THE JOURNAL OF IMMUNOLOGY

Activating Transcription Factor 3 Is a Positive Regulator of Human *IFNG* Gene Expression

Sanna Filén, Emmi Ylikoski, Subhash Tripathi, Anne West, Mari Björkman, Joel Nyström, Helena Ahlfors, Eleanor Coffey, Kanury V. S. Rao, Omid Rasool and Riitta Lahesmaa

J Immunol 2010;184;4990-4999; Prepublished online 19 March 2010;
doi:10.4049/jimmunol.0903106
http://www.jimmunol.org/content/184/9/4990

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Activating Transcription Factor 3 Is a Positive Regulator of Human *IFNG* Gene Expression

Sanna Filén,*,† Emmi Ylikoski,*,‡ Subhash Tripathi,* Anne West,* Mari Björkman,* Joel Nyström,* Helena Ahlfors,*,§ Eleanor Coffey,* Kanury V. S. Rao,¶ Omid Rasool,* and Riitta Lahesmaa*,‖

IL-12 and IL-18 are essential for Th1 differentiation, whereas the role of IFN-α in Th1 development is less understood. In this microarray-based study, we searched for genes that are regulated by IFN-α, IL-12, or the combination of IL-12 plus IL-18 during the early differentiation of human umbilical cord blood CD4$^+$ Th cells. Twenty-six genes were similarly regulated in response to treatment with IL-12, IFN-α, or the combination of IL-12 plus IL-18. These genes could therefore play a role in Th1 lineage decision. Transcription factor activating transcription factor (*ATF*) *3* was upregulated by these cytokines and selected for further study. Ectopic expression of *ATF3* in CD4$^+$ T cells enhanced the production of IFN-γ, the hallmark cytokine of Th1 cells, whereas small interfering RNA knockdown of *ATF3* reduced IFN-γ production. Furthermore, ATF3 formed an endogenous complex with JUN in CD4$^+$ T cells induced to Th1. Chromatin immunoprecipitation and luciferase reporter assays showed that both ATF3 and JUN are recruited to and transactivate the *IFNG* promoter during early Th1 differentiation. Collectively, these data indicate that ATF3 promotes human Th1 differentiation. ***The Journal of Immunology*****, 2010, 184: 4990–4999.**

Naive CD4$^+$ Th lymphocytes are capable of differentiating into functionally distinct subsets. These include IFN-γ, IL-2, and TNF-β–secreting Th1 cells, IL-4, IL-5, and IL-13–secreting Th2 cells (1), IL-17, IL-17F, IL-21, IL-22, and TNF-secreting Th17 cells, (2–6), and naturally occurring CD4$^+$CD25$^+$ Foxp3$^+$ T regulatory cells (7–11). The differentiation process is initiated by Ag encounter (i.e., activation via TCR/CD28) and controlled by the cytokine environment (reviewed in Ref. 12). IL-12R–mediated STAT4 signaling is the key event in Th1 lineage commitment, as shown by the studies using knockout mice deficient for IL-12 (13), IL-12Rβ2 (14), or Stat4 (15), which all exhibit impaired Th1 responses and reduced IFN-γ production. IL-18 is a cytokine that is also intrinsically involved in Th1 differentiation. It synergizes with IL-12 in the induction of IFN-γ, although it is unable to drive Th1 polarization on its own (16).

The precise role of type I IFNs in STAT4-dependent Th1 differentiation and IFN-γ production has yet to be resolved. Initially, it was shown by Brinkmann et al. (17) that human CD4$^+$ T cells produce increased levels of IFN-γ in the presence of IFN-α, which suggests that type I IFNs favor Th1 differentiation. Later, IFN-α/β in synergy with IL-18 was reported to induce IFN-γ production by T cell blasts (18). Furthermore, in the same report, IFN-α was shown to increase *IFNG* mRNA synthesis in human T cells. There is also clear evidence that type I IFNs are able to tyrosine phosphorylate and activate STAT4 in primary human T cells (19, 20). However, the ability of IFN-α to optimally drive the in vitro polarization of human Th1 cells has been called into question (21). Although IL-12 induced sustained phosphorylation of STAT4, the response to IFN-α was only transient and not sufficient for efficient Th1 polarization.

We hypothesized that genes regulated by several Th1-inducing cytokines are likely to be important for Th1 differentiation. To study this, we profiled the cytokine-induced (IFN-α, IL-12, or the combination of IL-12 plus IL-18) gene expression during the early stages of human umbilical cord blood CD4$^+$ T cell differentiation. Oligonucleotide microarrays with probes for ~22,000 genes were used. This resulted in the identification of 26 genes with a similar expression pattern in response to the studied cytokines. Several of the genes identified have not been previously associated with Th1 differentiation, although the expected regulation of known marker genes was also observed. Activating transcription factor (*ATF*) *3* was upregulated by all the cytokines studied. It is a member of the ATF/CREB family of transcription factors, and its functional role in human Th cell differentiation has not previously been addressed. In this report, we show that in Th1-promoting conditions, ATF3 positively regulated *IFNG* gene expression and Th1 differentiation.

Materials and Methods

Primary CD4$^+$ T cell isolation and culture

Lymphocytes were isolated from heparinized human umbilical cord blood samples (Turku University Hospital, Turku, Finland) or from buffy coats from healthy blood donors (Red Cross Finland Blood Service, Helsinki, Finland) by Ficoll-Paque gradient centrifugation (Amersham Pharmacia

*Turku Centre for Biotechnology, University of Turku and Åbo Akademi University; †Drug Discovery Graduate School and ‡Turku Graduate School for Biomedical Sciences, University of Turku; §National School for Informational and Structural Biology, Åbo Akademi University, Turku, Finland; ¶International Centre for Genetic Engineering and Biotechnology, New Delhi, India; and ‖Immune Disease Institute, Harvard Medical School, Boston, MA 02115

Received for publication September 28, 2009. Accepted for publication February 17, 2010.

This work was supported by the National Technology Agency of Finland, the Academy of Finland, Turku University Hospital Fund, Turku University Foundation, Ida Montin Foundation, Oskar Öflund Foundation, and Hengityssairauksien Tutkimussäätiö.

The sequences presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession number GSE20198.

Address correspondence and reprint requests to Dr. Riitta Lahesmaa, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, P.O. Box 123, FI-20521 Turku, Finland. E-mail address: riitta.lahesmaa@btk.fi

The online version of this article contains supplemental material.

Abbreviations used in this paper: act, anti-CD3/anti-CD28 activation; ATF, activating transcription factor; ChIP, chromatin immunoprecipitation; N.D., not detected; o/n, overnight; siRNA, small interfering RNA; SLR, signal $\log_2$ ratio.

