

Letter to the Editor

Profiling of human CD4⁺ T-cell subsets identifies the T_H2-specific noncoding RNA GATA3-AS1

To the Editor:

Host defense against diverse pathogens involves the recruitment and differentiation of CD4⁺ T-cell effector subsets. After recognition of antigens presented by antigen-presenting cells, naive CD4⁺ T (NT) cells can differentiate into T effector subsets, including T_H1 cells, T_H2 cells, T_H17 cells, and regulatory T (Treg) cells.¹ T_H1 and T_H2 cells are characterized by releasing IFN- γ or IL-4, IL-5, and IL-13, respectively.¹ T_H1 cells are involved in cellular immunity against intracellular microorganisms, whereas T_H2 cells are involved in allergy and responses to helminth infection.¹ T-bet controls T_H1 cell differentiation, whereas T_H2 cells are regulated by GATA binding protein 3 (GATA3). T_H17 cells produce IL-17 and IL-17F and are critical in mucosal and epithelial host defense.¹ Treg cells are characterized by the expression of the key transcription regulator forkhead box protein 3 (FOXP3) and express IL-10 and TGF- β . Treg cells play important roles in the maintenance of immune homeostasis.¹ Distinct

CD4⁺ T-cell lineages are achieved and maintained through epigenetic processes that require chromatin remodeling by recruiting chromatin remodeling complexes after activation of lineage-specific transcription factors during differentiation.²

Gene expression microarray (GEM) studies indicate that thousands of genes are involved in CD4⁺ T-cell differentiation. GEM analyses of CD4⁺ T effector subsets might help to identify novel lineage-specific markers and regulatory factors, which could improve our understanding of T-cell differentiation and how it relates to human diseases. However, previous GEM studies have not analyzed the expression of long intergenic non-protein-coding RNAs (lincRNAs). LincRNAs have been shown to regulate gene transcription and differentiation of embryonic stem cells through physically binding to multiple chromatin regulatory proteins involved in chromatin remodeling complexes,³ indicating that lincRNAs might play a role in CD4⁺ T-cell differentiation. Furthermore, GEM studies have been primarily undertaken with *in vitro*-polarized human CD4⁺ T cells and mouse CD4⁺ T cells, which might not fully resemble the characteristics of human CD4⁺ T effector cells *in vivo*.

