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

Jan Piet van Hamburg[1,2], Marjolein J. W. de Bruijn[1,3], Claudia Ribeiro de Almeida[1], Marloes van Zwam[1], Marjan van Meurs[1], Edwin de Haas[1], Louis Boon[4], Janneke N. Samsom[5] and Rudi W. Hendriks[1,3]

[1] Department of Immunology, Erasmus MC Rotterdam, Rotterdam, The Netherlands
[2] Department of Rheumatology, Erasmus MC Rotterdam, Rotterdam, The Netherlands
[3] Department of Pulmonary Medicine, Erasmus MC Rotterdam, Rotterdam, The Netherlands
[4] Bioceros B.V., Utrecht, Erasmus MC Rotterdam, Rotterdam, The Netherlands
[5] Division of Gastroenterology and Nutrition, Laboratory of Paediatrics, Erasmus MC Rotterdam, The Netherlands

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 naïve primary T cells *in vitro* in the presence of transforming growth factor-β 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, transforming growth factor-β/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 IFN-γ 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.

Key words: Animal models · Autoimmunity · Cytokines · T-helper cells · Transcription factors

Supporting Information available online

Introduction

T-helper cells are classically divided into two functionally distinct subsets, termed T-helper-1 (Th1) and Th2 [1, 2]. Th1 cells, producing IFN-γ 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 IFN-γ/STAT1/T-bet. Th2 cells, producing IL-4, IL-5 and IL-13, are not only 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

Correspondence: Dr. Rudi W. Hendriks
e-mail: r.hendriks@erasmusmc.nl

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-12R β2 chain expression [8–10]. Only if T-bet is sufficiently induced in naïve 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 IFN-γ, 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 independent 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 stages [15, 16], β-selection [17] and $CD4^+$ 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 [22, 23]). In mouse, transforming growth factor-β (TGF-β) and IL-6, an acute phase protein induced during inflammation, act together to induce Th17 differentiation [24–26]. Because TGF-β 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, TGF-β 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 [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 suppress Th17 development [20, 21]. But since IFN-γ 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 IFN-γ 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 naïve primary CD4 T cells from CD2-GATA3 transgenic 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 transgenic mice contained normal numbers of $IL\text{-}17^+$ cells in gut-draining lymphoid tissue, they were not susceptible to Th17-mediated EAE *in vivo*.

Results

Transgenic GATA3 induces IL-4 and limits IL-2 production in T-helper cells

To investigate how the presence of GATA3 affects differentiation of naïve T cells toward 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 Supporting Information Fig. 1), and contained reduced numbers of CD4 T cells and particularly CD8 T cells in spleen and lymph nodes (see Supporting Information Fig. 1). Peripheral T cells manifested an increased surface expression of the CD3/TCR-αβ-complex and a small but consistent decrease in CD5, which is a negative regulator of TCR signaling (Supporting Information Fig. 1). We previously found that when total peripheral CD4 T-cell fractions from CD2-GATA3 transgenic FVB mice were cultured under Th1 conditions, Th2 cytokine production was increased and IFN-γ 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 IFN-γ 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 IFN-γ production. To this end, we activated sorted splenic naïve $CD62L^+CD4^+$ T cells from wild-type (WT) and CD2-GATA3 transgenic C57BL/6 mice with

plate-bound anti-CD3/anti-CD28 under Th17 conditions, *i.e.* in the presence of TGF-β, IL-6, anti-IFN-γ 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-IFN-γ and anti-IL-12) polarizing conditions to confirm that the CD2-GATA3 transgene had the capacity to induce IL-4 and to limit IFN-γ production during differentiation of naïve 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 of ~2–3 higher than those in WT Th2 cultures (Fig. 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 (Fig. 1A). Whereas WT Th0 cultures contained detectable fractions of IFN-γ-producing cells, CD2-GATA3 transgenic Th0 cultures contained significant proportions of $IL\text{-}4^+$ cells, as determined by intracellular flow cytometry (Fig. 1B). Under Th1 polarizing conditions, a large majority of WT T cells produced IFN-γ. In CD2-GATA3 transgenic Th1 cultures, IFN-γ 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 WT Th17 cultures, $IL\text{-}4^+$ or $IFN\text{-}\gamma^+$ cells were not detected, but in CD2-GATA3 transgenic Th17 cultures ~16% of cells produced IL-4 (Fig. 1B).

CD2-GATA3 transgenic Th cultures showed decreased production of IL-2, when compared with WT (Fig. 1C), irrespective of polarization conditions. This reduction in 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 WT (Fig. 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-labeling (Fig. 1D and E).