Biotech, Uppsala, Sweden). $CD4^+$ T cell enrichment was performed using anti-CD4–conjugated magnetic beads (Dynal, Oslo, Norway). Cells were cultured in Yssel's medium (22) supplemented with 1% human AB serum (Red Cross Finland Blood Service). Cells were activated with either plate-bound anti-CD3 (2.5 μg/ml for coating) and 0.5 μg/ml soluble anti-CD28 (Immunotech, Marseille, France) or with 100 ng/ml PHA (Murex Diagnostics, Chatillon, France) and irradiated CD32/B7 transfected mouse L fibroblasts. A total of 2.5 ng/ml IL-12 (R&D Systems, Minneapolis, MN), 25 ng/ml IL-18 (Medical & Biological Laboratories, Nagoya, Japan), and 100 U/ml highly purified human leukocyte IFN-α (Red Cross Finland Blood Service) were used to induce Th1 polarization and 10 ng/ml IL-4 (R&D Systems) to promote Th2 differentiation. For neutral Th0 conditions, no polarizing cytokines were added. Where indicated, 3 μg/ml neutralizing anti–IFN-γ (clone 25718, R&D Systems) was added to the culture media. To promote proliferation, 17 ng/ml IL-2 (R&D Systems) was added on the second day of culture to 7-d cultures.

Microarray studies

Cells were harvested at different time points and lyzed in TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA was further purified using an RNeasy minikit (Qiagen, Valencia, CA). Sample preparation for GeneChip oligonucleotide array hybridizations was performed according to Affymetrix's instructions using 5 μg total RNA as starting material. Samples were hybridized to HG-U133A arrays (Affymetrix, Santa Clara, CA). Microarray Suite 5.1 software (Affymetrix) was used for data analysis. Data was normalized using the global normalization method and the expression analysis settings predefined by the manufacturer. Changes in gene expression levels between two samples were considered significant if $-1 \geq$ signal $\log_2$ ratio (SLR) ≥ 1, which equals a 2-fold change.

Quantitative real-time RT-PCR (TaqMan)

Total RNA was isolated from cells cultured in polarizing conditions with an RNeasy Mini Kit (Qiagen), and cDNA synthesis was performed from 0.5 μg DNase I treated total RNA using SuperScript II (Invitrogen). The primer and probe sequences used in TaqMan analysis were as follows: pIFNG-fwd: 5′-ctcgaaacagcatctgactcctt-3′; pIFNG-rev: 5′-tgtccaacgcaaagcaataca-3′; probe-IFNG: 5′-(FAM)-tgctggcgacagttcagccatcac-(TAMRA)-3′; pEEF1A1-fwd: 5′-ctgaaccatccaggccaaat-3′; pEEF1A1-rev: 5′-gccgtgtggcaatccaat-3′; and probe-EEF1A1: 5′-(FAM)-agcgccggctatgcccctg-(TAMRA)-3′. TaqMan analyses were performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) as described earlier (23). All analyses were performed in duplicate in two separate runs using samples from at least three cultures. Housekeeping gene *EEF1A1* was used as a reference for normalization (24).

Western blotting

Cells were lyzed in SDS sample buffer (62 mM Tris-HCl [pH 6.8], 2% w/v SDS, 10% v/v glycerol, 50 mM DTT, and 0.1% w/v bromphenol blue), and proteins were separated by SDS-PAGE by a standard Laemmli method (25). The separated proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Buckinghamshire, U.K.) and blocked with 5% nonfat dried milk and 0.1% Tween 20/TBS. All Ab incubations were performed in blocking solution, and the following Abs/antiserum were used: 1:1000 anti-ATF3 (rabbit IgG, C-19; Santa Cruz Biotechnology, Santa Cruz, CA), 1:800 anti-JUN (mouse IgG2a, clone 3; BD Biosciences, Franklin Lakes, NJ), 1:7500 goat anti-rabbit Ig-HRP (BD Biosciences). and 1:10,000 goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). The ECL detection system (Amersham Biosciences) was used for visualization of proteins. β-actin was blotted to control for equal loading with 1:10,000 anti–β-actin (AC-15; Sigma-Aldrich, St. Louis, MO).

Plasmid constructs and small interfering RNA oligonucleotides

ATF3 was cloned into pcDNA3.2/V5/GW/D-TOPO vector (Invitrogen) according to the manufacturer's instructions using PCR primers pATF3-fwd: 5′-caccatgatgcttcaacacc-3′ and pATF3-rev: 5′-ttagctctgcaatgttccttc-3′ and cDNA prepared from total RNA isolated from human T cells as template. The Gateway Vector Conversion System (Invitrogen) was used to modify pIRES2-H-$2K^k$ vector (26) into Gateway destination vector pIRES2-GW-H-$2K^k$. *ATF3* was subcloned from pcDNA3.2 vector into pIRES2-GW-H-$2K^k$ via pDONR/Zeo vector (Invitrogen) generating vector pATF3-IRES2-H-$2K^k$. PCR primers pJUN-fwd-EcoRI: 5′-atcgaattcatgactgcaaagatggaaacgac-3′ and pJUN-rev-BamHI: 5′-atcggatcctcaaaatgtttgcaactgctgcg-3′ were used to directly clone *JUN* into EcoRI-BamHI–digested pIRES2-H-$2K^k$ to create plasmid pJUN-IRES2-H-$2K^k$.

Cloning of *IFNG* promoter constructs illustrated in Fig. 7*A* was started by amplifying a 1.25-kb fragment from human *IFNG* promoter with primers IFNG_fwd: 5′-atgtgcccattaagcgtttg-3′ and IFNG_rev: 5′-agctgatcaggtccaaagga-3′ using human gDNA as template. Nested sets of primers were used to clone *IFNG* promoter fragments of differing length to KpnI-HindIII–digested pGL4.10[*luc2*] vector (Promega, Madison, WI). The sequences of the nested primers were: IFNG_1147_f: 5′-atatggtaccgaccaaattgagatgcac-3′; IFNG_940_f: 5′-atatggtacctacaccaccatgcctg-3′; IFNG_730_f: 5′-atatggtaccatgtcaaatacctttattaac-3′; IFNG_510_f: 5′-atatggtaccttactaaccaactctgat-3′; IFNG_292_f: 5′-atatggtaccgggaggtacaaaaaaattt-3′; IFNG_+38_r: 5′-atataagcttcttctaatagctgatcttca-3′; IFNG_1147_as: 5′-atataagcttgaccaaattgagatgcac-3′; and IFNG_+38_as: 5′-atatggtacccttctaatagctgatctt-3′. Deletion mutations of the potential ATF3/JUN binding sites were generated on pIFNG1147-GL4.10 using Phusion Site-Directed Mutagenesis Kit (Finnzymes, Espoo, Finland). The primers used for the generation of the mutations were: IFNG_del1_f: 5′-cctccctagtagctgagattacaggca-3′; IFNG_del1_r: 5′-agaatggcttgaacccagaaggtg-3′; IFNG_del2_f: 5′-attttaccagggcgaagtggggag-3′; IFNG_del2_r: 5′-tcattcacaaagcacattgatcatttgc-3′; IFNG_del3_f: 5′-cgtcaaaggacccaaggagtctaaagg-3′; IFNG_del3_r: 5′-cattatgcccacctgtgccattct-3′; IFNG_del4_f: 5′-cctcaggagacttcaattaggtataaatacc-3′; IFNG_del4_r: 5′-tcacaagtttttttaagatgagatggtg-3′; IFNG_del5_f: 5′-agtgccttcaaagaatcccaccag-3′; and IFNG_del5_r: 5′-caccattcaaggactggaaatttttttg-3′.