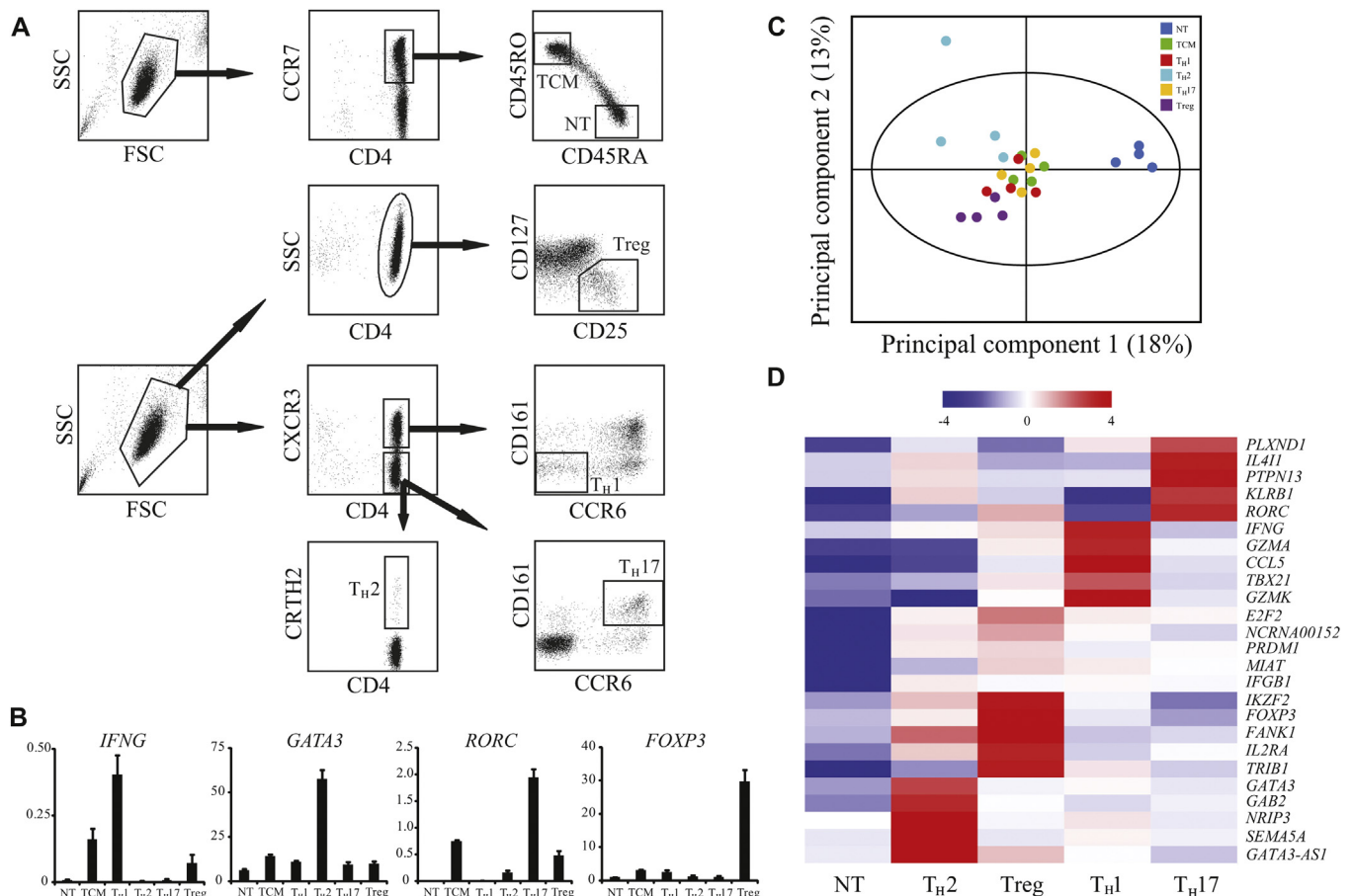


FIG 1. Analyses of human primary CD4⁺ T-cell subsets. **A**, Illustration of flow cytometric sorting. **B**, mRNA expression of *IFNG*, *GATA3*, *RORC*, and *FOXP3*. **C**, Principal component analysis of CD4⁺ T-cell subsets. **D**, Heat map presentation of subset-specific genes. High and low expression is shown in red and blue, respectively. TCM, CD4⁺ T central memory cells.