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/IFN-γ double positive cells and Th17 cultures contained $IL\text{-}4^+$ 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 WT mice and CD2-GATA3 transgenic mice contained equal proportions of IL-17-expressing cells at day 7 (Fig. 2A), but the CD2-GATA3 transgenic IL-17-producing cells co-expressed IL-4 and IL-13 (Fig. 2B). The proportions of $IL\text{-}13^+$ cells in CD2-GATA3 transgenic Th17 cultures were in the same ranges as those in CD2-GATA3 transgenic Th0, Th1 and Th2 cultures (Supporting Information Fig. 2). 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 $IL\text{-}10^+$ cells in CD2-GATA3 transgenic Th0 cells (Fig. 2C and D). In the presence of the CD2-GATA3 transgene the proportions of $IL\text{-}10^+$ cells were generally increased in Th1 and Th2 cultures. We found only a modest increase in CD2-GATA3 transgenic Th17 cultures, when compared with WT cultures (Fig. 2C and D). The presence of GATA3-induced expression of IL-5, irrespective of polarization conditions (Supporting Information Fig. 2 and Fig. 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 (Fig. 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 TGF-β, anti-IFN-γ and anti-IL-4) for 7 days (Supporting Information Fig. 3).

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 $IL\text{-}17^+$ cells in the CD2-GATA3 transgenic Th17 cultures, we found that they expressed IL-21 and TNF-α, albeit at lower levels when compared with WT Th17 cultures (Fig. 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-IFN-γ antibodies – enforced GATA3 expression does not significantly affect the generation of IL-17-producing cells *in vitro*. The CD2-GATA3 transgenic cells cultured in the presence of TGF-β 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).

Transgenic GATA3 affects transcription of various regulators of Th17 cells

In our Th17 culture system, in the presence of anti-IFN-γ and anti-IL-4 antibodies, the proportions of IL-17-producing cells in WT and CD2-GATA3 transgenic cultures were similar at day 7 (see Fig. 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 WT and

