATA3 in CD2-GATA3 transgene muizen op het CD4/CD8 dubbelpositieve (DP) T-cel stadium onderzocht. Dit is gedaan door het vergelijken van het genexpressieprofiel van DP T-cellen van wild-type muizen met het profiel van CD2-GATA3 transgene muizen. Dit onderzoek was gericht op het DP stadium, omdat in de normale T-cel ontwikkeling de expressie van GATA3 hier sterk geïnduceerd wordt en omdat GATA3 op dit stadium een essentiële rol vervult in de ontwikkeling van CD4 T-cellen (**Hoofdstuk III**). Door de vergelijking van deze genexpressie profielen, zijn er nieuwe mogelijke GATA3 targetgenen geïdentificeerd, die betrokken zijn bij de regulatie van celgrootte, de inductie van lymfomen en de selectie van CD4 T-cellen. Hierbij zijn genen gevonden die deel uitmaken van het V(D)J recombinatie systeem, zoals Rag1, Rag2, TdT en genen die coderen voor verscheidene signaleringsmoleculen. Daarnaast vonden we dat GATA3 betrokken was bij transcriptieregulatie van genen die een belangrijke rol spelen in celdeling en celoverleving, zoals Mad4, c-Myc, Cop9, Caf1 en Btg1. GATA3 bleek verder in staat te zijn om in beperkte mate Notch1 transcriptie te remmen en om transcriptie van Pin1, een remmer van Notch1 signalering, te versterken. Omdat zowel *GATA3*-deficiëntie en versterkte expressie van geactiveerd Notch1 een blokkade tot gevolg heeft van de overgang van DP T-cellen naar SP cellen in de thymus, hebben wij de hypothese opgesteld dat GATA3 een essentiële rol vervult in het beëindigen van Notch1 signalering in ontwikkelende CD4 T-cellen in de thymus.

In de GATA3 geïnduceerde lymfomen zijn verhoogde genexpressieniveaus gevonden van c-Myc, een oncogen en regelaar van celgrootte. Dit zou een verklaring kunnen zijn voor de celgrootte toename van CD2-GATA3 transgene DP T-cellen (**Hoofdstuk IV**). Verder vonden we secundaire effecten, zoals een extra kopie van chromosoom 15, waarop het c-Myc gen is gelokaliseerd, activerende Notch1 mutaties en een verlaagde expressie van E2A in de GATA3 geïnduceerde lymfomen. Om deze redenen concludeerden wij dat GATA3 samenwerkt met c-Myc en Notch1, in de inductie van lymfomen in de CD2-GATA3 transgene muizen. Deze bevindingen kunnen van belang zijn voor bepaalde vormen van kanker bij de mens waarbij gevonden is dat GATA3 tot expressie komt, zoals T-cel acute

lymfoblastische leukemie (T-ALL) en borstkanker.

Een andere geïdentificeerde GATA3 target is CD5, dat functioneert als een negatieve regulator van TCR signalering (**Hoofdstuk V**). *GATA3* deficiëntie resulteerde in verhoogde CD5 expressie op de celmembraan. Dit in tegenstelling tot de verlaagde CD5 expressie en verhoogde expressie van de TCR, in aanwezigheid van versterkte GATA3 expressie tijdens CD4 T-cel selectie. Het is aangetoond dat GATA3 expressie wordt geïnduceerd tijdens TCR signalering, specifiek in de context van MCH klasse II, gedurende het proces van positieve selectie van DP T-cellen naar CD4 T-cellen. Uit deze bevindingen concludeerden wij dat GATA3 tijdens CD4 positieve T- cel selectie een positief feedbackmechanisme initieert, resulterende in een versterkte TCR expressie op de celmembraan.

Als gevolg van stimulering door een antigeen, differentiëren naïeve CD4 T-cellen naar effector CD4 T-cellen. Deze cellen werden in het verleden geclassificeerd als twee functioneel verschillende subsets, namelijk Th1 en Th2. Th1 cellen produceren voornamelijk IFNγ en zijn betrokken bij de eliminatie van ziekteverwekkers die zich binnen de cel bevinden. Th2 cellen produceren IL-4, IL-5 en IL-13 en zijn van essentieel belang bij het opruimen van parasitaire wormen en zijn betrokken bij allergische reacties. Recentelijk zijn er nieuwe effector CD4 T-cel subsets geïdentificeerd. Dit zijn de $CD25^+$ regulatoire T (Treg) cellen en een helper T-cel subset die IL-17 kan produceren en om deze reden Th17 is genoemd. Treg cellen zijn betrokken bij de suppressie van de effector T-cel functie en zijn essentieel voor de tolerantie tegen lichaamseigen antigenen. Th17 cellen zijn meer geassocieerd met auto-immuunziekten, zoals multiple sclerosis (MS) en reumatoïde artritis (RA).

