

GATA-3 in Human T Cell Helper Type 2 Development

Alla Skapenko,^{1,2} Jan Leipe,^{1,2} Uwe Niesner,³ Koen Devriendt,⁴
Rolf Beetz,⁵ Andreas Radbruch,³ Joachim R. Kalden,² Peter E. Lipsky,⁶
and Hendrik Schulze-Koops^{1,2}

¹Nikolaus Fiebiger Center for Molecular Medicine, Clinical Research Group III, and ²Department of Internal Medicine III, University of Erlangen-Nuremberg, 91054 Erlangen, Germany

³Deutsches Rheuma-Forschungszentrum, 10117 Berlin, Germany

⁴Department of Clinical Genetics, Centre for Human Genetics, University of Leuven, 3300 Leuven, Belgium

⁵University Children's Hospital, 55101 Mainz, Germany

⁶National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892

Abstract

The delineation of the *in vivo* role of GATA-3 in human T cell differentiation is a critical step in the understanding of molecular mechanisms directing human immune responses. We examined T cell differentiation and T cell-mediated effector functions in individuals lacking one functional GATA-3 allele. CD4 T cells from GATA-3^{+/-} individuals expressed significantly reduced levels of GATA-3, associated with markedly decreased T helper cell (Th)2 frequencies *in vivo* and *in vitro*. Moreover, Th2 cell-mediated effector functions, as assessed by serum levels of Th2-dependent immunoglobulins (Igs; IgG4, IgE), were dramatically decreased, whereas the Th1-dependent IgG1 was elevated compared with GATA-3^{+/+} controls. Concordant with these data, silencing of GATA-3 in GATA-3^{+/+} CD4 T cells with small interfering RNA significantly reduced Th2 cell differentiation. Moreover, GATA-3 mRNA levels increased under Th2-inducing conditions and decreased under Th1-inducing conditions. Taken together, the data strongly suggest that GATA-3 is an important transcription factor in regulating human Th2 cell differentiation *in vivo*.

Key words: Th1/Th2 cells • cellular differentiation • transcription factors • T lymphocytes • siRNA

Introduction

CD4 T cells potentiate cellular or humoral immune responses through the action of Th1 and Th2 cells, respectively. The molecular mechanisms by which antigenic stimulation of the T cell receptor and signals derived from costimulatory molecules integrate to drive the differentiation of naive T cell precursors into the Th1 or Th2 direction has been the focus of intensive research over recent years. It has become clear that T cell differentiation is a complex process regulated by a network of transcription factors (1). It appears, however, that the master transcription factor in the Th1/Th2 decision is GATA-3. GATA-3 is expressed in naive T cells and is

strongly up-regulated during Th2 development, but subsides to a minimal level during *in vitro* Th1 differentiation (2). Ectopic expression of GATA-3 induces Th2-specific cytokine expression not only in developing Th1 cells, but also in otherwise irreversibly committed Th1 cells and Th1 clones (3). GATA-3 fully reconstitutes Th2 development in signal transducer and activator of transcription (STAT)-6-deficient T cells (4). Furthermore, GATA-3 inhibits IFN- γ production, probably through down-regulation of STAT-4 expression (5).

However, the *in vivo* analysis of GATA-3 function with regard to T cell differentiation is difficult as GATA-3 also plays an important role during vertebrate embryogenesis and homozygous mutant mice lacking GATA-3 do not survive gestation (6). Moreover, RAG-2^{-/-} C57BL/6 mice reconstituted with GATA-3^{-/-} embryonic stem cells do not develop T cells as GATA-3 is a critical transcription factor for thymic T cell development (7). Thus, the role of GATA-3 in T cell differentiation *in vivo* is still

A. Skapenko and J. Leipe contributed equally to this work.

Address correspondence to Hendrik Schulze-Koops, Nikolaus Fiebiger Center for Molecular Medicine, Clinical Research Group III, University of Erlangen-Nuremberg, Glueckstrasse 6, 91054 Erlangen, Germany. Phone: 49-9131-853-3795; Fax: 49-9131-853-4770; email: Schulze-Koops@med3.imed.uni-erlangen.de

rather elusive. Moreover, the significance of GATA-3 in regulating human T cell differentiation has not been addressed in detail.