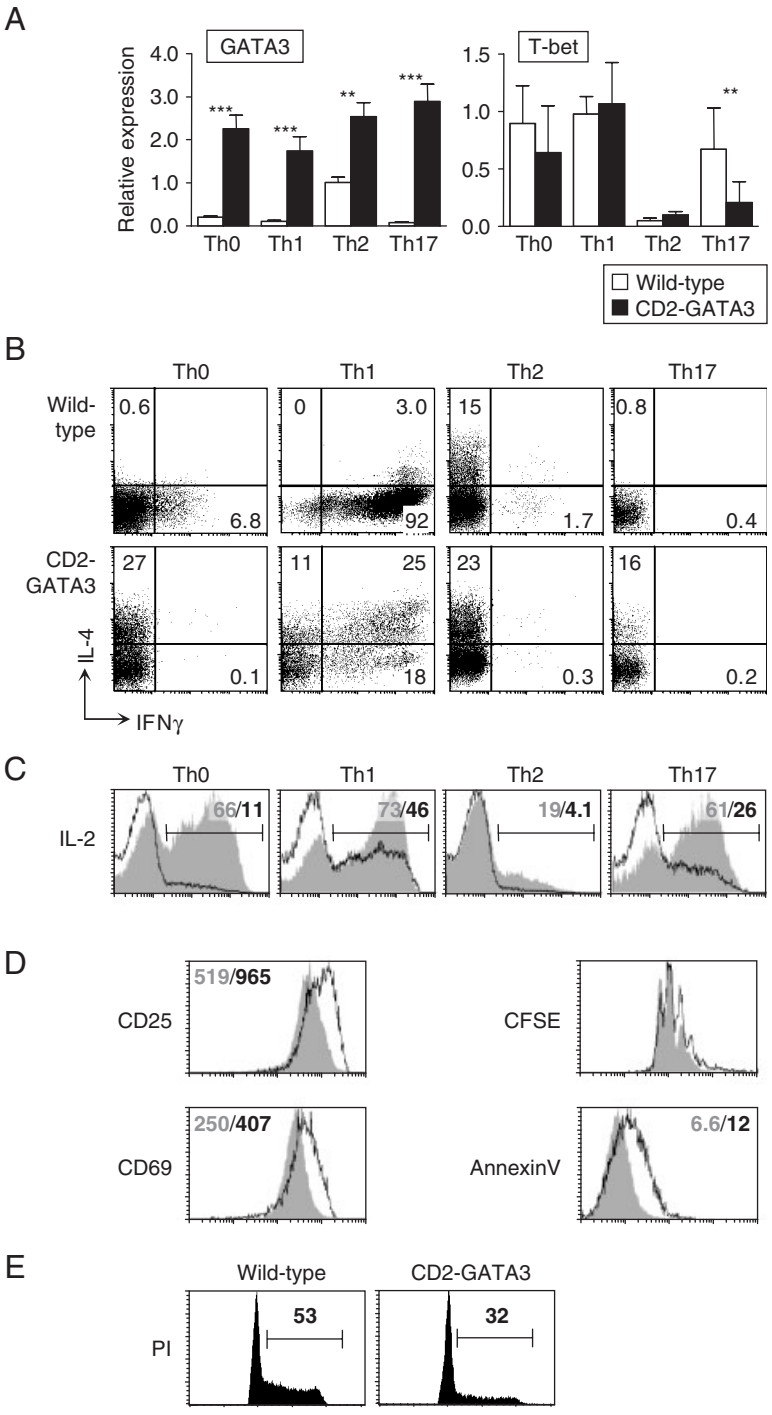


Figure 1. The CD2-GATA3 transgene induces IL-4 and limits IL-2 production in Th1, Th2 and Th17 cells. (A) Quantitative RT-PCR analysis of GATA3 and T-bet expression in different T effector cells from WT (white bars) and CD2-GATA3 transgenic mice (black bars), cultured for 7 days. Expression was normalized to GAPDH and expression levels of GATA3 in WT Th2 cells and T-bet in WT Th1 cells were set to 1. 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 IFN-γ in gated $CD4^+$ T cells from day 7 cultures. Numbers indicate percentages of total cells within the quadrants. (C and D) Flow cytometric analysis for intracellular IL-2 expression (C), CD25 and CD69 expression, Annexin and carboxy-fluorescein succinimidyl ester (CFSE) (D) in the indicated WT (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 WT (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. (B–D) Data shown are representatives of four mice analyzed per group.

CD2-GATA3 transgenic Th17 cultures were activated by anti-CD3/anti-CD28 stimulation for 4 h and mRNA expression levels were analyzed by quantitative RT-PCR and compared with those in Th0, Th1, Th2, Treg and CD8 cultures (Fig. 3 and Supporting Information Fig. 4).

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 (Supporting Information Fig. 4). GATA3 expression did not have detectable effects on the expression levels of NFATc1 or IRF-4, which was recently shown to be essential for Th17 differentiation [40] (Supporting Information Fig. 4).

JAK-STAT (signal transducer and activator of transcription) 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 (Fig. 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 (Fig. 3 and Supporting Information Fig. 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 (Supporting Information Fig. 4). IL-23-mediated phosphorylation of STAT3, and thus Th17 generation, is negatively regulated by the cytokine-inducible inhibitor SOCS3 (suppressor of cytokine signaling) [43]. We found that the presence of enforced GATA3 expression is associated with significantly reduced levels of SOCS3 transcripts (Fig. 3).