De differentiatie en handhaving van de individuele effector T-cel subsets is afhankelijk van specifieke transcriptiefactoren, zoals T-bet voor Th1 cellen, GATA3 voor Th2 cellen en FoxP3 voor Treg cellen. De transcriptiefactor RORγt is geïdentificeerd als de regulator van Th17 cellen. Het is bekend dat GATA3 direct de expressie van T-bet en van FoxP3 negatief kan beïnvloeden. De effecten van GATA3 op Th17 waren echter nog onbekend. Uit *in vitro* experimenten met naïeve CD2-GATA3 transgene CD4 T-cellen, gekweekt onder Th17 polariserende condities, bleek dat de differentiatie van IL-17 producerende cellen mogelijk was (**Hoofdstuk VI**). Ondanks het feit dat de IL-17 expressie niet negatief beïnvloed werd door versterkte GATA3 expressie *in vivo*, vonden we dat GATA3 transgene muizen niet gevoelig waren voor de inductie van experimentele auto-immuun encephalomyelitis (EAE), een muizenmodel voor MS. De geïnduceerde expressie van cytokinen, zoals IL-10 (waarvan bekend is dat het de Th17 effector functie kan remmen) en de verlaagde transcriptie van Th17 geassocieerde factoren, zoals RORγt, zouden het effect van GATA3 op Th17 geassocieerde pathologie kunnen verklaren.

De effecten van versterkte GATA3 expressie zijn ook onderzocht in antigeen geïnduceerde artritis (AIA), een muizen model voor RA (**Hoofdstuk VII**). In dit geval bleek GATA3 beschermend te werken tegen ernstige gewrichtsontstekingen en boterosie. Deze beschermende werking was geassocieerd met een verlaagde expressie van IL-17, maar niet van IFNγ, door zowel perifere als in het gewricht geïnfiltreerde CD4 T-cellen. Daarnaast was er een sterk verhoogde productie van

Th2 cytokinen, zoals IL-4, door CD2-GATA3 transgene CD4 cellen. Het is bekend dat IL-4 beschermend werkt tegen artritis, echter het geven van neutraliserende IL-4 antilichamen, was niet voldoende om artritis te induceren in CD2-GATA3 transgene muizen.

Het onderzoek, beschreven in dit proefschrift, geeft meer inzicht in de moleculaire mechanismen die ten grondslag liggen aan de functie van GATA factoren in T-cellen en in hun ontwikkeling. Dit kan implicaties hebben voor de verbetering van klinische strategieën om T-cellen te genereren uit HSC, met name in de context van beenmerg- of stamceltransplantaties. Bovendien kan de identificatie van mogelijke GATA3 targets, nieuwe targets opleveren voor toekomstige medicijnen en specifieke anti-tumor therapieën. Deze zouden in de plaats kunnen komen voor de huidige intensieve behandeling van kanker, zoals T-ALL en borstkanker. Versterkte expressie van GATA3 was beschermend tegen EAE en AIA. Dit betekent dat de modulatie van specifieke transcriptiefactoren tot nieuwe therapeutische toepassingen zou kunnen leiden in de behandeling of het voorkomen van auto-immuunziekten, zoals MS en RA.

ABBREVIATIONS

AGM	aorta-gonads-mesonephros
AIA	antigen induced arthritis
ALL	acute lymphoblastic leukemia
AMKL	acute megakaryoblastic leukemia
AML	acute myeloid leukemia
APC	allophycocyanin
bHLH	basic-helix-loop-helix
BM	bone marrow
BTG	B cell translocation gene
CAF	CCR4-associated factor 1
CD	cluster of differentiation
CFSE	carboxy fluorescein succinimidyl ester
CFU-S	colony forming unit-spleen
CIA	collagen induced arthritis
CLP	common lymphoid progenitor
CNS	central nervous system
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CTLA	cytotoxic T lymphocyte antigen
DC	dendritic cell
DN	double negative
DP	double positive
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
Ery	erythrocyte
ER	estrogen receptor
ERK	extracellular signal regulated kinase
ES	embryonic stem
ETP	early T lineage progentor
E10.5	embryonic day 10.5
FACS	fluorescent activated cell sorting
FDG	fluorescein-di-β-D-galactopyranoside
FITC	fluorscein isocytothiocynate
FL	fetal liver
FOXP3	forkhead box P3
G	granulocyte
GAPDH	glycereraldehyde-3-phosphate dehydrogenase
GFI	growth factor independent
GFP	green fluorescent protein
HD	heterodimerization domain
HDR	hypopara-thyroidism, sensorineural deafness and renal anomalies
HES	hairy enhancer of split
HSC	hematopoietic stem cell
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IRF	Interferon regulatory factor
ISP	immature single positive
JAK	Janus kinase

LEF	lymphoid enhancer factor
LMO	LIM-only domain protein
LSK	Lin-Sca-1^{hi}c-kit^{hi}
LT-HSC	long term hematopoietic stem cell
HSC	hematopoietic stem cell
M	macrophage
mBSA	methylated bovine serum albumin
MDS	myelodysplastic syndrome
Meg	megakaryocyte
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
MUC	mucin
NFAT	nuclear factor of activated T cells
NK	natural killer cell
NKT	natural killer T cell
NOTCH	neurogenic locus notch homolog
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein
PEST	proline-, glutamic acid-, serine- and threonine-rich domain
PI3K	phosphoinositide 3-kinase
PML	promyelocytic leukemia
P-Sp	para-aortic splanchnopleura
PTEN	phosphatase and tensin homolog
RA	rheumatoid arthritis
RAG	recombination activating gene
RAR	retinoic acid receptor
ROG	repressor of GATA
ROR	retinoid related orphan receptor
RT-PCR	real-time polymerase chain reaction
SKY	spectral karyotyping
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signaling
Sp	splanchnopleura
SP	single positive
STAT	signal transducer and activator of transcription
ST-HSC	short term hematopoietic stem cell
TAL	T cell acute leukemia
T-BET	T-box transcription factor
TCF	T cell factor
TCR	T cell receptor
TdT	terminal deoxynucleotidyltransferase
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor
Treg	regulatory T cell
V(D)J	variable (diversity) joining
WT	wild type
YS	yolk sac
5-FU	5-fluorouracil
7-AAD	7-Amino-Actinomycin D

DANKWOORD

Het is dan eindelijk zover, het proefschrift is af! Het tot stand komen van dit proefschrift kon natuurlijk niet plaatsvinden zonder de hulp en betrokkenheid van een groot aantal collega's, samenwerkingsverbanden, vrienden en familie. Als blijk van dank zou ik graag een aantal mensen persoonlijk willen bedanken.