To delineate the *in vivo* role of GATA-3 in human T cell differentiation we analyzed previously described individuals lacking one functional GATA-3 allele (GATA-3^{+/-}; references 8 and 9). We demonstrate that CD4 T cells from GATA-3^{+/-} individuals express decreased levels of GATA-3 associated with decreased Th2 cell frequencies *in vivo* and *in vitro* and reduced Th2-dependent effector functions *in vivo*. On the other hand, inhibition of GATA-3 by GATA-3-specific small interfering RNA (siRNA) in control CD4 T cells (GATA-3^{+/+}) resulted in dramatically reduced Th2 cell differentiation, emphasizing the significance of GATA-3 in human Th2 cell development. Taken together, the data indicate that GATA-3 is critically involved in regulating human Th2 cell differentiation.

Materials and Methods

Reagents and Antibodies. The following mAbs and reagents were used for purification, stimulation, and staining: anti-CD16 (3g8FcIII), anti-CD45RO (UCHT-1), anti-CD3 (OKT3), anti-CD8 (OKT8), and anti-HLA-DR (L243; American Type Culture Collection); FITC-conjugated anti-CD3, PE-labeled anti-CD4, and FITC-labeled anti-CD4 (Sigma-Aldrich); anti-CD19 and FITC-labeled anti-HLA-DR (DakoCytomation); PE-labeled anti-IL-4 (MP4-25D2), FITC-labeled anti-IFN- γ (4S.B3), and anti-CD28 (CD28.2; BD Biosciences); polyclonal goat anti-mouse Igs (ICN Biomedicals); anti-CD45RA (111-1C5; provided by R. Vilella, Hospital Clinic, Barcelona, Spain); human recombinant IL-4 and neutralizing mAb to IL-4 (Endogen); human recombinant IL-12 (provided by the Genetics Institute); and human recombinant IL-2 (GIBCO BRL).

Study Population. Three individuals (a 16-yr-old male, an 18-yr-old male, and a 30-yr-old female) in whom genetic abnormalities in one GATA-3 allele have been identified (8–10) were analyzed in the study (see Table I). These individuals have normal peripheral blood T cell and CD4 T cell counts. They do not have any signs of immunological incompetence and have undergone successful immunizations. GATA-3 haploinsufficiency was reported in some tissues resulting clinically in hypoparathyroidism, sensorineural deafness, and renal insufficiency (HDR syndrome; reference 8). Young healthy volunteers of comparable age and gender not taking any medications were used as control GATA-3^{+/+} individuals.

Determination of Ig Levels. Serum Ig levels were determined by standard ELISA procedures in the central clinical laboratory facility of the University of Erlangen-Nuremberg. The normal ranges are defined by the 2.5 and 97.5 percentiles of age-matched healthy individuals.

Cell Purification. CD4 naive or memory T cells were isolated from peripheral blood of GATA-3^{+/-} or control individuals by negative selection using mAbs to CD8, CD19, CD16, HLA-DR, CD45RO, or CD45RA as previously described (11, 12). Homogeneity and purity of the recovered cells were assessed by flow cytometry.

Cell Cultures. All cell cultures were performed in RPMI 1640 medium supplemented with 100 IU/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine (all GIBCO BRL), 10% normal human serum, and 10 U/ml recombinant human

IL-2. CD4 naive T cells were stimulated with 1 μ g/ μ l immobilized anti-CD3. For Th2-inducing conditions, 1 μ g/ μ l anti-CD28 and IL-4 (31.25 ng/ml, where indicated) were added. For Th1-inducing conditions, 40 ng/ml IL-12 and 10 μ g/ μ l anti-IL-4 were added to the cell culture. CD4 memory cells were primed under Th2-inducing conditions with 1 μ g/ μ l anti-CD28 in the presence or absence of 31.25 ng/ml IL-4 (12). After 5 d cells were analyzed for cytokine mRNA by RT-PCR. T cell effector functions were assessed after an additional 2.5 d of rest.

siRNA Transfection. Freshly isolated memory CD4 T cells were transfected with 4 μ M GATA-3 SMARTpool siRNA or, as control, lamin siRNA (Dharmacon) using the human T cell nucleofector kit (Amaxa) according to the manufacturer's instructions. The transfection efficiency was close to 100% as assessed by using FITC-labeled 30 mer oligos.