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

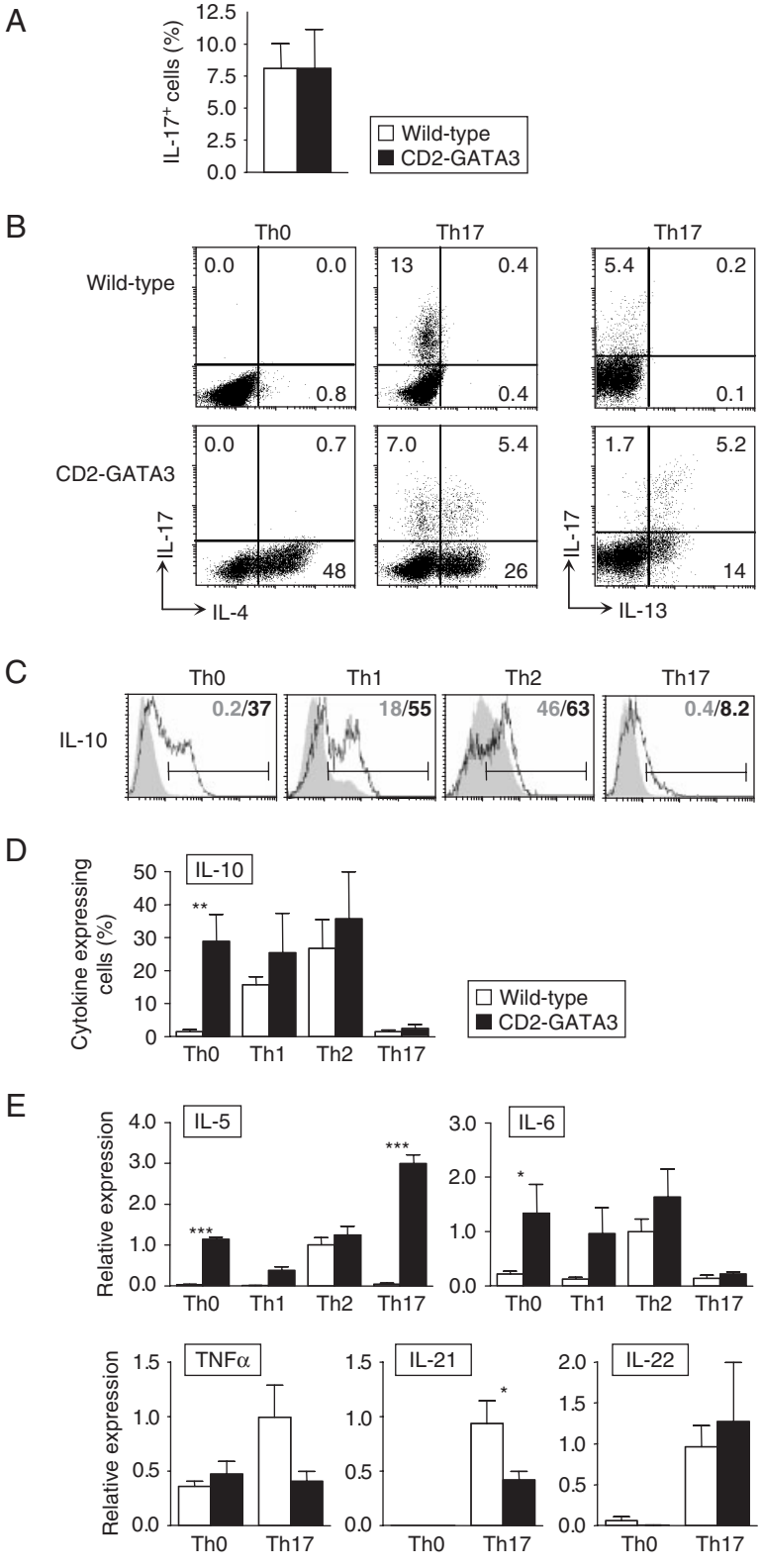
As enforced GATA3 expression resulted in increased IL-4 and reduced IL-2 expression, both of which are negative regulators of Th17 differentiation [24, 28, 37], we explored the effects of GATA3, IL-2 and IL-4 on Th17-cell generation in more detail.

To this end, we stimulated primary $CD62L^+CD4^+$ T cells with plate-bound anti-CD3/anti-CD28 and cultured them with TGF-β/IL-6 and anti-IFN-γ 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 h before intracellular cytokine staining, which yielded higher proportions of $IL\text{-}17^+$ cells, when compared with anti-CD3 re-stimulation (compare Fig. 2A with Fig. 4). Hereby, clearance of IL-4 had only marginal effects on the generation of $IL\text{-}17^+$ cells in WT cells, probably due to the limited IL-4 expression of differentiating Th17 cells in the presence of TGF-β and IL-6. At day 3, the CD2-GATA3 transgenic Th17 cultures contained significantly reduced proportions of $IL\text{-}17^+$ T cells: ∼12% in the absence and ∼22% in the presence of anti-IL-4 (Fig. 4A and B). Although addition of external IL-4 reduced the proportions of $IL\text{-}17^+$ cells expression in WT Th17 cultures, it had only marginal effects on IL-17 expression in CD2-GATA3 transgenic cultures, probably due to substantial transgenic IL-4 production of differentiating GATA3-expressing Th17 cells (Fig. 4A and B).

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



Downregulation of STAT3/4, SOCS3, NFATc2 and RORγt by GATA3 is IL-4-independent

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 IFN-γ (Fig. 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 (Supporting Information Fig. 5A). 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 (Supporting Information Fig. 5B).