Als eerste wil ik natuurlijk Rudi bedanken voor de directe begeleiding en ondersteuning van dit project. Beste Rudi, het is alweer een hele tijd geleden dat ik bij je langs kwam met de vraag voor of er een stageplaats in het kader van mijn opleiding aan de Hogeschool Rotterdam, een stageplaats beschikbaar was. Tijdens deze stageperiode, onder de enthousiaste begeleiding van Marie-José Blankwater vanuit de Hogeschool, is de basis ontstaan van een langdurige samenwerking. Tijdens het gehele promotietraject heb je me altijd, desnoods na middernacht via de mail, met raad en daad ter zijde gestaan, zelfs met persoonlijk advies. Ik denk dat jouw enthousiasme en gedrevenheid een voorbeeld mag zijn voor elke wetenschapper. Ik hoop en verwacht dat onze plezierige samenwerking ook in de toekomst voortgezet zal worden.

Dit promotieonderzoek op de afdeling Immunologie was mede mogelijk door de ondersteuning van prof. dr. R. Benner. Beste Rob, ik wil je vanaf deze plaats erg bedanken voor het in mij gestelde vertrouwen en de geboden mogelijkheden tijdens het onderzoek en de afronding van dit proefschrift.

Natuurlijk wil ik ook mijn labgenoten van de groep Rudi bedanken voor de plezierige tijd op het lab en daarbuiten. Marjolein, ik kan me nog herinneren dat ik, in een goede duopresentatie met Esther, op de Hogeschool Utrecht een poster heb gepresenteerd, om toekomstige stagiaires te werven voor het onderzoek op ons lab. Het viel me al direct op dat jij meer dan gemiddelde interesse had in ons onderzoek. Ik denk dat ik van geluk mag spreken dat je jouw stageperiode bij ons hebt doorgebracht en dat je daarna zelfs als analist aan de slag bent gegaan. Volgens mij moet ik maar niet gaan berekenen hoeveel PCR's, sequentieanalyses, T-helperkweken en nog veel meer experimenten door jou zijn uitgevoerd. Zonder deze hulp zou het proefschrift er heel anders hebben uitgezien. Ik ben je erg dankbaar voor deze samenwerking en vind het dan ook bijzonder dat jij mijn paranimf wilt zijn.

Sabine, volgens mij zijn we in dezelfde maand begonnen, jij als AIO en ik als stagiair. Mede door jouw hulp heb ik een goede start kunnen maken op het moment dat ik terugkwam als AIO binnen de groep. Rogier, als T-cel AIO heb ik niet direct met je samengewerkt, maar dat neemt niet weg dat we heel goed met elkaar overweg konden. Al was het zetten van vanillethee op de schrijfkamer toch wel een klein dieptepunt. Gemma, bedankt voor het wegwijs maken in de wereld van de moleculaire technieken en jouw hulp bij experimenten. Ook het opstarten van de micro-array studie is mede door jou erg soepel verlopen. Kam-Wing, I would like to thank you for our nice collaboration on the GATA3 project. I think it's about time that we are going to buy something of our coffee points, which we collected by drinking al these cups of senseo coffee. Perhaps, we can select something that helps us to start up the beer brewing process again. Petra, gelukkig had ik nog een rustpunt in de schrijfkamer

en konden er ook serieuze gesprekken gevoerd worden. Veel succes nog met het opstarten van het onderzoek in Amsterdam. Als je het over een kritisch persoon hebt, die niet te beroerd is om over een schouder mee te kijken, dan heb je het natuurlijk over Pieter Fokko. Het zou zomaar kunnen dat 1 oktober een extra bijzonder tintje krijgt met een geboorte. Veel geluk en plezier gewenst de komende periode. Iemand waarmee altijd een leuk gesprek te voeren valt, is Van. Veel succes met het submitten van de artikelen en met de afronding van jouw promotie. Claudia, trust me, I don't think it will last long anymore until a CTCF article is accepted. And whenever you look back in time after obtaining your PhD, you will notice that everything ended up nicely (even after reaching the peak). De sfeer binnen de groep werd nog eens extra positief beïnvloed door de aanwezigheid van enthousiaste stagiaires, als Esther, Bart, Annemieke en "hoe gaat het" Laurens.

Tijdens het acceptatieproces van een artikel wil het wel eens voorkomen dat een reviewer vraagt om tamelijk ingewikkelde EAE experimenten uit te voeren. Gelukkig kon er in dat geval teruggevallen worden op de expertise binnen de unit Immuunregulatie. Marloes, Marjan, Annet en Jon, jullie wil ik bedanken voor het meedenken en voor de hulp bij de uitvoering van de experimenten, die zelfs in het weekend plaatsvonden. Deze experimenten hadden niet eens plaats kunnen vinden als Louis Boon niet zo vrijgevig was geweest met het verstrekken van antilichamen.