Small interfering RNA (siRNA) oligonucleotides targeting *ATF3* (ATF3-siRNA3: 5′-cauuugauauacaugcucatt-3′; ATF3-siRNA4: 5′-gaaaccucuuuauccaacatt-3′; and ATF3-siRNA-new2: 5′-gaaggaacauugcagagcu-3′) and control (scramble-siRNA1: 5′-gcgcgcuuuguaggauucguu-3′) (all from Sigma/Proligo, Evry Cedex, France) were used in knockdown studies.

Nucleofection, dead cell removal, and enrichment of transfected cells

The protocol for nucleofection, dead cell removal, and enrichment of transfected cells is described elsewhere (26). Briefly, 5 μg plasmids pATF3-IRES2-H-$2K^k$ and empty vector pIRES2-H-$2K^k$ or 1.5 μg oligonucleotide siRNAs targeting *ATF3* or control (scramble) were used per 4 to 5×10^6 cells suspended in 100 μl Opti-MEM I (Invitrogen). Postnucleofection, the cells were let to rest 20 h in RPMI 1640 supplemented with 10% FBS, penicillin plus streptomycin, and 2 mM L-glutamine. When the cells were nucleofected with plasmid DNA, a Dead Cell removal kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-H-$2K^k$–coated magnetic beads (Miltenyi Biotec) were used to remove dead cells and enrich the transfected cells. Cells transfected with oligonucleotide siRNAs were not sorted. In vitro Th1 differentiation of the transfected cells was performed by using anti-CD3/anti-CD28 activation and IL-12 as a polarizing cytokine as described above.

Jurkat cell transfections

Jurkat E6-1 or Jtag cell lines were maintained in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and 2 mM L-glutamine. A total of 5×10^6 cells were transfected by electroporation with 5–15 μg plasmid DNA, and the cells were rested overnight (o/n). The next day, the cells were activated with 50 ng/ml PMA (Calbiochem, San Diego, CA) and 1 μg/ml PHA (Sigma-Aldrich). When the effect of *ATF3*/*JUN* overexpression on *IFNG* gene transcription was studied, the cells were harvested after 24 h of activation and H-$2K^k$–positive cells were sorted using magnetic beads as described elsewhere (26). Cells used in transactivation assays were not H-$2K^k$ selected.

Flow cytometric analysis of intracellular cytokines

Sample preparation for flow cytometric analysis of intracellular cytokine staining was performed as previously described (26). Briefly, cells harvested 7 d after culture were stimulated with 5 ng/ml PMA (Calbiochem) and 0.5 pg/ml ionomycin (Sigma-Aldrich) in Yssel's medium. A portion of the cells was incubated in Yssel's medium only and used as an unstimulated control. After 2 h of stimulation, 10 μg/ml brefeldin A (Alexis Biochemicals, Lausanne, Switzerland) was added to all samples, and incubation was continued for 3 h. Cells were washed and fixed with 4% (w/v) paraformaldehyde/PBS and permeabilized with 0.5% (w/v) saponin/PBS. Anti-human–IFN-γ-FITC (B27; Caltag Laboratories, Burlingame, CA) was used for staining of the intracellular IFN-γ, and mouse IgG1-FITC (Caltag Laboratories) was used as an isotypic control. Cells were analyzed with an FACSCalibur Flow Cytometer (BD Biosciences).

IFN-γ cytokine assay

Human umbilical cord blood $CD4^+$ T cells that were nucleofected with siRNA oligonucleotides were induced toward Th1 as described above. Supernatants were collected after 24 h of culture for analysis of IFN-γ production. After 7 d of stimulation, the cells were treated with 5 ng/ml PMA and 0.5 pg/ml ionomycin for 24 h and the supernatants collected. A

Bio-Plex Cytokine Assay kit (Bio-Rad, Hercules, CA) was used for IFN-γ detection according to the manufacturer's instructions, and the assays were analyzed with Luminex 100 IS 2.2 (Luminex, Austin, TX).

Nuclear extracts and ATF3/JUN coimmunoprecipitations

Human umbilical cord blood $CD4^+$ T cells were isolated, activated with anti-CD3 and anti-CD28, and cultured in the presence or absence of IL-12 or IL-4 or in the absence of cytokines as described above. Cells were harvested after 48 h, and nuclear extracts were prepared as described below. The cells were washed twice with PBS and lyzed in 250 μl lysis buffer (21.8 mM HEPES [pH 7.9], 0.2% Tween-20, 1 mM NaF, 1 mM Na_3VO_3, 1 mM DTT, 1 mM PMSF, and protease inhibitor Complete Mini [Roche, Basel, Switzerland]). After 2 min incubation, cells were centrifuged, and cytoplasmic fractions were collected. A total of 100 μl nuclear lysis buffer (21.8 mM HEPES (pH 7.9), 430 mM NaCl, 1% glycerol, 1 mM NaF, 1 mM Na_3VO_3, 1 mM DTT, 1 mM PMSF, and protease inhibitor Complete Mini [Roche]) was added, and the samples were incubated on ice for 1 h. Thereafter, the samples were sonicated, centrifuged, and the soluble fractions collected. Nuclear extracts were precleared with 100 μl protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) for 2 h at 4˚C. The samples were spun, and supernatants were collected. A total of 1.5 μg anti-ATF3 Ab and 10 μl JUN antiserum (polyclonal anti-JUN was raised in rabbits inoculated with rGST-human-JUN [5–89 aa]) were used for immunoprecipitation; 1.5 μg anti-RANKL Ab (rabbit IgG, FL-317; Santa Cruz Biotechnology) was used as control. Ab incubations were done o/n at 4˚C. A total of 50 μl protein G-Sepharose beads were added for collecting the protein-Ab complexes, and the samples were incubated 1 h at 4˚C. The beads were washed three times in 1 ml PBS, and 20 μl SDS sample buffer was added for eluting the proteins in 95˚C for 5 min.