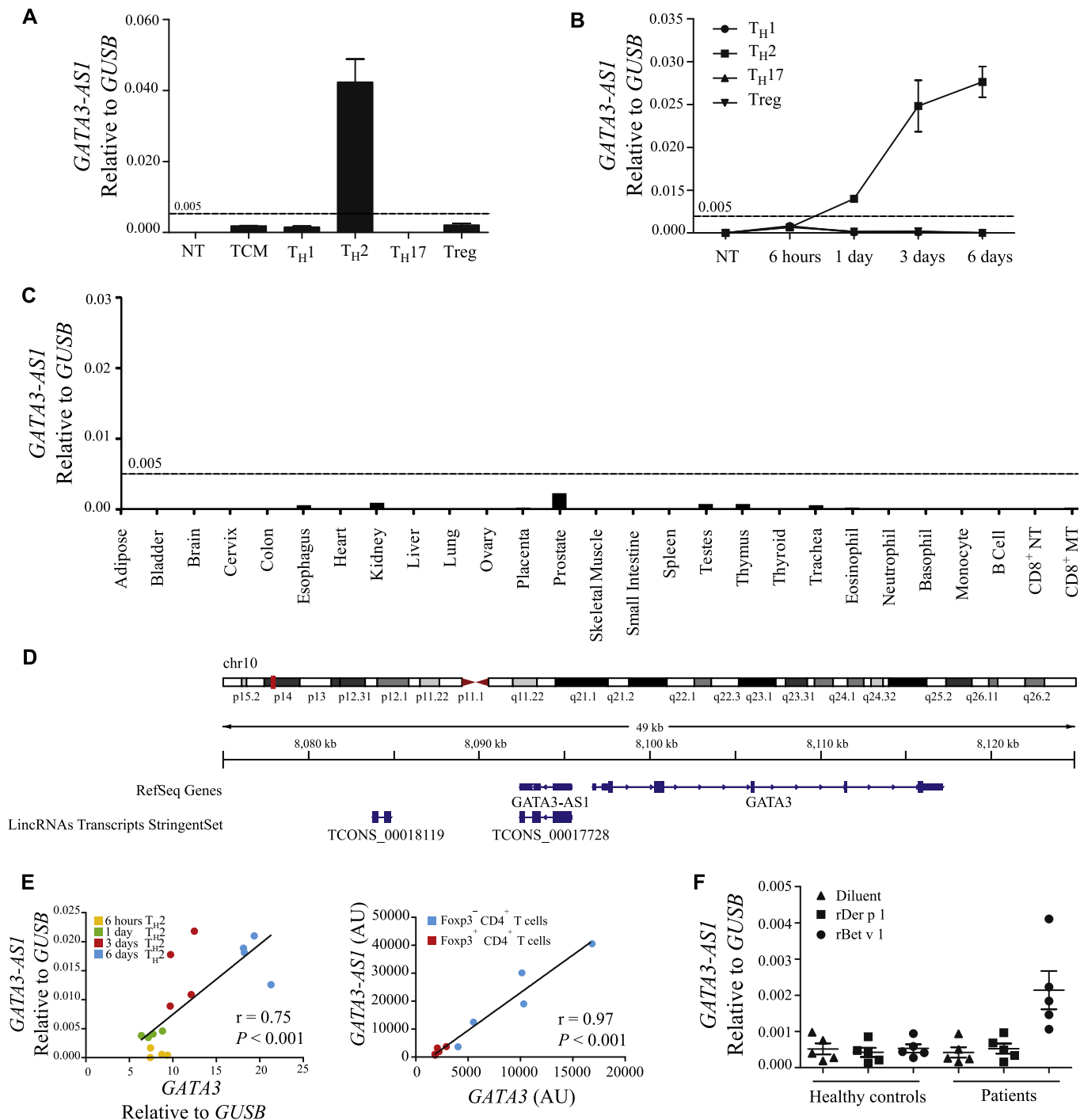


FIG 2. GATA3-AS1 is a T_H2-specific large intergenic noncoding RNA. **A**, GATA3-AS1 in human primary CD4⁺ T-cell subsets. **B**, GATA3-AS1 in polarized CD4⁺ T-cell subsets. **C**, GATA3-AS1 in human tissues and immune cells. **D**, GATA3-AS1 in human genome. **E**, Correlation between GATA3-AS1 and GATA3. **F**, GATA3-AS1 in *in vitro* cultured CD4⁺ T cells from allergic patients and control subjects.

In this study we performed genome-wide profiling of mRNA and lincRNA in human primary NT cells, CD4⁺ T central memory cells, and T_H1, T_H2, T_H17, and Treg cells (accession no. GSE43005).^{4,6} The aims were to (1) identify novel CD4⁺ T lineage-specific markers *in vivo* and (2) test whether T_H2-specific markers were relevant for allergy, a model T_H2 disease. The primary CD4⁺ T-cell subsets were purified from buffy coats of 4

healthy donors by means of flow cytometric sorting and confirmed by using real-time PCR analysis of lineage-specific genes (Fig 1, A and B). Principal component analysis of the GEM data showed a clear separation between NT cells and primary CD4⁺ T effector cell subsets along the first component (18%), whereas the difference between effector subsets were explained in the second component (13%; Fig 1, C).

Next, we defined subset-specific genes of each CD4⁺ T-cell subset, including NT, T_H1, T_H2, T_H17, and Treg cells, as genes that differed in expression (a fold change ≥ 2 or ≤ -2 and a *P* value $\leq .05$) in 1 subset against the other 4 subsets. This led to the identification of 330 genes in the NT subset, 24 genes in the T_H1 subset, 72 genes in the T_H2 subset, 22 genes in the T_H17 subset, and 104 genes in the Treg subset (see Tables E1–E5 in this article's Online Repository at www.jacionline.org). Top subset-specific genes included T-box 21 (*TBX21*) and *IFNG* in T_H1 cells; *GATA3*, semaphorin 5A (*SEMA5A*), *GATA3* antisense RNA 1 (*GATA3-AS1*), and GRB2-associated binding protein 2 (*GAB2*) in T_H2 cells; killer cell lectin-like receptor subfamily B, member 1 (*KLRB1*), RAR-related orphan receptor C (*RORC*), and plexin D1 (*PLXND1*) in T_H17 cells; and interleukin 2 receptor, alpha (*IL2RA*), fibronectin type III and ankyrin repeat domains 1 (*FANK1*), IKAROS family zinc finger 2 (*IKZF2*), and *FOXP3* in Treg cells. A heat map presentation of selected top subset-specific genes is shown in Fig 1, D.