Om achter het mechanisme van het ontstaan van tumoren in de GATA3 transgene muizen te komen, heb ik met veel plezier samengewerkt met Berna Beverloo en Ellen van Drunen om de SKY techniek uit te voeren. Zeker dankzij de enthousiaste begeleiding van Ellen zijn er toch wel erg mooie plaatjes van gekleurde chromosomen gemaakt. Soms kan het zoeken naar specifieke lymfeklieren een hele specialistische opgave zijn. Dankzij de hulp van Janneke Samsom en Colin de Haar, is het toch gelukt om de met het blote oog niet te vinden lymfeklieren te isoleren. Verder wil ik Janneke bedanken voor het kritisch lezen van de manuscripten.

Voor dit onderzoek zijn er enorme aantallen T-cellen gesorteerd en bij deze wil ik Edwin en Tom bedanken voor de soms dagenlange sorteringen. Tevens wil ik de dierverzorgers en in het bijzonder Hans Diepstraten van het EDC bedanken voor de goede zorg en het checken van de muizen. Zonder deze hulp zou het onderzoek naar de ontwikkeling van de tumoren in GATA3 transgene muizen een stuk moeilijker zijn verlopen. Om een proefschrift uiteindelijk in deze vorm te krijgen, heb ik de onmisbare hulp van Tar gehad bij het bewerken van het grote aantal figuren en van Wendy, Daniëlle en Marcia voor de lay-out.

De schrijfruimte op de afdeling heb ik in de loop der jaren met meerdere mensen gedeeld. In het bijzonder denk ik terug aan de gezellige gesprekken met Evelien tijdens het drinken van een kopje koffie. Ook zal ik de kennismaking met de wervelwind, genaamd Evert, niet snel vergeten. Verder wil ik de mensen van de afdeling bedanken voor de goede samenwerking en plezierige tijd.

Tijdens het promotieonderzoek is er nauw samengewerkt met de werkgroep van Erik Lubberts van de afdeling Reumatologie. Dit ging zelfs zo goed dat dit voor mij is uitgemond in een nieuwe baan. En zoals het er nu naar uitziet zelfs in een publicatie. Het is volgens mij erg bijzonder dat er binnen deze baan ruimte werd geboden om

het proefschrift af te ronden. Verder wil ik iedereen binnen de Lubberts groep, Erik, Patrick, Anne-Marie, Ferry, Jeer en Sonja bedanken voor de prettige en enthousiaste werkssfeer. Ik kijk met plezier uit naar de komende periode van onderzoek.

Prof. dr. J.N.J. Philipsen, dr. H.R. Delwel en dr. B. Blom wil ik bedanken voor het kritisch doorlezen van dit proefschrift, als leden van de kleine commissie. Daarnaast wil ik prof. dr. J.P. Disanto, prof. dr. E.A. Dzierzak, prof. dr. J.D. Laman en dr. E. Lubberts bedankten voor de toezegging van deelname aan de grote commissie.

Het uitvoeren van een promotieonderzoek is een langdurig proces waarbij de steun van vrienden en familie onmisbaar is. Hierbij wil ik mijn ouders bedanken voor de steun en betrokkenheid tijdens al die jaren. Ik ben er ook trots op dat jullie, Martin en Marcella, mijn broer en zus, paranimfen willen zijn tijdens de promotie.

Als laatste wil ik natuurlijk mijn lieve vriendin Anne bedanken voor de betrokkenheid en heel veel geduld tijdens de laatste zware loodjes van de promotie. Zoals je altijd zegt, “een beetje positieve energie doet wonderen”.

CURRICULUM VITAE

Jan Piet van Hamburg (Jan Pieter) was born on January 10th 1979 in Oud-Beijerland. He attended secondary school (HAVO) at 'C.S.G. Willem van Oranje' in Oud-Beijerland and finished it in 1996. In the same year he started studying Biology and Medical Laboratory Research at the Higher Laboratory Education, Rotterdam. During this study he conducted research on the characterization of the regulation and function of Bruton's tyrosine kinase during B cell development (Dr. R.W. Hendriks, Department of Immunology, Erasmus MC, Rotterdam). In 2000, he obtained his Bachelor of Applied Science degree and he started his study Biology at the University of Leiden. During this study he investigated the role of the endosperm in seed enlargement and embryo development in Arabidopsis thaliana. (Dr. R. Offringa, Institute of Molecular Plant Sciences, University of Leiden). During his study he successfully completed the programme Science Bases Business, for which strategic marketing research was performed to explore new applications for the cDNA-AFLP technology. (Dr. G. Simons, Keygene N.V., Wageningen). In 2002 he obtained his Master of Science degree and in the same year he started his PhD project at the Department of Immunology at the Erasmus MC, Rotterdam. In October 2007 he started his postdoc project entitled 'The identification of human T helper 17 cells in rheumatoid arthritis' (Dr. E. Lubberts, Department of Rheumatology, Erasmus MC, Rotterdam).