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

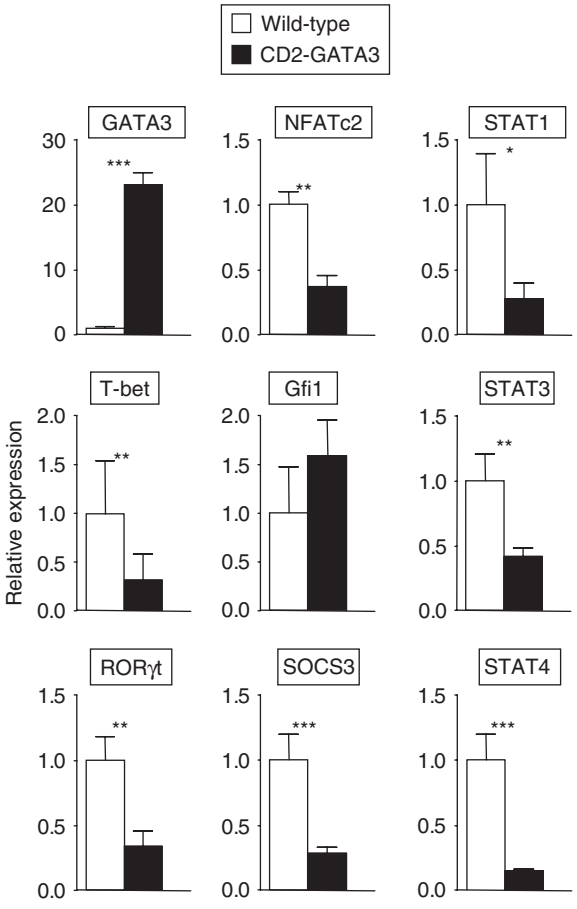


Figure 3. Enforced GATA3 expression affects factors involved in Th17 differentiation. Quantitative RT-PCR analysis of expression of the indicated genes in WT (white bars) and CD2-GATA3 transgenic (black bars) Th17 cultures at day 7. Expression levels were normalized for GAPDH and values in Th17 cultures from WT mice were set to 1. Mean values and SEM are given for 6–11 mice analyzed per group (*$p<0.05$; **$p<0.01$; ***$p<0.001$; *t*-test).

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 (Fig. 5A), but also IL-4 production was induced (Fig. 5B). Thus, enforced GATA3 expression allowed differentiation of Th17 cells *in vivo*.

Transgenic GATA3 allows *in vivo* Th17 differentiation but protects against 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/CFA immunization, five out of eight WT mice developed EAE. In contrast, no clinical disease symptoms were found in ten CD2-GATA3 transgenic mice (Fig. 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 WT and in CD2-GATA3 transgenic mice (Fig. 6B), although the values in CD2-GATA3 transgenic mice were slightly higher at day 14, and slightly lower at day 28, as compared

◀ **Figure 2.** Enforced GATA3 expression induces Th17 cells with Th2 characteristics. (A) Flow cytometric analysis of IL-17 production of WT (white bar) and CD2-GATA3 transgenic (black bar) Th17 cells. Mean values and SEM are from 17 mice per group. (B) Intracellular flow cytometric analysis of the indicated cytokines in WT and CD2-GATA3 transgenic T-cell cultures at day 7. Numbers indicate the percentages of total cells within the quadrants. (C) Flow cytometric analysis for intracellular IL-10 in WT (gray filled histogram) and CD2-GATA3 transgenic (black line) T-cell cultures. Numbers indicate the percentages of IL-10$^+$ cells. Data are representatives of 4–6 mice per group. (D) Quantification of flow cytometric analysis for intracellular IL-10 in gated CD4$^+$ T cells from WT (white bars) and CD2-GATA3 transgenic mice (black bars). Mean values and SEM are from 4 to 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 WT Th2 and expression levels of TNF-α, IL-21 and IL-22 in WT Th17 cells, were set to 1. Mean values and SEM are for 5–7 mice per group (*$p<0.05$;***$p<0.001$; *t*-test).

with WT 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 WT mice (both proportions were <0.2%) (Fig. 6C).