Of note, we found that lincRNA *GATA3-AS1*, also termed *FLJ45983*, was specifically expressed in primary T_H2 cells (Fig 1, D). This T_H2-specific expression pattern was confirmed by using real-time PCR (Fig 2, A). To characterize its expression during T-cell differentiation, we polarized NT cells toward T_H1, T_H2, T_H17, and Treg cells, which were validated by means of real-time PCR (see Fig E1 in this article's Online Repository at www.jacionline.org). *GATA3-AS1* was increased specifically in *in vitro*-polarized T_H2 cells from 24 hours after polarization (Fig 2, B). Further analysis of *GATA3-AS1* in 27 human tissues and immune cell types showed that *GATA3-AS1* was largely undetectable or had very limited expression (Fig 2, C), supporting its T_H2-specific expression pattern.

Significantly, the transcription start sites of *GATA3-AS1* and *GATA3* are 1220 bp apart (Fig 2, D), suggesting a potential shared bidirectional promoter.⁷ Indeed, *GATA3-AS1* and *GATA3* were significantly correlated (Pearson $r = 0.75$, $P < .001$; Fig 2, E, left panel). This result was supported by an independent correlation analysis ($r = 0.97$, $P < .001$; Fig 2, E, right panel) by using the public GEM data of sorted FOXP3⁺ Treg and FOXP3[−] ex-Treg cells, the latter of which expressed T_H2 signature genes, including *GATA3*, *IL4*, *IL5*, and *IL13*, on loss of FOXP3 (accession no. GSE26190).⁸ Taken together, this indicates that the expression of *GATA3-AS1* and *GATA3* might be coregulated by the same regulatory elements and might have shared functions in T_H2 cell responses.

Finally, we examined whether *GATA3-AS1* would increase in patients with a T_H2-associated disease, namely seasonal allergic rhinitis.⁹ We stimulated PBMCs from 5 patients with seasonal allergic rhinitis who are allergic to birch pollen but not to house dust mite and 5 healthy control subjects and analyzed *GATA3-AS1* in enriched total CD4⁺ T cells. An increase in *GATA3-AS1* was observed in recombinant Bet v 1-challenged CD4⁺ T cells from patients but not healthy control subjects (Fig 2, F). This is consistent with increased expression of *GATA3-AS1* in Bet v 1-challenged, Bet v 1-specific T-cell clones (see Fig E2 in this article's Online Repository at www.jacionline.org). Elucidation of the functions of *GATA3-AS1* is warranted in future studies.

In summary, this first systematic genome-wide analysis of human primary CD4⁺ T-cell subsets helped to identify novel CD4⁺ T lineage-specific genes. LincRNA *GATA3-AS1* might serve as a specific indicator of T_H2 response and T_H2-associated diseases and might be involved in T_H2 cell differentiation. Our study will provide an important reference for mRNA and lincRNA studies of human T-cell subsets *in vivo* and how these relate to diseases.

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TITLE PAGE**Letter to editor****Profiling of human CD4⁺ T subsets identifies a Th2-specific non-coding RNA
GATA3-AS1**

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35 Methods**36 Ethics statement**

37 The usage of blood from patients and healthy controls has been approved by the ethics
38 committee of the Linköping University. We obtained written informed consent from
39 all subjects. The written consent was obtained on a special form with information
40 about the study and the conditions of the study, according to instructions from the
41 ethics committee.

42

43 Subjects

44 Five patients with seasonal allergic rhinitis (SAR), who are allergic to birch pollen but
45 not to house dust mite, and 5 healthy controls were included. ImmunoCap Rapid
46 (Phadia, Thermo Fisher Scientific, Uppsala, Sweden) test revealed that all patients
47 displayed birch pollen-specific but no house dust mite-specific IgE. All healthy
48 controls are negative to all tested allergen sources including birch pollen and house
49 dust mite. The median (range) age of patients was 23 (21-31) and 3 were women. The
50 median (range) age of healthy controls was 22 (22-29) and 3 were women.