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APPENDIX

Chapter 1: Figure 3

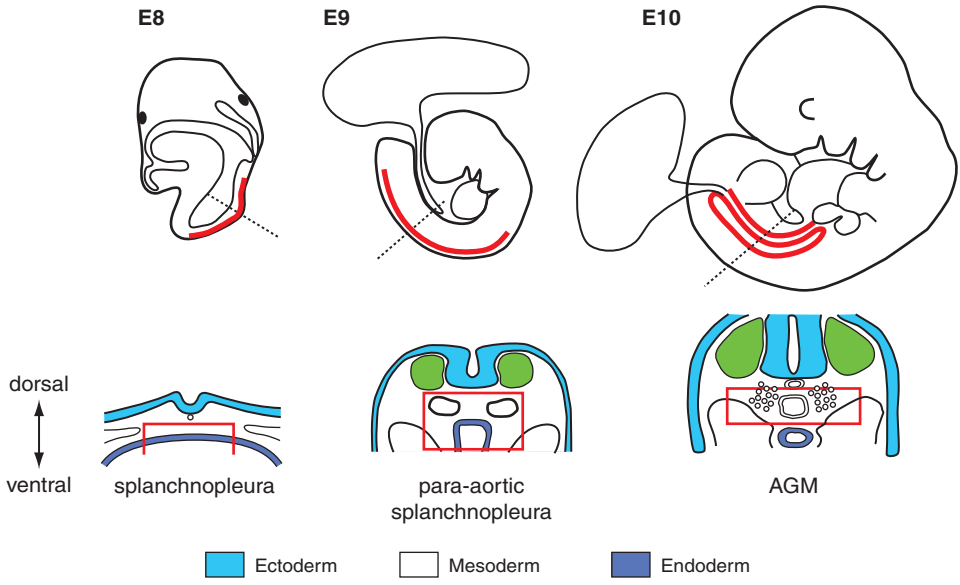


Figure 3. Location and development of the intra-embryonic sites with lympho/hematopoietic potential in the mouse embryo. The top panel shows a schematic representation of a whole mouse embryo at E8 (left), E9 (middle) and E10 (right). Cross sections marked by dotted lines of the corresponding stages are shown in the lower penal. Red boxes in the lower panel are the embryonic sites in which lympho/hematopoietic activity and progentors can be detected. At E8, splanchnopleura (Sp) is defined as the mesoderm that is associated with the endoderm. The dorsal aortae will develop from this region. At E9, when the paired aortae have developed, this embryonic region is defined as the para-aortic splanchnopleura (P-Sp). At E10, the paired aortae fuse, forming a single aorta and the development of the gonads and mesonephros become apparent. Collectively, this region is referred to as the aorta, gonads and mesonephros (AGM) region.