Chromatin immunoprecipitation assay

Freshly isolated human umbilical cord blood $CD4^+$ T cells were induced toward Th1 and Th2 as described above. Poststimulation, the protein-DNA complexes were cross-linked with 1/10 volume of cross-linking solution (50 mM HEPES-KOH [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 11% formaldehyde) for 10 min and quenched with 1/20 volume of 2.5 mM glycine solution for 10 min, after which the cells were washed twice with ice-cold PBS. The cells were pelleted, and nuclear fractions were prepared. The cell pellet was resuspended in 30 ml lysis buffer 1 (50 mM HEPES KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and 0.25% Triton X-100 with protease inhibitor Complete Mini [Roche]) and mixed for 10 min at 4˚C. Postcentrifugation at 2000 × *g* for 10 min at 4˚C, the cell pellet was resuspended in 24 ml lysis buffer 2 (200 mM NaCl, 1 mM EDTA [pH 8], 0.5 mM EGTA, and 10 mM Tris [pH 8], with protease inhibitor) at room temperature for 10 min. Postcentrifugation at 2000 × *g* for 10 min at 4˚C, the pellet was resuspended in 10 ml lysis buffer 3 (1 mM EDTA [pH 8], 0.5 mM EGTA [pH 8], and 10 mM Tris [pH 8], with protease inhibitor). The chromatin was sheared by sonication, and the fragmentation was analyzed with agarose gel electrophoresis. The majority of the DNA fragments were in the 400–1000 bp range. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was collected for chromatin immunoprecipitation (ChIP). A total of 20 μl chromatin supernatant was retained as the DNA input control in the following PCR reaction.

Magnetic beads were used for ChIP, and 50 μl sheep anti-rabbit IgG-conjugated Dynabeads (Dynal) were conjugated with 10 μg anti-ATF3 (C-19; Santa Cruz Biotechnology), 10 μl polyclonal anti-JUN, or 5 μg control rabbit IgG (sc-2027; Santa Cruz Biotechnology) in PBS containing 5 mg/ml BSA o/n at 4˚C. Beads were collected by centrifugation, washed three times with PBS/BSA, and resuspended in 100 μl PBS/BSA for immunoprecipitation. Equal volumes of soluble chromatin were divided into sample and control and adjusted to 0.1% Triton X-100, 0.1% sodium deoxycholate, and 1 mM PMSF, mixed with 100 μl magnetic beads prebound to the Ab and incubated at 4˚C o/n. The magnetic beads were collected, and the supernatant was removed. To remove nonspecifically bound material, the beads were resuspended to 1 ml RIPA buffer (50 mM HEPES [pH 7.6], 1 mM EDTA, 0.7% sodium deoxycholate, 1% Nonidet P-40, and 0.5 M LiCl, with protease inhibitor mixture) at 4˚C. The beads were collected and washed with RIPA buffer a total of 10 times. After washing once with 1 ml TE buffer, the beads were collected by centrifugation at 2000 × *g* for 3 min and resuspended in 50 μl elution buffer (50 mM Tris [pH 8], 10 mM EDTA, and 1% SDS). To elute precipitated chromatin from the beads, the tubes were incubated at 65˚ for 30 min with constant agitation and centrifuged for 1 min at 2000 × *g*. A total of 40 μl eluted supernatants were removed and mixed with 120 μl TE buffer. Similarly, 20 μl chromatin input control was mixed with 140 μl TE buffer. Cross-links were reversed by incubation at 65˚C o/n.

Proteins in the DNA sample were removed by incubation with 120 μl proteinase K solution (2% glycogen, 5% proteinase K stock solution, and TE) for 2 h at 37˚C. The DNA was then extracted twice with phenol and once with 24:1 chloroform/isoamyl alcohol. The sample was adjusted to 200 mM NaCl. After ethanol precipitation, the DNA was dissolved in 30 μl TE containing 10 μg DNase-free RNase A and incubated for 2 h at 37˚C. The DNA was further purified with a Qiagen PCR kit (Qiagen). Primers F1: 5′-cttgcagtgagccgagattg-3′, R1: 5′-ccagcctaggtgacagagtaagat-3′, F2: 5′-gaccaaattgagatgcaccgtagg-3′, R2: 5′-cacttcgccctggtaaaatgtt-3′, F3: 5′-cattctttccttgctttctggtc-3′, and R3: 5′-tgcttcttttacatatgggtcctg-3′ were used for PCR amplification.

Transactivation assays

Jurkat Jtag cells (27) were used in transactivation assays, and they were transfected as described above. *IFNG* promoter constructs were cotransfected with either pATF3-IRES2-H-$2K^k$ or pJUN-IRES2-H-$2K^k$ plasmids alone or with both. A total amount of 15 μg plasmid DNA/5 × 10^6 cells was used in transfections. The values of luciferase assays of the mutation analysis were normalized by the transfection efficiency values (i.e., the percentage of H-$2K^k$–positive cells). Britelite plus (PerkinElmer, Waltham, MA) was used to measure luciferase activity, and the results were read with a Victor 1420 Multilabel Counter (PerkinElmer).

Prediction of ATF3/JUN binding sites

Putative ATF3/JUN binding sites in the human *IFNG* promoter (−1147 to +38) were analyzed with both MatInspector (Genomatix Software, Munich, Germany) and AliBaba 2.1 (Biobase, Wolfenbuettel, Germany).

Accession numbers

The Affymetrix gene expression data were deposited to the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) with accession number GSE20198.

Results

IFN-α–induced IFNG *gene expression peaks at 6 h of stimulation*

Because the induction of *IFNG* gene expression by type I IFNs has been controversial, we were interested in studying how potent human leukocyte IFN-α is in inducing *IFNG* transcription in human umbilical cord blood $CD4^+$ T cells. $CD4^+$ cells, purified using magnetic selection, were activated with immobilized anti-CD3 and soluble anti-CD28 and cultured in the presence or absence of 100 U/ml IFN-α. In addition, 3 μg/ml neutralizing Ab against IFN-γ was used to inhibit the induction of *IFNG* gene by autocrine IFN-γ production. Anti–IFN-γ (9 μg/ml) has previously been shown to effectively block IFNGR-induced T-bet expression and STAT1 phosphorylation in human $CD4^+$ T cells (28). The 3 μg/ml anti–IFN-γ used in this study was also effective in blocking T-bet induction after 24 h of cell culture in Th1-inducing conditions (Supplemental Fig. 1). Cells were harvested for quantitative real-time RT-PCR analysis of *IFNG* mRNA at the time points of 0, 6, 12, 24, and 48 h. Although anti-CD3/anti-CD28 activation was a potent inducer of *IFNG* transcription, IFN-α receptor signaling greatly enhanced the gene induction, which peaked first at 6 h, was diminished at 12 h, and induced again at 48 h poststimulation (Fig. 1). The expression peak at 6 h was concordant with the finding that IFN-α was able to induce only transient STAT4 phosphorylation (21).