Next, we sorted T-helper cells with the $CD62L^-CD4^+$ activated/memory phenotype from spleens from WT 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 (Fig. 6D), in agreement with our findings in *in vitro* Th17 cultures (Fig. 3). In addition, we found that T-bet and TNF-α 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 TGF-β/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-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*,

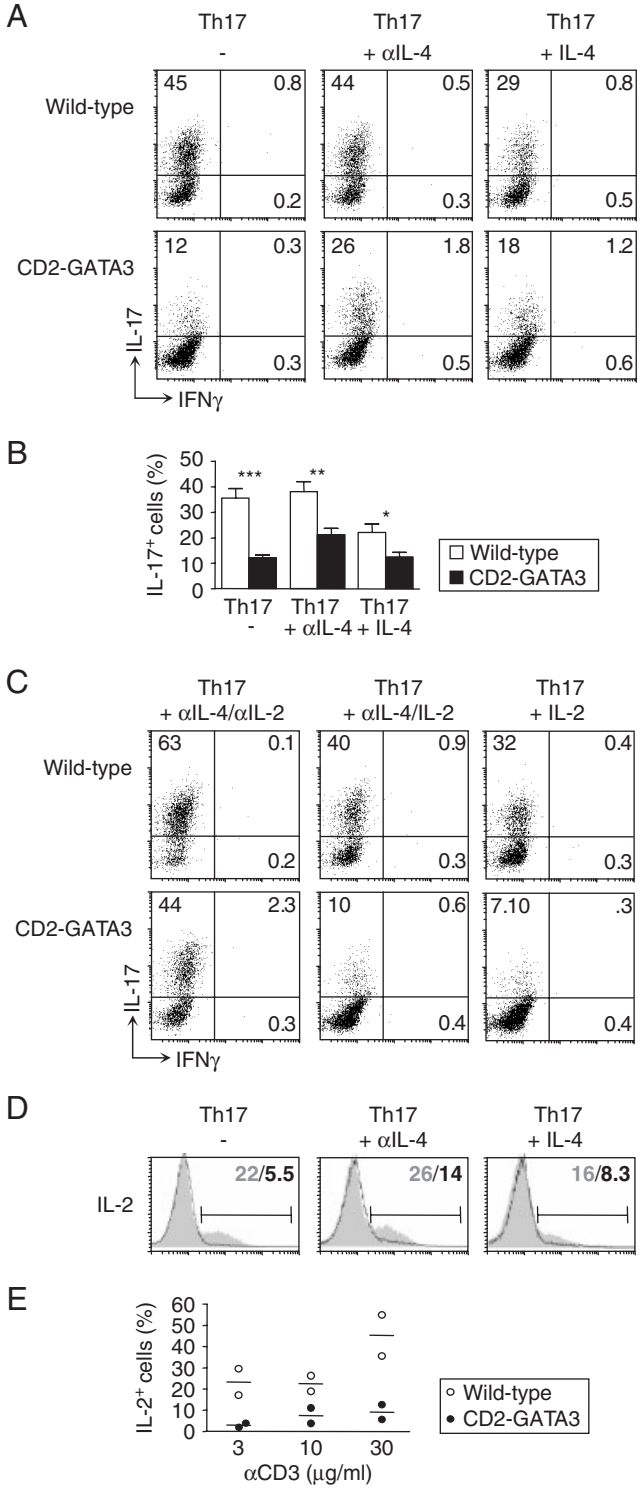


Figure 4. Enforced GATA3 expression affects Th17 differentiation *via* IL-4 and IL-2. (A–C) IL-17/IFN-γ expression profiles of $CD4^+$ T cells in Th17 cultures from the indicated mice. Cells were cultured for 3 days with the indicated antibodies or cytokines. Numbers indicate percentages of total cells within the quadrants (A, C) or mean values and SEM of the percentage of $IL\text{-}17^+$ 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 WT (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 of $IL\text{-}2^+$ 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 $IL\text{-}2^+$ cells.