51

52 Cell sorting strategy and flow cytometry

53 For the purification of human primary CD4⁺ T subsets, peripheral blood mononuclear
54 cells (PBMCs) were enriched from buffy coat of four healthy donors using
55 Lymphoprep (Axis-Shield PoC, Oslo, Norway) according to the manufacturer's
56 protocol. Next, total CD4⁺ T cells were purified from PBMCs by Magnetic-activated
57 cell sorting (MACS) negative sorting using a Total CD4⁺ T cells Isolation Kit II from
58 Miltenyi Biotec (Bergisch-Gladbach, Germany). For the isolation of naïve CD4⁺ T

cells ($CD4^+CCR7^+CD45RA^+CD45RO^-$) and $CD4^+$ central memory T cells ($CD4^+CCR7^+CD45RA^-CD45RO^+$), purified total $CD4^+$ T cells were labeled with Pacific Blue™ anti-human CD4, PE anti-human CCR7, FITC anti-human CD45RO and APC anti-human CD45RA. For the isolation of primary Th1 cells ($CD4^+CXCR3^+CCR6^-CD161^-$), Th2 cells ($CD4^+CXCR3^-CRTH2^+$), Th17 cells ($CD4^+CXCR3^-CCR6^+CD161^+$) and Treg cells ($CD4^+CD25^+CD127^{low}$), total $CD4^+$ T cells were labeled with Pacific Blue™ anti-human CD4, FITC anti-human CD161, PE anti-human CRTH2, PerCP/Cy5.5 anti-human CXCR3, PE/Cy7 anti-human CCR6, APC anti-human CD127 and APC/Cy7 anti-human CD25. All antibodies were purchased from Biolegend (San Diego, CA, USA) except that PE anti-human CRTH2 was purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany).

For the analysis of *GATA3-AS1*, eosinophils ($SSC^{high}CD16^-$), neutrophils ($SSC^{high}CD16^+$), basophils ($CD123^+HLA-DR^-$), monocytes ($SSC^{low}CD14^{high}$), B cells ($CD19^+CD20^+$), naïve $CD8^+$ T cells ($CD8^+CD45RA^+CD45RO^-$) and $CD8^+$ memory T cells ($CD8^+CD45RA^-CD45RO^+$) were sorted from one buffy coat as previously reported.¹ Mouse anti-human CD8-Pacific Blue™, mouse anti-CD123-APC, mouse anti-HLA-DR-PE and mouse anti-human CD45RO-PerCP/Cy7 were purchased from BD Pharmingen (San Diego, CA, USA). Mouse anti-human CD14-FITC, mouse anti-human CD16-APC, mouse anti-human CD19-PerCP/Dy5 and mouse anti-human CD20-APC were purchased from Immuno Tools GmbH (Friesoythe, Germany). Mouse anti-human CD45RA-APC was purchased from Biolegend (San Diego, CA, USA).

The flow cytometry sorting was performed using FACS Aria flow cytometer (BD

Biosciences, San Jose, CA, USA). The purity of the sorted cells, confirmed by flow cytometric analysis, was above 99%. The data was analyzed by FlowJo 7.6 (Treestar, Inc., San Carlos, CA).

***In vitro* polarization of human Th1, Th2, Th17 and Treg cells**

Naïve CD4⁺ T cells were isolated from buffy coat of four healthy donors using a naïve CD4⁺ T cell isolation kit (Miltenyi, Bergisch-Gladbach, Germany). Naïve CD4⁺ T cells were stimulated with plate-bound anti-CD3 (500 ng/mL), soluble anti-CD28 (500 ng/mL), in the presence of IL-12 (5 ng/mL), IL-2 (10 ng/mL) and anti-IL-4 (5 µg/mL) for Th1, IL-4 (10 ng/mL), IL-2 (10 ng/mL) and anti-IL-12 (5 µg/mL) and anti-IFN-γ (5 µg/mL) for Th2, TGF-β (1 ng/mL), IL-1β (10 ng/mL), IL-6 (25 ng/mL), IL-21 (25 ng/mL) and IL-23 (25 ng/mL) for Th17, and TGF-β (10 ng/mL) and IL-2 (10 ng/mL) for Treg.^{2, 3} Cells were cultured for six days in Iscove's modified Dulbecco medium (IMDM) supplemented with 2 mM L-glutamine (PAA Laboratories, Linz, Austria), 10% heat-inactivated FCS (PAA Laboratories, Linz, Austria), 5 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri, USA) and 50 µg/mL gentamicin (Sigma-Aldrich, St. Louis, Missouri, USA).