Microarray studies revealed a set of genes regulated in a similar manner by IL-12, IL-18, and IFN-α

To identify which genes are regulated in a similar fashion by IFN-α, IL-12, and the combination of IL-12 and IL-18, microarray studies were conducted using HG-U133A GeneChip arrays. Human umbilical cord blood $CD4^+$ cells were cultured as described in *Materials and Methods*, and total RNA was isolated from cells harvested at different time points. During the first hours of cell culture, few changes in gene expression can be observed in the

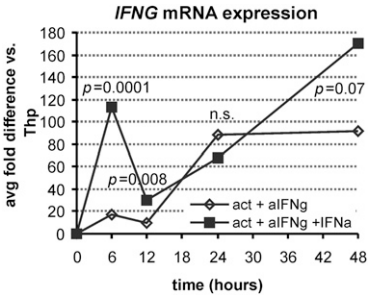

FIGURE 1. IFN-α–induced *IFNG* expression peaks at 6 h. Human cord blood CD4$^+$ T cells were activated with anti-CD3/anti-CD28 (act) in the presence/absence of human leukocyte IFN-α. Neutralizing anti–IFN-γ was added to both cultures. *IFNG* gene expression was studied using quantitative real-time RT-PCR. Results were normalized against the reference gene *EEF1A1* and shown as fold difference versus naive Thp cells. Data are averages from three independent cell cultures. Statistical significance was determined using Student *t* test.

presence of IL-12 (29) due to the absence of IL12Rβ2 on the cell surface of naive Thp cells. Accordingly, the time point of 48 h was chosen for studies on the effect of IL-12 and IL-12 plus IL-18 on gene expression. However, IFN-α signaling is fully functional already in naive Thp cells and, thus, time points of 2, 6, and 48 h were chosen for IFN-α–treated samples.

When the IL-12 plus IL-18–induced gene expression changes were directly compared with those induced by IL-12 (anti-CD3/anti-CD28 activation plus IL-12 plus IL-18 versus anti-CD3/anti-CD28 activation plus IL-12), no reproducible changes between two independent analyses were observed using an SLR cutoff level of $-1 \geq \text{SLR} \geq 1$ (i.e., a 2-fold change). However, IL-18 seemed to function as an enhancer of the effects caused by IL-12. In general, it was observed that most of the genes regulated by the combination of IL-12 and IL-18 behaved similarly. The response was only less pronounced than that resulting from IL-12 treatment alone. Activation of cells with a combination of IL-12 and IL-18 caused the expression of 50 genes to change significantly and reproducibly when compared with activation only. The data are provided as supplemental data (Supplemental Table I).

IFN-α induced significant changes in the expression of 172 genes ($-1 \geq \text{SLR} \geq 1$) in at least two of the three time points studied. The expression of 66 of these changed 4-fold or more, and among these, there were several genes known to be regulated by IFN-α, as well as genes previously unknown to be targets of IFN-α receptor signaling. These data are provided as supplemental data (Supplemental Table II).

We were most interested in elucidating which genes are regulated in a similar manner by IFN-α, IL-12, and the combination of IL-12 plus IL-18. We hypothesized that such genes could play an important role in the polarization of Thp cells along the Th1 lineage. Direct comparisons between the sets of genes that were found to be regulated in response to IFN-α with the genes regulated by the combination of IL-12 and IL-18 revealed that the expression patterns of 26 genes were similar. These genes are presented in Fig. 2 and Supplemental Table III.

LAIR2, *SOCS2*, and *AQP3* were downregulated in response to IL-12, IL-18, and IFN-α receptor signaling and have previously been associated with Th1/Th2 differentiation (30, 31). *CECR1* was also downregulated in response to IL-12, IL-12 plus IL-18, and IFN-α and to our knowledge has not previously been associated with Th functions. Genes that were upregulated in response to IL-12, IL-12 plus IL-18, and IFN-α treatment and previously known to be differentially regulated in Th1/Th2 cells included *NFIL3*, *LIF*, *NKG7*, *IL18RAP*, *1L12RB2*, *GZMB*, *IFNG, GZMH*, *ADAM19*, *BCL6*, *ATF3*

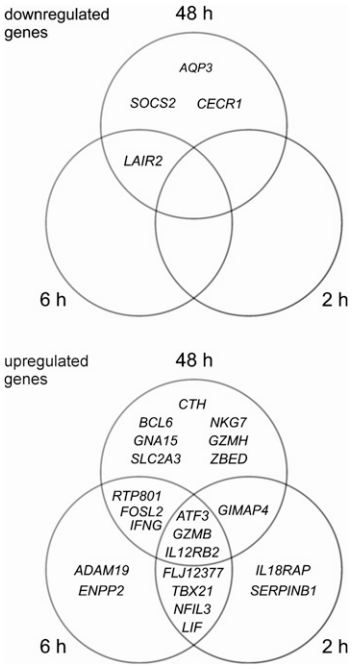

FIGURE 2. Twenty-six genes were similarly regulated by IFN-α and the combination of IL-12 plus IL-18. Human CD4$^+$ cells were activated with anti-CD3 and anti-CD28 Abs and induced to differentiate with IL-12 plus IL-18 or IFN-α. Cells treated with IL-12 plus IL-18 were harvested after 48 h and cells treated with IFN-α after 2, 6, or 48 h. The genes regulated in response to IFN-α treatment at different time points were directly compared with those regulated by IL-12 plus IL-18 after 48 h. Genes showing similar regulation in response to the different treatments were selected for further examination. The Venn diagrams show how the genes with similar expression patterns were regulated by IFN-α during the time-scale studied.

GIMAP4, *SLC2A3*, *FOSL2*, *ZBED2*, *CTH*, and *TBX21* (28–30, 32–40, 41). Genes upregulated and novel in this context were *FLJ12377*, *GNA15*, *SERPINB1*, *DDIT4*, and *ENPP2*.