Intracellular Cytokine Analysis. Cytoplasmic cytokine production was determined by intracellular staining as previously described (11). In brief, 2 \times 10⁶ cells were stimulated with 1 mM ionomycin (Calbiochem) and 20 ng/ml PMA (Sigma-Aldrich) for 5 h in the presence of 2 μ M monensin (Sigma-Aldrich). Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.1% (wt/vol) saponin (Sigma-Aldrich) in 2% FCS/PBS (both GIBCO BRL). Nonspecific binding sites were blocked with 4% mouse and rat serum. Cytoplasmic IL-4 and IFN- γ were detected by staining with directly labeled mAb against human cytokines for 25 min on ice. Cells were washed with 0.1% saponin/2% FCS/PBS, resuspended in 2% FCS/PBS, and analyzed by flow cytometry. An aliquot of the cells were stained with irrelevant mAbs as control for determining background fluorescence.

Western Blot Analysis. 2 \times 10⁶ cells were lysed in 20 μ l 1 \times SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue). Proteins were separated in a 10% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher & Schuell). The blots were incubated with specific antibodies for GATA-3 (H48; Santa Cruz Biotechnology, Inc.), STAT-6 (S20; Santa Cruz Biotechnology, Inc.), and actin (Sigma-Aldrich), followed by horseradish peroxidase-conjugated anti-rabbit Ig (New England Biolabs, Inc.).

Preparation of Total RNA and RT-PCR. Total RNA was extracted using the RNeasy Minikit (QIAGEN) with an additional DNA digestion step (RNase Free DNase set; QIAGEN). RT (TaqMan Reverse Transcription Reagent; Applied Biosystems) was performed in a conventional thermocycler (10 min at 25°C, 40 min at 48°C, and 5 min at 95°C) with 1 μ g total RNA in a 1:1 mixture of oligo(dT) and random hexamer primers in a final volume of 20 μ l. Real-time PCR was performed with the Light-Cycler and the FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The primer sequences for GATA-3 and the housekeeping gene EF-1-a were deduced from published sequences (GATA-3: 5'-GAACCGCCCCCTCATTAAG-3' and 5'-ATT-TTTCGGTTTCTGGTCTGGAT-3', EF-1-a: 5'-GTTGATA-TGGTTCCTGGCAAGC-3' and 5'-GCCAGCTCCAGCAG-CCTTC-3'; reference 13). Data were evaluated using the Light-Cycler software version 3.5.28 (Roche Diagnostics) and the second derivative maximum algorithm. Specificity of the PCR product was confirmed by agarose gel electrophoresis and melting curve analysis. Serial dilutions of human CD4 cells were used to generate the standard curves.

Statistical Analysis. Differences in data distribution were analyzed by the two-tailed Student's *t* test.

Table I. *GATA-3 Genetic Abnormalities in GATA-3^{+/-} Individuals*

	Mutation	Phenotype
Individual no. 1	49-bp intragenic deletion	Frameshift, premature stop codon
Individual no. 2	900-kb deletion	Deletion of one allele
Individual no. 3	Terminal deletion of chromosome 10p	Deletion of one allele

Results and Discussion

The Loss of One Functional GATA-3 Allele Results in Reduced Th2 Cell Differentiation and Decreased Th2 Effector Functions In Vivo. Indications of protein functions in vivo have largely been derived from gene knockout approaches. However, the analysis of proteins critically involved in embryogenesis or early organ development is difficult to pursue if the target disruption of the particular gene results in the death of the embryo. To evaluate the role of GATA-3 in human Th2 cell differentiation in vivo, we analyzed three individuals lacking one functional GATA-3 allele (Table I). CD4 T cells from those individuals contained significantly less GATA-3 protein than CD4 T cells from controls (Fig. 1 A). Ex vivo analysis of cytokine production by CD4 memory T cells from the peripheral blood of the GATA-3^{+/-} individuals revealed that the frequencies of IL-4-producing Th2 cells were at the lower end of or below the normal range (Fig. 1 B). In contrast, the frequencies of IFN- γ -producing Th1 cells were within the normal

range (Fig. 1 B). The decreased Th2 cell frequencies in the peripheral blood of the GATA-3^{+/-} individuals resulted in a shift of the Th2/Th1 balance, reflecting a Th1 bias in these individuals (Table II).