Stimulation of PBMCs with allergen

PBMCs were incubated with diluent, recombinant Bet v 1 (rBet v 1, ALK Abello', Hørsholm, Denmark; 5 µg/mL), or recombinant Der p 1 (rDer p 1, Biorbyt, Riverside, UK; 5 µg/mL) for seven days in RPMI 1640 supplemented with 2 mM L-glutamine (PAA Laboratories, Linz, Austria), 5% human AB serum (Lonza, Switzerland), 5 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri, USA) and 50 µg/mL gentamicin (Sigma-Aldrich, St. Louis, Missouri, USA). Next, CD4⁺ T cells were

enriched by MACS negative sorting using a Total CD4⁺ T cells Isolation Kit II from Miltenyi Biotec (Bergisch-Gladbach, Germany).

Allergen-specific T cell clones

Two Bet v 1-specific CD4⁺ T cell clones were stimulated with rBet v 1 (Biomay AG, Vienna, Austria; 5ug/mL) or rHev b 1 (Biomay AG, Vienna, Austria; 5ug/mL) in the presence of autologous EBV-transformed B cells in a ratio of 1:2 for 3 days.⁴ Thereafter, CD4⁺ T cells were purified by MACS negative sorting using a Total CD4⁺ T cells Isolation Kit II from Miltenyi Biotec (Bergisch-Gladbach, Germany).

Gene expression microarray analysis

Total RNA was extracted from naive CD4⁺ T cells, CD4⁺ T central memory T cells, Th1, Th2, Th17 and Treg subsets using a miRneasy Mini Kit (Qiagen, Valencia, CA, USA). RNA concentrations were analyzed with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). The cRNA was prepared using a LowInput QuickAmp Labeling Kit (Agilent Technologies, Palo Alto, Calif, USA). Gene expression microarray analysis was performed using SurePrint G3 Human Gene Expression 8X60K microarray according to the manufacture's instruction (Agilent Technologies, Palo Alto, Calif, USA). Probes that were detected in at least one condition (four replicates), namely no less than 4 out of 24 samples, were included for analysis. The microarray data was quantile normalized using R. A two-tailed Student's t test and a fold-change ≥ 2 or ≤ -2 and a P value ≤ 0.05 was used to identify genes that differed in expression.

Quantitative real time PCR

Total RNA from 20 different human tissues was obtained from the FirstChoice® Human Total RNA Survey Panel (Ambion, Austin, TX, USA). The cDNA synthesis was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The expression of *IFNG* (Hs00989291_m1), *GATA3* (Hs00231122_m1), *RORC* (Hs01076122_m1), *IL17* (Hs00174383_m1), *FOXP3* (Hs01085834_m1), and *GATA3-AS1* (Hs01651755_m1) was analyzed by real-time PCR performed on an ABI 7900HT Real-Time PCR cycler with the Taqman Gene expression Master Mix and Taqman probe (Applied Biosystems, Foster City, CA, USA). *GUSB* was used as an internal control. The gene expression was calculated relative to *GUSB* by using a delta CT method. One-way ANOVA followed by Tukey's post-hoc test was used to compare differences between multiple groups.