Chapter 2: Figure 5

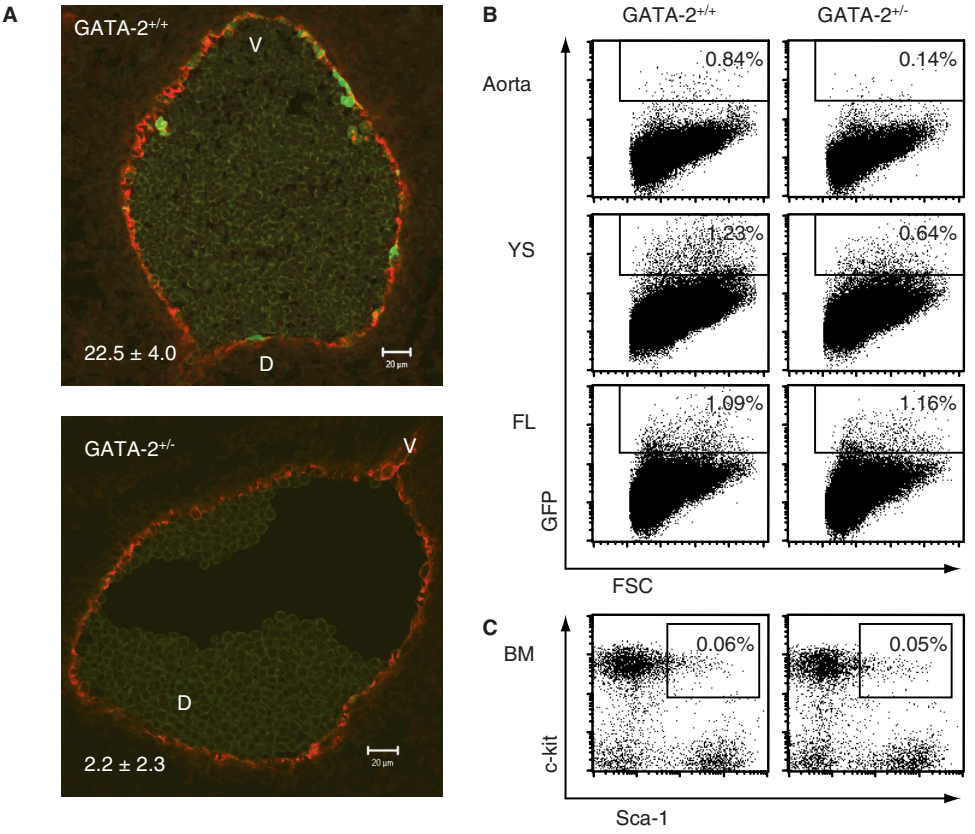


Figure 5. Phenotypic analysis of HSCs in *GATA2*$^{+/-}$ embryos and adults. *GATA2:Ly-6A GFP* compound transgenic embryos were generated by timed pluggings. (A) Representative transverse sections through the E11 dorsal aorta of a *GATA2*$^{+/+}$*:Ly-6A GFP* embryo (45 somite pairs; top panel) and a *GATA2*$^{+/-}$*:Ly-6A GFP* embryo (43 somite pairs; bottom panel). Sections were taken from the caudal end of the AGM, at the height of the hindgut and stained with anti-CD34 antibody. In total, 4 embryos were analysed (2 embryos and a total of 37 sections from each genotype) and cells counted in the aortic endothelium throughout the levels containing the gonads and mesonephroi. CD34^{+} endothelial cells served as a control for section quality and normalization. Red fluorescence (CD34) and green fluorescence (GFP). The percentage of GFP^{+}/CD34^{+} endothelial cells ± SEM is shown on the bottom left and is significantly reduced in the *GATA2*$^{+/-}$ embryos; $p < 0.05$. Flow cytometric analysis of phenotypically defined HSCs was performed on (B) embryonic hematopoietic tissues and (C) adult BM. Expression of the Ly-6A GFP HSC marker was analysed on E11 aorta, YS and FL cells. Adult BM cells were analysed for the percentage of cells in the Lin^{-} fraction that are Sca-1^{+}c-kit^{+}. Percentages of GFP^{+} cells in the embryonic tissue and Sca-1^{+}c-kit^{+} cells enclosed in each gate are shown.

Chapter 3: Figure 1

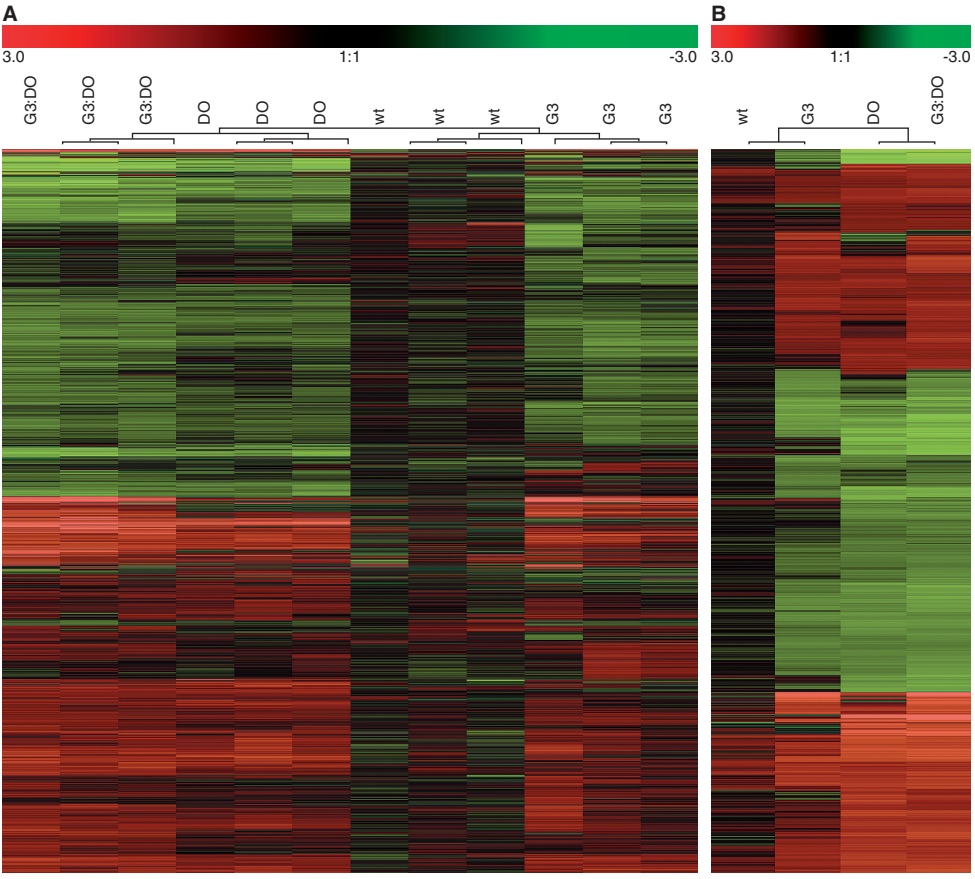
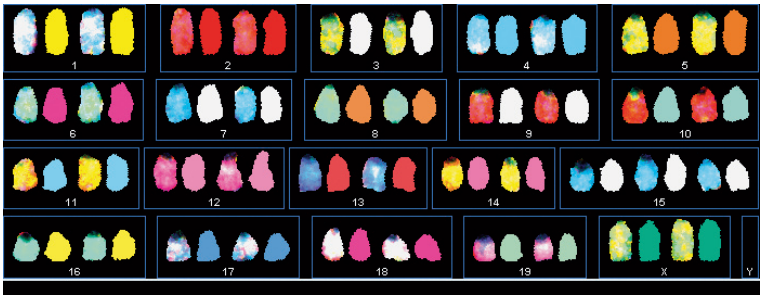


Figure 1. Identification of specific gene expression signatures in DP cells as a result of enforced GATA3 expression. Gene expression profiles were generated from FACS-sorted DP cells from wild-type (wt) mice and CD2-GATA3 transgenic (G3), DO11.10 TCR transgenic (DO) and CD2-GATA3; DO11.10 double transgenic (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 matrixes indicate the average linkage clustering of respectively individual mice and genes. Green and red colors indicate the level of down-regulation or up-regulation 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 values numbers represent ^{2}log values.