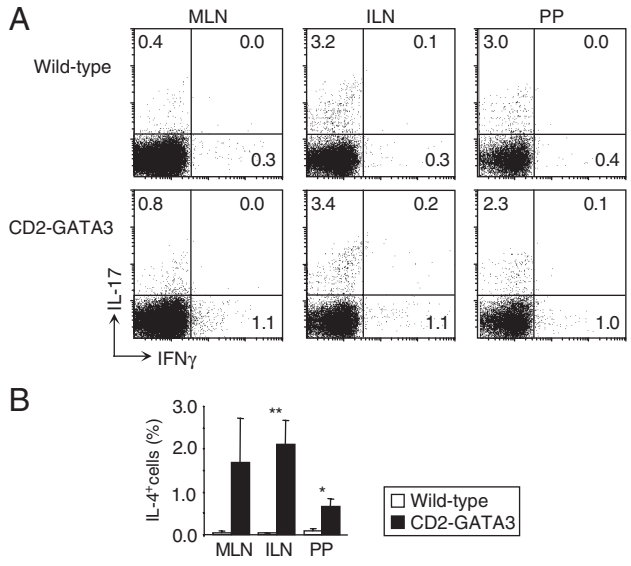


Figure 5. IL-17-producing cells in CD2-GATA3 transgenic mice *in vivo*. (A) Flow cytometric analysis for intracellular IL-17 and IFN-γ expression in gated $CD4^+$ T cells from mesenteric lymph nodes (MLN), iliacal lymph nodes (ILN) and Peyer's patches (PP) from WT and CD2-GATA3 transgenic mice. Numbers indicate the percentage of cells within the quadrants. Data are representatives of six mice analyzed per group. (B) Quantification of flow cytometric analysis for intracellular IL-4 in gated $CD4^+$ T cells from the indicated tissues from WT (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).

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 IFN-γ (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 WT Th2 cell cultures. Thus, in CD8 T-cells transgenic GATA3 could counteract the repression of Th2 cytokine production by repressor of GATA. 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 naïve $CD8^+$ 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 transgenic mice is consistent with our findings of (i) significant IL-4 production in lymph nodes from CD2-GATA3 transgenic 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 (Fig. 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 TGF-β 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 $IL\text{-}10^+$ CD4 T cells during *in vivo* EAE induction in CD2-GATA3 transgenic mice (Fig. 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* (Fig. 2C). Therefore, it is more likely that the induction of $IL\text{-}10^+$ 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 WT mice (J. P. van Hamburg, 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 WT 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