To assess T cell-mediated effector functions in vivo we measured concentrations of different Ig classes and IgG subclasses in the serum. Although the levels of IgM and IgA were within the normal range in all GATA-3^{+/-} individuals, the levels of the Th2-dependent IgE were in the lower range near the bottom of the normal limits (Fig. 1 D). In contrast, the total IgG concentrations were within or above the normal range (Fig. 1 E). Interestingly, although the concentrations of the Th1-dependent Ig, IgG1, were increased in the serum of all GATA-3^{+/-} individuals, the levels of the Th2-dependent IgG4 were dramatically decreased (Fig. 1 E). The levels of IgG2 and IgG3 were distributed within the normal limits (Fig. 1 E). Thus, these data indicate that the reduction of the GATA-3 protein level in CD4 T cells in vivo resulted in Th1-shifted CD4 T cell differentiation and effector functions in humans. Moreover, this is the first conclusive demonstration that Th1-biased immunity in vivo in humans is associated with increased levels of IgG1 and reduced production of IgE and IgG4.

CD4 T Cells Lacking One GATA-3 Allele Manifest Reduced Th2 Cell Differentiation In Vitro. Next, we analyzed the ability of CD4 T cells lacking one functional GATA-3 allele to differentiate into the Th2 direction in vitro. Naive CD4 T cells were isolated from the peripheral blood of the GATA-3^{+/-} individuals and cultured under Th2-inducing conditions. In accordance with the in vivo data, Th2 cell differentiation from CD4 cells of the GATA-3^{+/-} individuals was less effective compared with the control group,