ATF3 protein is differentially expressed in Th1 and Th2 cells

ATF3 was chosen for further studies because its function in human Th1 and Th2 differentiation has previously not been examined. Western blot analysis showed that although ATF3 protein was virtually absent in naive cord blood CD4$^+$ Thp cells, its expression was rapidly induced after 2 h of TCR activation with anti-CD3 and anti-CD28 mAbs (data not shown). Inducing Th1 polarization with IFN-α, IL-12, or with IL-12 plus IL-18 further upregulated ATF3 protein expression, whereas addition of IL-4 downregulated the ATF3 protein expression. This differential regulation of ATF3 protein became detectable after 12 h of culture and was prominent at 24 h and 48 h (Fig. 3). The presence of neutralizing anti–IFN-γ in the

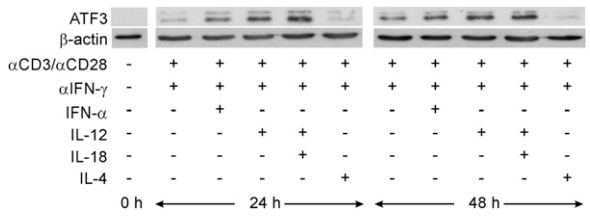

FIGURE 3. ATF3 protein is differentially expressed during the early stages of Th1 or Th2 cell polarization. Human cord blood CD4$^+$ T cells were cultured under the conditions indicated for 0, 24, or 48 h. Proteins were separated on a standard SDS-PAGE gel, transferred to nitrocellulose membrane, and blotted with rabbit anti-ATF3. β-actin expression was used as a loading control. Data are representative of four independent analyses.

culture media demonstrated that the observed regulation of ATF3 expression was not dependent on autocrine IFN-γ production.

ATF3 is a positive regulator of IFN-γ production and T-bet and JUN expression

To study the effects of ATF3 overexpression on Th1 differentiation, human peripheral blood $CD4^+$ T cells were transiently transfected either with pATF3-IRES2-H-$2K^k$ vector or with an empty vector, pIRES2-H-$2K^k$. Dead and apoptotic cells were removed, after which the cells expressing the H-$2K^k$ selection marker were sorted with magnetic beads. Overexpression of ATF3 protein was detected by Western blotting and was shown to be dependent on the amount of pATF3-IRES2-H-$2K^k$ used for nucleofections (Supplemental Fig. 2). For differentiation studies, the cells were cultured in Th1-promoting conditions (i.e., in the presence of anti-CD3/anti-CD28 and IL-12) for 7 d. The ability of the cells to produce IFN-γ after restimulation with PMA plus ionomycin was studied using intracellular cytokine staining and flow cytometry (Fig. 4*A*). The percentage of IFN-γ–positive cells, especially cells that stained highly positive for IFN-γ, was higher in the cell population expressing ectopic *ATF3* than in the mock-transfected cells. The two distinct IFN-γ–positive cell populations are typically detected by intracellular staining and flow cytometry (21, 42, 43) and can be considered as IFN-γ^+ (high) and IFN-γ^+ (low) cell populations.

To confirm the role of ATF3 in IFN-γ expression by an alternative approach, specific siRNA oligonucleotides targeting *ATF3* mRNA were designed. Scrambled sequence was used as a control. Human cord blood $CD4^+$ cells were transiently transfected with the siRNA oligonucleotides and subsequently induced to differentiate toward Th1 by anti-CD3/anti-CD28 activation and IL-12.