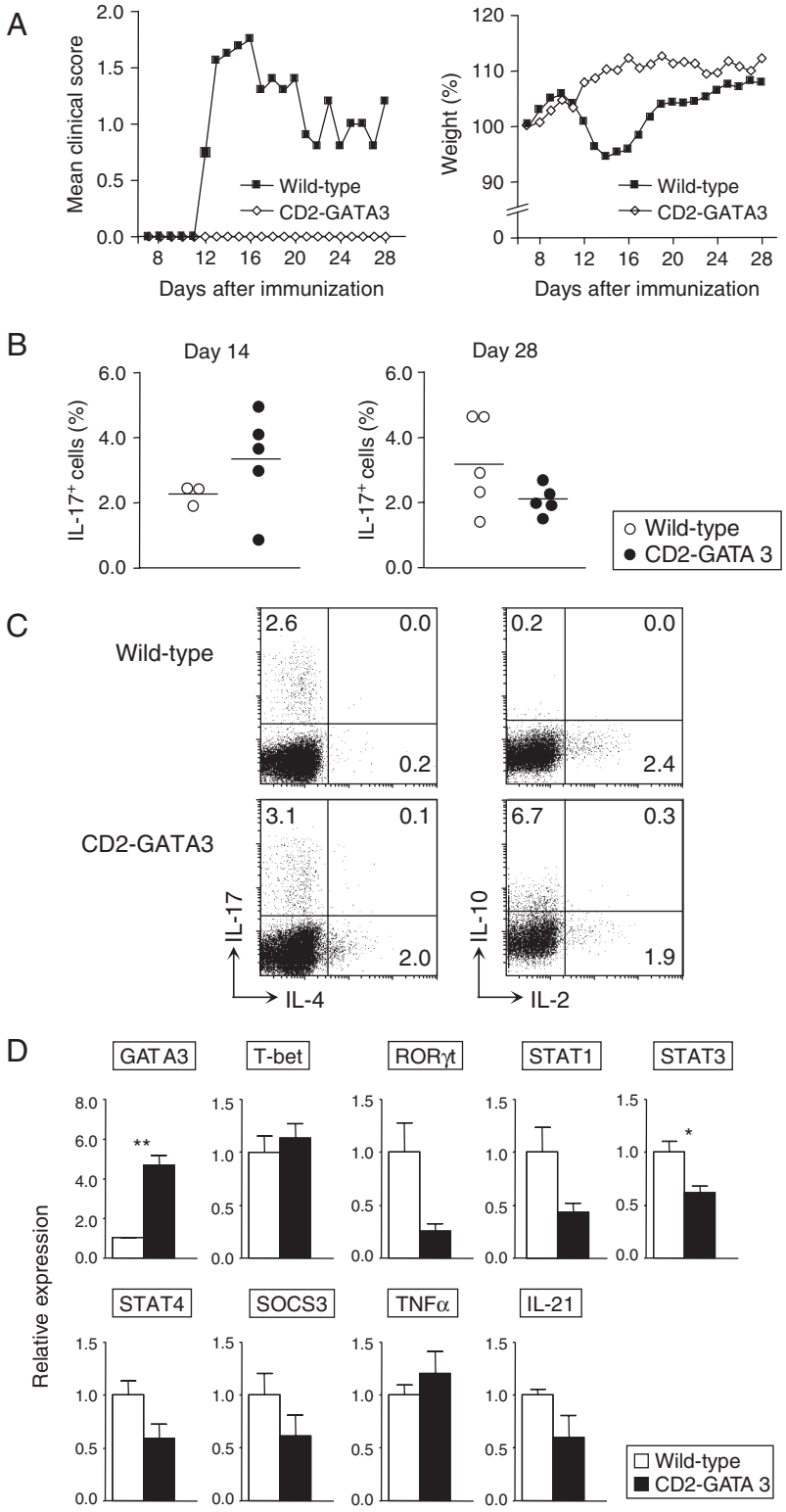


Figure 6. CD2-GATA3 transgenic mice are not susceptible to EAE induction. (A) Induction of EAE by immunization with MOG_{35-55} peptide. Mean clinical scores and weight are given for 8–10 mice per group. (B) Flow cytometric analysis of intracellular IL-17 expression in gated LN $CD4^+$ T cells from WT and CD2-GATA3 transgenic mice 14 and 28 days after EAE induction. Mean and individual data points are displayed, indicating the percentage of IL-17^+ cells. (C) Flow cytometric analysis for the indicated cytokines in gated LN $CD4^+$ T cells from WT 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 WT (white bars) and CD2-GATA3 transgenic (black bars) mice 14 days after EAE induction. Expression levels were normalized for GAPDH and values of WT mice were set to 1. Mean values and SEM are given for three mice analyzed per group (*$p<0.05$; **$p<0.01$; *t*-test).

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 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 IFN-γ-producing cells, which suppress development of Th17 cells from naïve precursor cells [20, 21]. Taken together, GATA3 has various stimulatory and inhibitory effects on Th17 differentiation (see Fig. 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 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 (Supporting Information Fig. 4), 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.

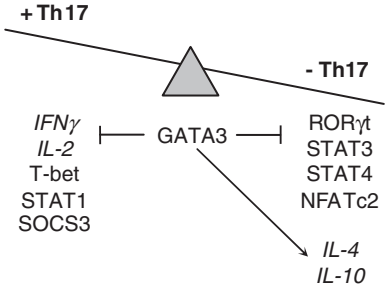


Figure 7. Regulatory effects of GATA3 on Th17 differentiation. Model showing effects of GATA3 on Th17 differentiation. GATA3 has positive effects by limiting IFN-γ and IL-2 production and inhibiting expression of T-bet, STAT1, and the Th17 repressor protein SOCS3. On the other hand, GATA3 constrains 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.

Materials and methods

Mice

CD2-GATA3 transgenic mice [36] were backcrossed on the C57BL/6 background for at least eight generations and genotyped by PCR. GATA3 primers were: 5′-CAGCTCTGGACTCTTCC CAC-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, except PE-conjugated anti-Granzyme B (GB12) and biotinylated anti-IL-13 (BAF413), which were from Caltag Laboratories (Burlingame, CA, USA) and R&D Systems (Minneapolis, MN, USA), 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 4 h. 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, USA) or FlowJo™ (Tree Star, Ashland, OR, USA) 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

Naïve CD62L$^+$CD4$^+$ or CD8$^+$ T cells were purified from spleens obtained from 8-wk-old mice and activated/memory

$CD62L^-CD4^+$ were purified from spleens from mice subjected to EAE induction. Cells were sorted, using an FACSVantage VE equipped with Diva Option and BD FACSDiva software (BD Bioscience). Purity of obtained fractions was typically >98%.

$CD62L^+CD4^+$ or $CD8^+$ T-cell fractions were cultured at concentrations of 1×10^6 cells/mL in Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, MD, USA), containing 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO, USA), 5×10^{-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°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-IFN-γ (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-IFN-γ. 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, USA). 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 1 μg was used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) 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, USA) and probes were chosen from the universal probe library (Roche Applied Science) or designed manually and purchased from Eurogentec (Seraing, Belgium) (Supporting Information Table 1). Quantitative real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). 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-wk-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.

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Abbreviation: **Gfi-1:** growth factor independent-1

Full correspondence: Dr. Rudi W. Hendriks, Department of Pulmonary Medicine, Erasmus MC Rotterdam, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
Fax: +31-10-7044728
e-mail: r.hendriks@erasmusmc.nl

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