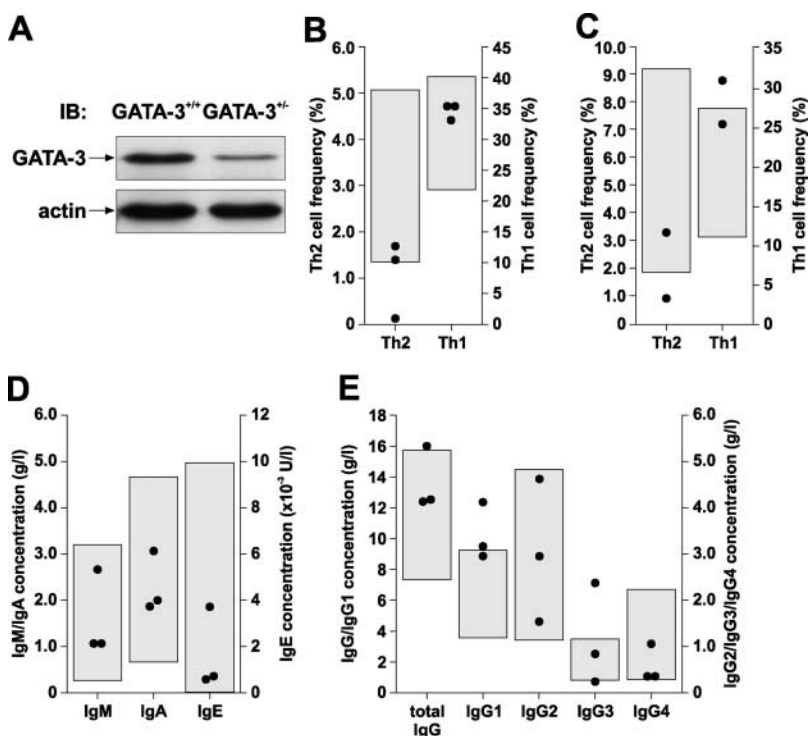


Figure 1. CD4 T cells from GATA-3^{+/-} individuals express reduced levels of GATA-3 associated with decreased Th2 cell differentiation and reduced Th2 effector functions. (A) Whole cell lysates of CD4 T cells from GATA-3^{+/-} (individual no. 1) and GATA-3^{+/+} (control) individuals were analyzed by Western blotting with mAbs to GATA-3 and actin. One representative of three independent experiments with similar results is shown. (B) Freshly isolated memory CD4 T cells from GATA-3^{+/-} individuals were stimulated in vitro with PMA and ionomycin and analyzed for cytoplasmic IL-4 (Th2) and IFN- γ (Th1). Dots indicate Th2 and Th1 cell frequencies of the individual GATA-3^{+/-} individuals, whereas the shaded areas denote mean \pm SD of 20 age-matched control individuals. (C) Freshly isolated naive T cells of GATA-3^{+/-} individuals were primed in vitro under Th2-inducing conditions, stimulated with PMA and ionomycin, and assessed for cytoplasmic IL-4 and IFN- γ . Dots indicate Th2 and Th1 cell frequencies of two GATA-3^{+/-} individuals. The third GATA-3^{+/-} individual was not analyzed because of limited numbers of available cells. Shaded areas denote the normal range of control individuals. (D and E) Serum Ig levels of GATA-3^{+/-} individuals. Shaded areas reflect the normal limits.

Table II. *Th2/Th1 Ratio*^a

	In Vivo	In Vitro ^b
Control group	0.108 ± 0.057	0.350 ± 0.261
Individual no. 1	0.037	0.108
Individual no. 2	0.006	0.038
Individual no. 3	0.049	ND

^aData are presented as absolute values or mean ± SD.

^bEffector cells were generated from naive CD4 T cells.

whereas Th1 cell differentiation was increased (Fig. 1 C). This resulted in a markedly decreased Th2/Th1 ratio when compared with the control group (Table II). Therefore, reduced GATA-3 protein levels in human CD4 T cells diminishes the efficiency of Th2 cell differentiation in vitro while favoring Th1 development.

Th1-dominated immunity has been implicated in the immunopathology of several autoimmune diseases (14). Moreover, impaired Th2 cell differentiation has been documented at the onset of autoimmune rheumatoid inflammation and has been suggested to contribute to the development of chronic unresolved Th1 inflammation (12). However, despite the marked Th1 shift in the GATA-3^{+/-} individuals, they did not manifest clinical signs of T cell-mediated immunologic disorders at the time of analysis, such as autoimmune diseases (9, 10). Furthermore, the sera of the GATA-3^{+/-} individuals were negative for autoantibodies to kidney, adrenal gland, thyroid, parathyroid gland, and the mucosa of the stomach, and for autoantibodies to nuclear and mitochondrial antigens (not depicted). From the genetic point of view, induction of autoimmunity is a result of particular combinations of allelic variants or DNA mutations that affect the expression/function of genes involved in immune responses (15, 16). However, most isolated mutations have an almost undetectable effect with regard to autoimmunity and only in combination with allelic variants of other loci do they result in an autoimmune phenotype (15). Therefore, although GATA-3 is an important gene for directing the immune response, the decrease of its expression level appears not to be sufficient to induce autoimmune disorders in the genetic environment of the GATA-3^{+/-} individuals analyzed here.

The loss of one GATA-3 allele in humans results clinically in hypoparathyroidism, sensorineural deafness, and renal insufficiency (8). This raises the possibility that metabolic abnormalities caused by hypoparathyroidism and renal insufficiency might have affected lymphocyte responses in the GATA-3^{+/-} individuals. However, analysis of Th differentiation in vivo and in vitro and of serum Ig levels in three control patients with chronic renal insufficiency and in two patients with hypoparathyroidism did not reveal any differences compared with GATA-3^{+/+} individuals (not depicted). These data strongly support the view that Th1-