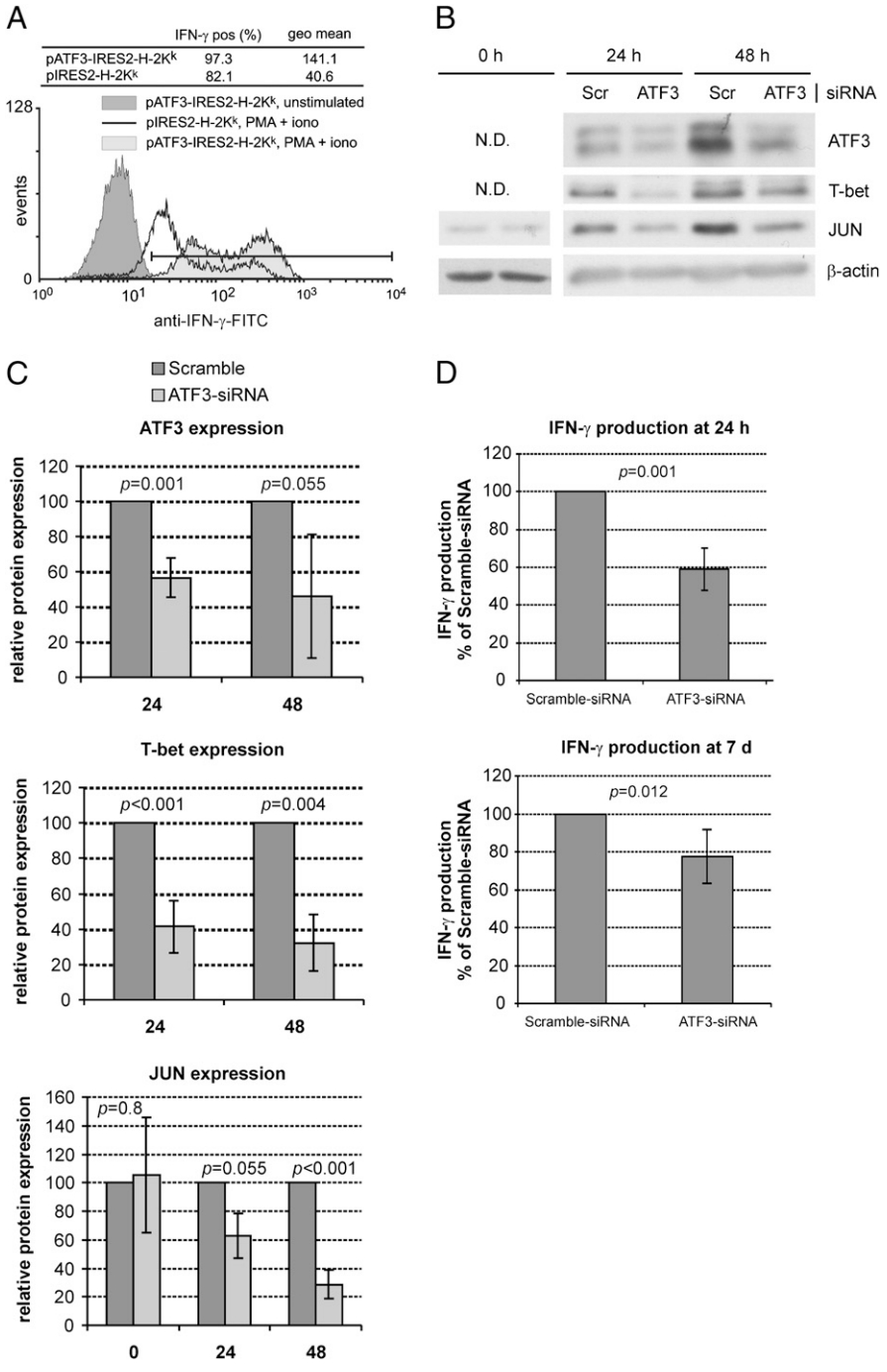

FIGURE 4. ATF3 promotes Th1 differentiation by inducing IFN-γ production. *A*, The *ATF3* gene was introduced into peripheral blood $CD4^+$ cells in pATF3-IRES2-H-$2K^k$ vector by nucleofection. Cells nucleofected with an empty vector pIRES2-H-$2K^k$ were used as controls. Dead cells were removed, and H-$2K^k$–positive transfected cells were sorted by magnetic beads. Cells were cultured for 7 d in Th1-polarizing conditions, after which half of the cells were restimulated with PMA plus ionomycin, whereas the other half was left unstimulated. Cells were stained for intracellular IFN-γ production and analyzed with a flow cytometer. The values represent the percentage of cells stained positive for IFN-γ production and the geometrical mean of fluorescence intensity. Data are representative of three independent analyses. *B–D*, Human cord blood $CD4^+$ T cells were transiently transfected with siRNA oligonucleotides targeted against ATF3. Scrambled sequence was used as a control. Subsequently, the cells were activated with anti-CD3/anti-CD28 and induced to differentiate toward Th1 in the presence of IL-12 for 7 d. *B*, ATF3 knockdown as well as T-bet and JUN expression was assayed by Western blotting after 0, 24, and 48 h of stimulation. Data are representative of three to five individual experiments and obtained using ATF3-siRNA-new2. *C*, The relative ATF3, T-bet, and JUN protein expression was quantified from three to five individual cultures, and the figure shows the average values with error bars indicating the SD. *D*, Supernatants were collected 24 h poststimulation for IFN-γ assay, and reduction in the amount of secreted INF-γ was observed in five out of seven independent experiments. The averages of these five are shown in the figure. Cells cultured for 7 d were restimulated with PMA and ionomycin for 24 h, after which the supernatants were harvested for IFN-γ assay. The figure shows average reduction of IFN-γ secretion from six out of eight independent experiments in which the reduction was observed. Error bars indicate SD. N.D., not detected.

Because ATF3 expression was not detectable by Western blotting in undifferentiated cells, the efficacy of the knockdown was examined from stimulated cells. The residual expression of ATF3 protein varied depending on the oligonucleotide used, but was on average 57% and 46% after 24 h and 48 h of differentiation, respectively (Fig. 4*B*, 4*C*).

The effect of ATF3 knockdown on IFN-γ production was measured from the supernatant by cytokine bead assay after 24 h of stimulation as well as after restimulation of cells cultured for 7 d (Fig. 4*D*). These experiments verified the role of ATF3 as a positive regulator of IFN-γ expression, because the selective knockdown of ATF3 resulted in reduced amounts of IFN-γ production. Consistently, the knockdown of ATF3 also resulted in diminished amounts of T-bet expression (Fig. 4*B*, 4*C*). Most likely, this was due to the decreased level of IFN-γ. However, it is also possible that ATF3 regulated T-bet expression directly by binding to its promoter or via other signaling routes.

JUN is a known positive regulator of ATF3 transcription (44–47), and there is also implication that ATF3 may control JUN expression (48). Furthermore, a recent report described ATF2 as a positive regulator of JUN expression in human T cells (49). Thus, we examined the effect of ATF3 knockdown on JUN expression also and discovered that the ATF3 knockdown resulted in decreased expression of JUN (Fig. 4*B*, 4*C*). Importantly, JUN expression was not affected by ATF3 knockdown at the time of activation (0 h sample), thus indicating that JUN was not an off-target of ATF3 siRNA.

ATF3 and JUN dimerize upon IL-12 stimulation

ATF3 homodimers are known to act as transcriptional repressors (50), but heterodimers ATF3/JUN or ATF3/JUND possess transcriptional activator function (51, 52). Our results clearly indicated that ATF3 was a positive regulator of *IFNG* transcription. This allowed us to hypothesize that to form an activating complex, ATF3 dimerizes with JUN, a transcription factor induced by Th1 differentiation (Supplemental Table I). To test the hypothesis, we conducted coimmunoprecipitation assays of nuclear extracts of cord blood $CD4^+$ cells induced to differentiate toward Th1 and Th2 lineages for 48 h. Both anti-ATF3 and anti-JUN Abs were used for immunoprecipitation, and the protein/Ab complexes were isolated using protein G-Sepharose beads. Proteins were eluted to SDS sample buffer and resolved by SDS-PAGE. Immunoblotting assays showed (Fig. 5*A*) that endogenous ATF3 and JUN coimmunoprecipitated in cells induced to polarize to Th1 direction but not in cells polarized to Th2 direction.

To test the hypothesis further, we cotransfected pJUN-IRES2-H-$2K^k$ and pATF3-IRES2-H-$2K^k$ vectors into Jurkat cells to study the resulting induction of *IFNG* transcription by quantitative real-time RT-PCR. Posttransfection, the cells were rested o/n and activated with PMA/PHA. The cells were harvested after 24 h of activation. Real-time RT-PCR analysis showed (Fig. 5*B*) that although both *JUN* and *ATF3* transfection enhanced *IFNG* transcription compared with the empty vector, the induction induced by cotransfection was greater than that induced by either of the transgenes alone.

ATF3 and JUN bind and transactivate human IFNG *promoter*

ChIP assays were performed to study the recruitment of ATF3 and JUN to the *cis* regulatory elements of the *IFNG* promoter. Abs specific for ATF3 and a JUN-specific antiserum was used. Primers were designed to amplify both the proximal and distal elements of the human *IFNG* promoter. Three different regions were studied: from −1763 to −1084, from −1147 to −293, and from −458 to +216. The ChIP assays were performed on nuclear fractions of