Chapter 4: Figure 5

A



B Sky analysis of CD2-GATA3 lymphoma

Case	Numerical	Structural
#b-9	+15	
#a-11*	-	-
#a-13*	+18, +19	+t(2;16), del(8)
#bhf-50	+15	der(9)
#b-38	-Y, +14	+1, dic(10), der(12;15)
#b-41	-	-
#a-45	+15	-
#bhf-53	-	-

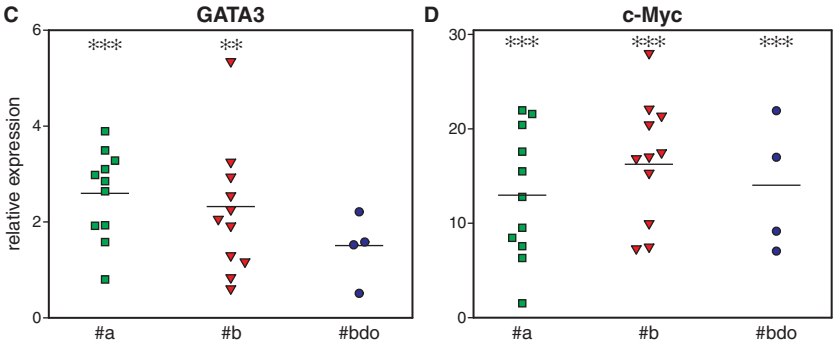
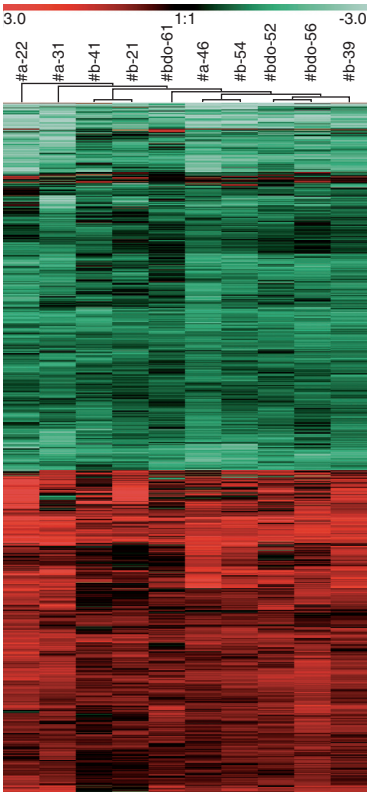


Figure 5. Chromosome 15 abnormalities and increased c-Myc expression in CD2-GATA3 lymphomas. (A) A representative metaphase of SKY analysis of a CD2-GATA3 lymphoma, indicating trisomy of chromosome 15 as a sole chromosomal abnormality (#b = CD2-GATA3#b mice). (B) Summary of SKY analysis performed on CD2-GATA3 lymphoma samples (* = lymphoma derived cell line, #a = CD2-GATA3#a and #bhf = female CD2-GATA3#b:HY:Rag2$^{-/-}$ mice). (C-D) Quantitative RT-PCR analysis of GATA3 (C) and c-Myc (D) expression in thymic lymphoma samples from the indicated mice. Average values and individual data points indicate the ratio between the expression of the indicated gene and Gapdh. Expression levels in wild-type DP cells were set to 1. For statistical analysis a t-test was performed, comparing gene expression in the indicated lymphoma to wild-type DP cells (* = p<0.05, ** = p<0.01, *** = p<0.001).

Chapter 4: Figure 6

A



B

Selection of differentially expressed genes in CD2-GATA3 lymphoma and their mean expression level

Gene	Mean	Gene	Mean
cluster 9: (down ≤ -1.2 fold, n =261 genes)		*cluster 8:* (up ≥ -1,4 fold, n = 54 genes)	
Rag2	-3.31	Apo E	2.83
Rag1	-2.48	c-Myc	2.22
Ror gamma	-2.27	Ccl8	2.16
Ets2	-2.22	Methyltransferase-like 1	2.01
Ccr9	-2.15	Gadd45 gamma	1.80
Itk	-2.11	Transferrin	1.78
c-Myb	-2.00	Ccl9	1.68
CD5	-1.96	Granzyme A	1.63
CD4	-1.96	Asparagine synthetase	1.63
Bc11B	-1.82		
Nfat-c3	-1.70	cluster 6:	
Cbl	-1.61	(up 1.0 -1.7 fold,	
Tcf1	-1.59	n = 177 genes)	
TEC	-1.55		
IL-16	-1.54	Transferrin receptor	1.63
Socs1	-1.54	spermidine synthase	1.59
Stat5B	-1.54	Heat shock 70 kda-like protein 1	1.56
Ifn gamma receptor alpha	-1.43	IL-17BR	1.53
IL-21R	-1.40	Cyclin D2	1.51
Slam	-1.40	Muc2	1.43
Cxcr4	-1.34	Hes1	1.26
CD3 zeta	-1.31		
IL-4R alpa	-1.29		
Nfat5	-1.25		
JunD	-1.22		

Figure 6. Gene expression profiles of CD2-GATA3 induced lymphoma. (A) Hierarchical clustering of both genes (rows) and thymic lymphoma (columns). Green and red colors indicate the down-regulation or up-regulation of the levels of genes expressed in lymphoma compared to sorted wild-type DP cells. The color scale above the matrix correlates with gene expression and the given values numbers represent ^{2}log values. In total 2845 genes were found to be differentially expressed ($p < 0.01$) in at least 2 lymphoma samples. Gene expression profiles were analyzed for 10 lymphoma samples from CD2-GATA3#b ($n = 4$, #b), CD2-GATA3#a, ($n = 3$, #a) and CD2-GATA3#b:DO11.10 mice ($n = 3$, #bdo). The dendogram above and on the left side of the matrix indicate the average linkage clustering of respectively lymphomas and genes. (B) From the differentially expressed genes shown in A, 3 clusters were specified. For each cluster a selection of genes and their mean expression level is given. Numbers represent ^{2}log values.