Gene expression microarray data

Microarray data can be found at the NCBI Gene Expression Omnibus (GEO) repository with accession number GSE43005. A public gene expression microarray data of sorted FOXP3⁺ Treg and FOXP3⁻ ex-Treg cells was retrieved from the GEO repository (accession number: GSE26190).⁵

Statistics

Principal component analysis (PCA) was performed using SIMCA-P+ 13 software (UMETRICS, Umeå, Sweden). Clustering analysis was performed using hierarchical clustering analysis in GeneSpring GX software (Agilent Technologies, Palo Alto, Calif, USA). Visualization of gene location was performed using the Integrative

159 Genomics Viewer (IGV) and the stringent set of human lincRNA was downloaded
160 from http://www.broadinstitute.org/genome_bio/human_lincrnas/lincRNA_catalog.^{6, 7}
161 Statistical analyses were performed by Graphpad Prism 5 (Graphpad Software, San
162 Diego, CA, USA). Data were expressed as the mean $\pm$ SEM. A *P* value less than 0.05
163 was considered significant.
164

165

166 **Figure legends**

167 **Figure E1. Identification of polarized CD4⁺ T effector subsets.** Naive CD4⁺ T cells
168 were polarized towards Th1, Th2, Th17 and Treg cells. The mRNA expression of
169 *IFNG*, *GATA3*, *RORC*, *IL17* and *FOXP3* was determined in polarized subsets at day 6.
170 N = 4.

171

172 **Figure E2. Expression of *GATA3-AS1* in Bet v 1-specific CD4⁺ T cell clone.** Bet v
173 1-specific T cell clone cells were co-cultured with EBV-transformed B cells in the
174 presence of diluent, rBet v 1 or and rHev b 1 for 3 days. The mRNA expression of
175 *GATA3-AS1* and *GATA3* in purified CD4⁺ T cells was analyzed by real-time PCR. N
176 = 2. Triangle and circle represent two T cell clones, respectively.

177

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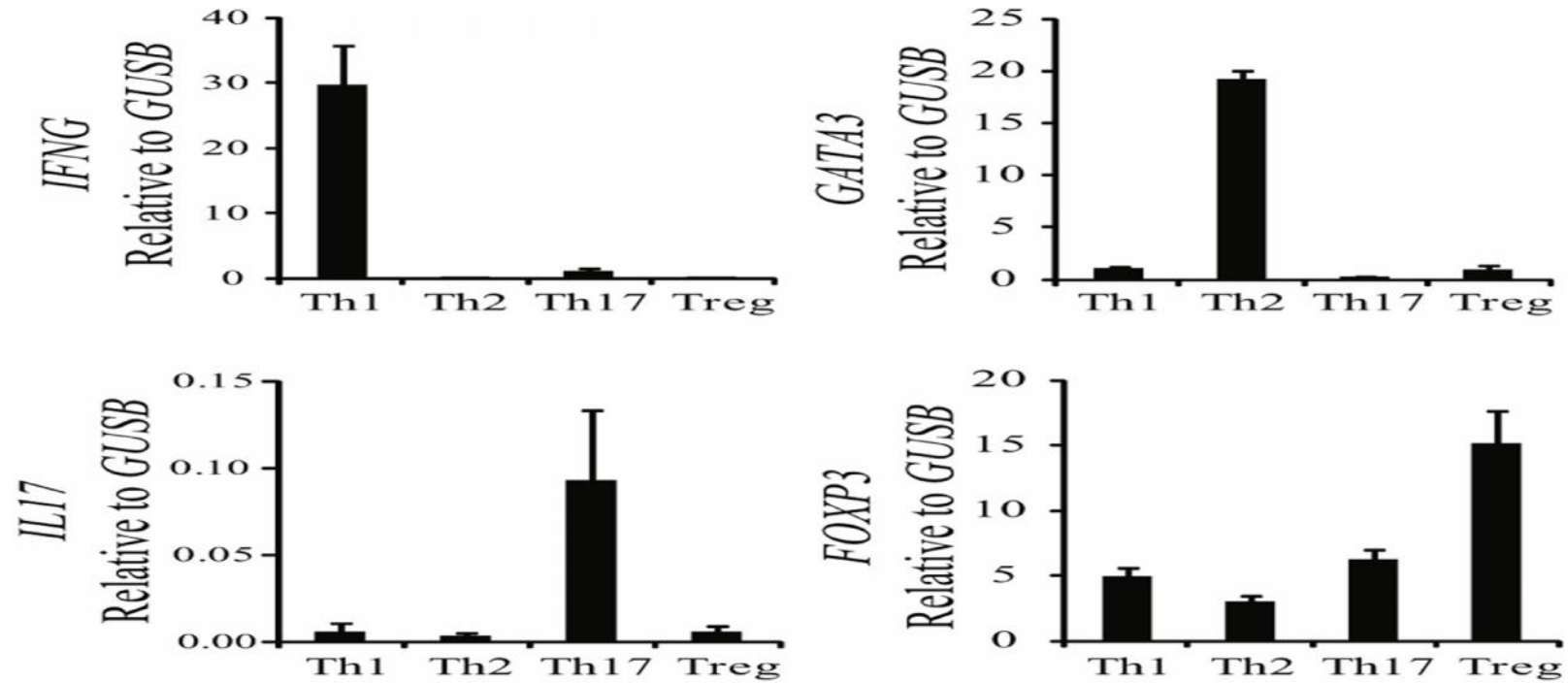