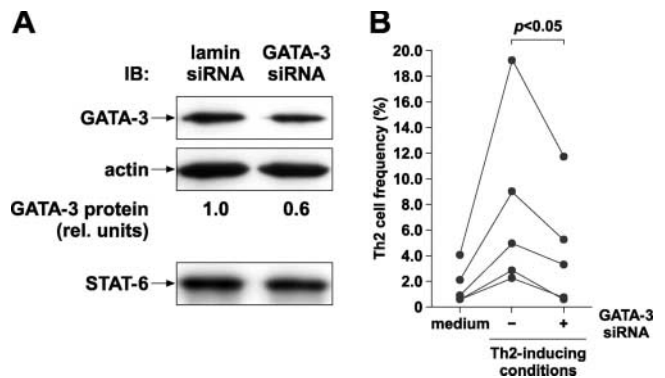


Figure 2. Silencing of GATA-3 inhibits human Th2 cell differentiation. (A) Freshly isolated CD4 memory T cells from GATA-3^{+/+} individuals were transfected with siRNA specific for GATA-3 or lamin (control), and GATA-3, actin, and STAT-6 protein levels were assessed 2 d later by Western blot analysis. GATA-3 protein levels were assessed by densitometric analysis after Western blot and normalized for actin. (B) After transfection, freshly isolated memory T cells from GATA-3^{+/+} individuals were cultured under Th2-inducing conditions. For control, untransfected cells were analyzed after priming in neutral (non-Th1/Th2-inducing) conditions (medium). Cytoplasmic IL-4 was determined in the generated effector populations as described in Materials and Methods. Data from five independent experiments with cells from individual donors are indicated by dots and connected with lines.

shifted immunity in GATA-3^{+/-} individuals is a consequence of insufficient GATA-3 protein expression.

Silencing of GATA-3 by GATA-3-specific siRNA Results in Impaired Th2 Cell Differentiation In Vitro. To substantiate the important role of GATA-3 in human Th2 cell differentiation in greater detail, we blocked GATA-3 expression by transfecting freshly isolated memory CD4 T cells from GATA-3^{+/+} control individuals with siRNA specific for GATA-3. After 2 d of culture in the presence of non-mitogenic concentrations of IL-2, the GATA-3 protein level was assessed by Western blot analysis. Transfection of memory CD4 T cells with GATA-3 siRNA resulted in a markedly decreased level of GATA-3 protein when compared with control siRNA (Fig. 2 A, lamin). In contrast, the protein level of STAT-6, another transcription factor important in Th2 cell differentiation, was not affected. Priming of GATA-3 siRNA-transfected memory CD4 T cells under conditions that were optimal for Th2 cell differentiation from human memory cells (12, 17) resulted in significantly reduced Th2 differentiation (Fig. 2 B), indicating a dominant role of GATA-3 in initiating the Th2 cell differentiation program. Thus, the results are concordant with the results from GATA-3^{+/-} CD4 T cells.

Expression of GATA-3 Is Up-regulated During Human Th2 Cell Differentiation. In the mouse GATA-3 is expressed at a high level during Th2 cell development (2). To analyze GATA-3 expression during human Th2 cell differentiation, CD4 naive T cells were isolated from GATA-3^{+/+} control individuals and cultured under Th2- or Th1-inducing conditions for 5 d. GATA-3 mRNA levels were assessed in the generated effector populations by highly sensitive quantitative real-time PCR. A more than twofold