Chapter 7: Figure 1

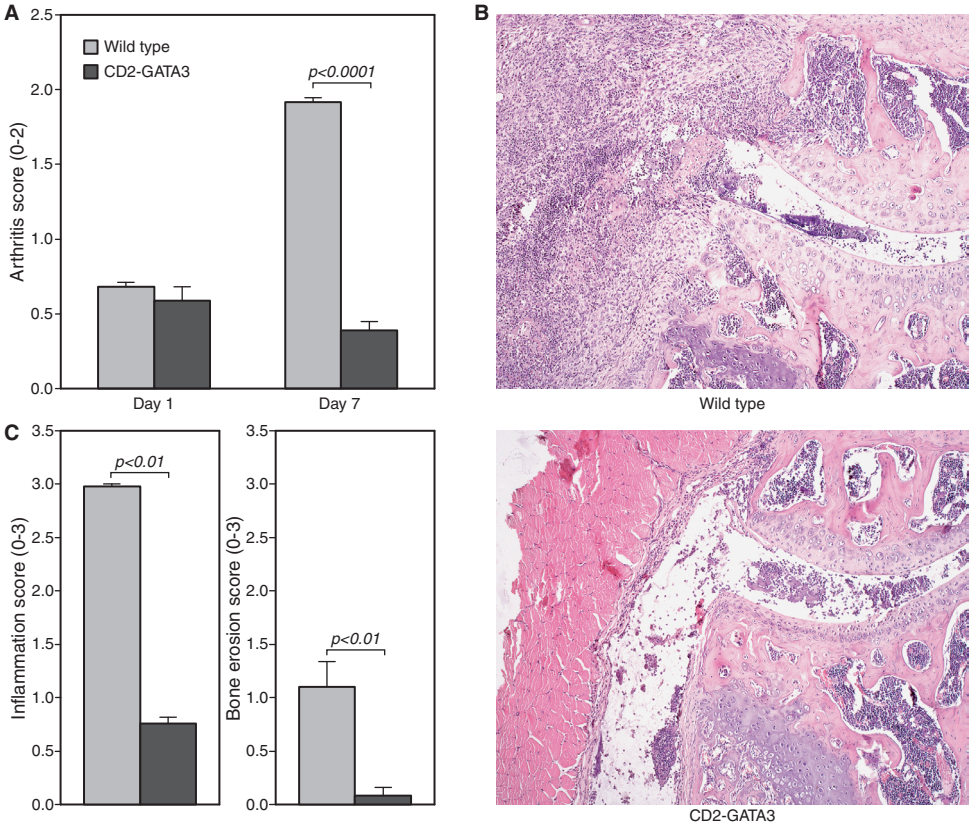


Figure 1. GATA3 protects against mBSA induced joint inflammation. (A) Macroscopical scores of wild-type and CD2-GATA3 tg mice at days 1 and 7 after intra-articularly mBSA injection in the knee joint. Mean values and SEM are given for 6 mice analyzed per group at day 1 and 11 mice per group at day 7. Data are obtained from at least 2 separate experiments. (B) Histological (H&E) staining of knee joint sections obtained from wild-type and GATA3 mice 7 days after mBSA injection. Data are representatives of 6 histological sections per group (original magnification 100x). (C) Inflammation and bone erosion scores of wild-type and CD2-GATA3 tg mice, 7 days after the induction of arthritis. Data are obtained from 6 histological sections per group.

Chapter 8: Figure 3

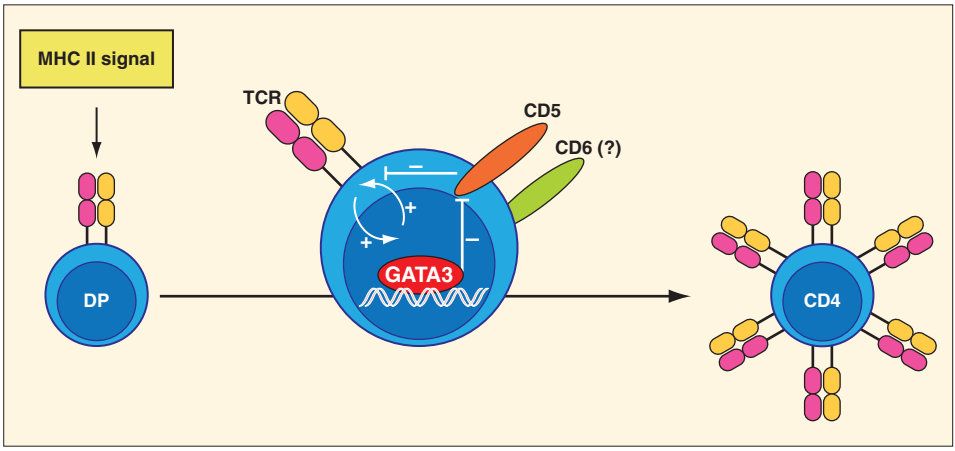


Figure 3. Schematic overview of the control of CD5 and TCR expression by GATA3 during CD4 selection. Upon TCR signaling in the context of an MHC class II signal, GATA3 expression is induced. On its turn GATA3 establishes a positive feedback loop that increases TCR surface expression in developing CD4 lineage cells. In this context, GATA3 acts as a negative regulator of CD5 and CD6 expression, resulting in increased TCR signal strength.