Fig E1

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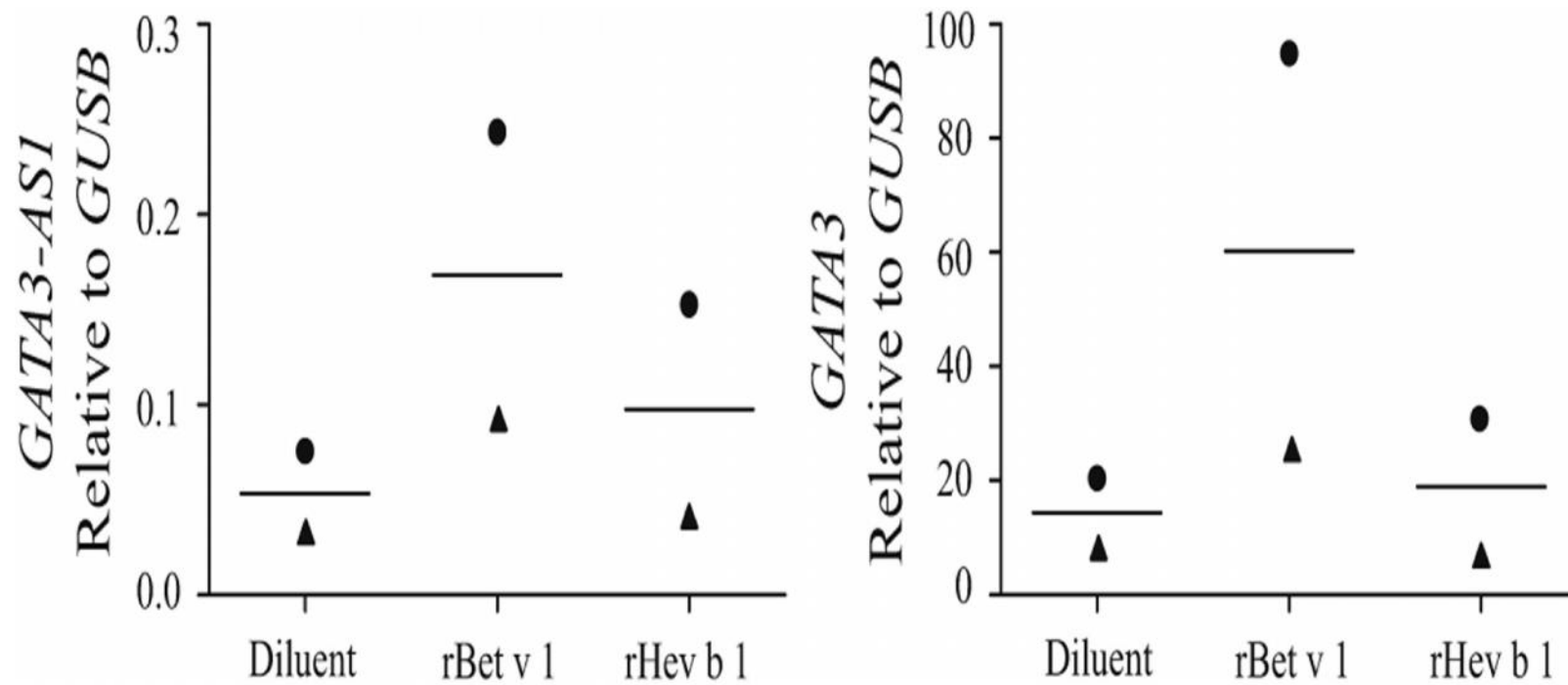


Fig E2

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