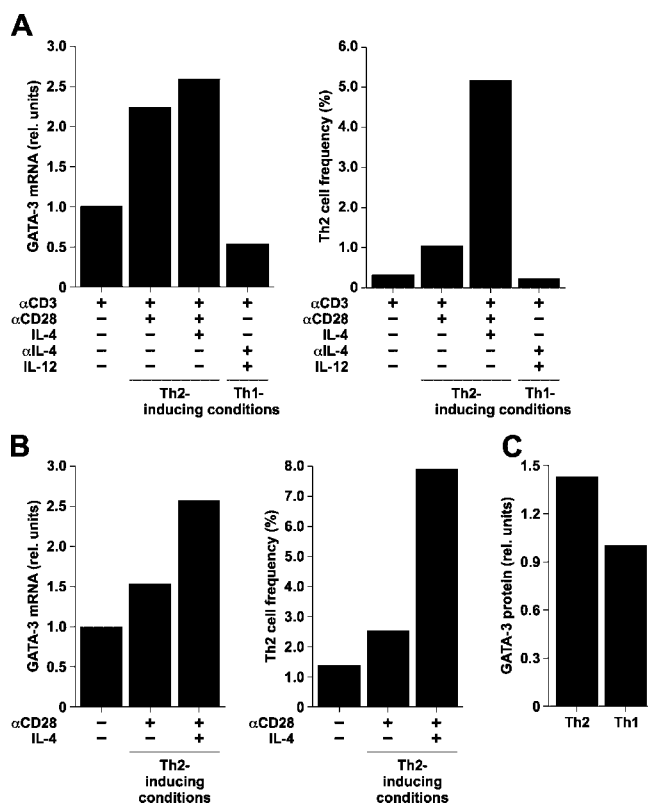


Figure 3. GATA-3 is regulated during human T cell differentiation in vitro. (A) Freshly isolated CD4 naive T cells from GATA-3^{+/+} individuals were primed for 5 d in the presence of mAbs to CD3, CD28, and/or IL-4 and/or recombinant IL-4 or IL-12 as indicated. GATA-3 mRNA was assessed in the generated effector populations by real-time PCR (left). Th2 cell frequencies were determined in the same populations after an additional 2.5 d of rest by assessing cytoplasmic IL-4 and IFN- γ after stimulation with PMA and ionomycin (right). (B) Freshly isolated CD4 memory T cells from GATA-3^{+/+} individuals were primed in the presence or absence of mAbs to CD28 and/or recombinant IL-4 as indicated and GATA-3 mRNA was assessed as in A (left). Th2 cell frequencies were determined as described in A in the same populations (right). (C) Th2 and Th1 effector memory T cells from GATA-3^{+/+} individuals were sorted based upon their ability to produce IL-4 and IFN- γ , respectively, to purity. GATA-3 protein levels in the sorted populations were assessed by densitometric analysis after Western blot and normalized for actin.

increase of the GATA-3 mRNA level was observed in cells primed with anti-CD3 and CD28 mAbs (Th2-inducing conditions) when compared with cells primed with anti-CD3 mAb alone (neutral conditions; Fig. 3 A). A further increase was induced when IL-4 was added to the priming culture (Fig. 3 A). In contrast, Th1-inducing conditions resulted in marked reduction of the GATA-3 mRNA level (Fig. 3 A). The levels of the GATA-3 mRNA correlated with the Th2 cell frequencies in the respective populations as assessed by flow cytometric analysis of cytoplasmic IL-4 (Fig. 3 A). An increase in the GATA-3 mRNA level was also observed during Th2 cell differentiation from human memory CD4 cells (Fig. 3 B). Thus, human Th2 cell differentiation is accompanied by an increase in the GATA-3 mRNA level. As a consequence, the significant increase in GATA-3 mRNA during human Th2 cell differentiation

resulted in increased GATA-3 protein levels in sorted IL-4-producing Th2 cells when compared with IFN- γ -producing Th1 cells (Fig. 3 C). Of interest, Th1 cells expressed a relatively high level of GATA-3 protein, which was not anticipated. Whether this relates to an important function of GATA-3 in maintaining T cell-specific transcriptional regulation as reported previously (13, 18, 19) or to a low turnover of GATA-3 protein remains to be elucidated.

The identification of Th2-specific transcription factors that control the production of IL-4 and, hence, the continued formation of Th2 cells might allow a selective manipulation of Th subsets, for example in human diseases that are characterized by unbalanced Th cell differentiation, such as autoimmune diseases or allergies. The determination of whether these transcription factors are also involved in regulating Th2 development in human cells is a first step to evaluate such a possibility. Using the unique opportunity to evaluate GATA-3^{+/-} individuals and using diverse in vitro assays we could for the first time conclusively demonstrate an important role of the transcription factor GATA-3 in human Th2 cell differentiation. As significantly reduced levels of GATA-3 expressed in CD4 T cells of GATA-3^{+/-} individuals resulted in decreased Th2 cell differentiation in vivo and in vitro associated with markedly diminished Th2 cell-mediated effector functions in vivo, targeting GATA-3 might be a successful means to modulate inappropriate Th2 effector functions.

The authors thank Daniela Thein for her expert technical assistance. We are grateful to Dr. R. Vilella for his generous gift of the anti-CD45RA mAb and to Drs. M. Pavel, M. Hammerschmidt, and S. Jank (all Erlangen) for providing blood of control patients.

This work was supported by the Deutsche Forschungsgemeinschaft (Schu 786/2-3), by the Federal Ministry of Education and Research (BMBF, Kompetenznetzwerk Rheumatologie, C2-5), and by the Interdisciplinary Center for Clinical Research (IZKF) at the University Hospital of the University of Erlangen-Nuremberg (project B27).

Submitted: 1 August 2003

Accepted: 1 December 2003

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