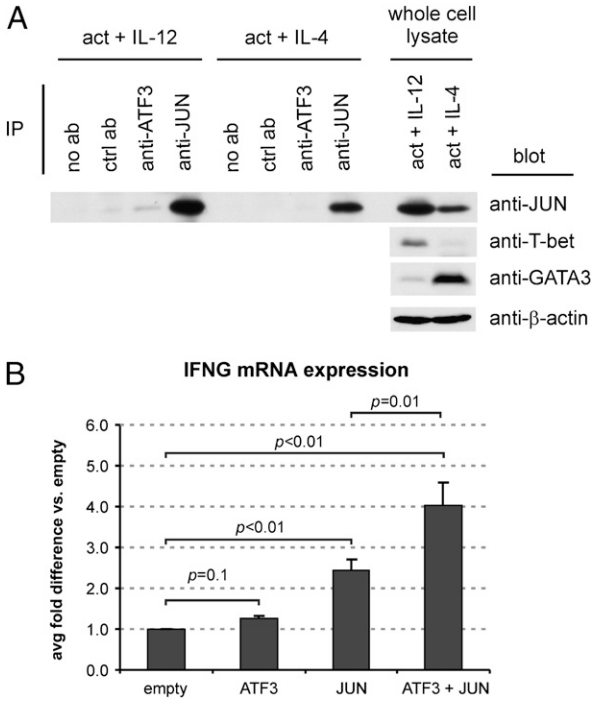

FIGURE 5. ATF3/JUN dimer is formed in $CD4^+$ cells induced to differentiate along Th1 lineage and induces *IFNG* expression in Jurkat cells. *A*, Human cord blood $CD4^+$ T cells were activated using anti-CD3 and anti-CD28 (act) and induced to polarize toward Th1 lineage by IL-12 or toward Th2 lineage by IL-4. After 48 h, the cells were harvested and nuclear fractions were prepared. Coimmunoprecipitations were performed using 1.5 μg anti-ATF3 Ab or 10 μl anti-JUN antiserum. A total of 1.5 μg anti-RANKL Ab was used as a control. One sample was treated with no Abs. Immunoprecipitated protein complexes were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-JUN mAb. As controls, T-bet and GATA3 were detected from total cell lysates (10% of input) to demonstrate that the cells have been induced to polarize properly. The results showed that JUN coimmunoprecipitated with ATF3 in cells induced to differentiate toward the Th1 lineage but not in cells induced to Th2. The membrane was also blotted with anti-ATF3, but the resulting bands were masked by unspecific signals resulting from protein G (data not shown). No mAbs for ATF3 were available at the time of study. Data are representative of three individual experiments. *B*, Jurkat cells were transfected with indicated plasmids and rested for 24 h. The cells were activated with PMA/PHA, harvested after 24 h, and H-$2K^k$–positive cells were selected using magnetic beads. Quantitative real-time RT-PCR analysis showed that although ectopic expression of either *ATF3* or *JUN* increased *IFNG* mRNA expression, cotransfection of both transgenes further increased *IFNG* expression. Data are the average of five individual experiments. Statistical significance was evaluated with Student *t* test. Error bars indicate SEM.

human umbilical cord blood $CD4^+$ T cells induced to differentiate toward Th0, Th1, and Th2 for 9 h as well as undifferentiated Thp cells to study the recruitment of ATF3 and JUN to the *IFNG* promoter in the early stages of Th differentiation. Reproducible binding of both ATF3 and JUN was observed with a primer pair that amplified a 855-bp fragment of the distal promoter (from −1147 to −293) (Fig. 6*A*). The band intensities were quantified with a densitometer, and the data from anti-ATF3– and anti-JUN–precipitated samples were normalized to control IgG. The data demonstrated that the recruitment of both ATF3 and JUN to this region was specific for Th1-promoting conditions and statistically significant ($p = 0.004$ and $p = 0.006$, respectively) (Fig. 6*B*). Some binding of ATF3 and JUN to the other regions (from −1763 to −1084 and from −458 to +216) was also detected, but this binding was neither reproducible nor specific for Th1-inducing conditions (data not shown).

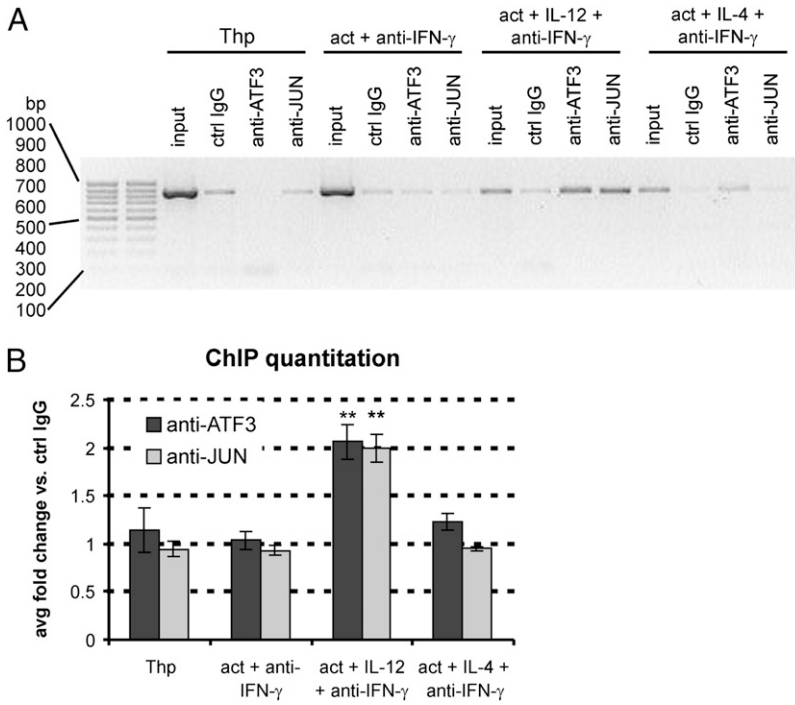

FIGURE 6. ATF3 and JUN are recruited to the *IFNG* promoter in Th1-promoting conditions. Umbilical cord blood $CD4^+$ T cells were induced to differentiate toward Th0, Th1, or Th2 in the presence of anti–IFN-γ for 9 h. ChIP was performed with both anti-ATF3 and JUN antiserum. Reproducible binding of ATF3 and JUN was detected in Th1- promoting conditions. PCR was performed using primers amplifying the *IFNG* promoter from −1147 to −293. *A*, A representative result from at least three independent experiments is shown. *B*, The band intensities were quantified with a densitometer, and the data from anti-ATF3 and anti-JUN precipitated samples were normalized against control IgG. Error bars indicate SEM. $**p < 0.01$. act, anti-CD3/anti-CD28 activation.

Although ATF3 and JUN were able to form a protein complex in Th1-inducing conditions, and they both were recruited to the *IFNG* promoter, the data do not enable us to make conclusions about the possibility that the two factors may bind in a cooperative fashion. To further study the potential of ATF3 and JUN to bind and transactivate the *IFNG* promoter, we designed and exploited luciferase reporter assays. As the ChIP analysis showed binding of both ATF3 and JUN on promoter areas 1.1 kb upstream of the *IFNG* gene transcription initiation site, the luciferase assays were designed to cover the promoter from −1147 to +38 (Fig. 7). The promoter fragments were cloned in front of the luciferase gene (*luc2*) (Fig. 7*A*), and the *IFNG* promoter constructs were cotransfected to Jurkat Jtag cells with either *ATF3* or *JUN* overexpression vectors or both of them. The resulting luminescence signals were compared with the signal obtained by cotransfection of the *IFNG* promoter constructs with an empty vector (Fig. 7*B*). When *ATF3* was cotransfected alone with any of the *IFNG* promoter constructs, the luciferase activity was increased only by 1.2- to 1.3-fold, which may indicate that ATF3 is not able to bind the promoter alone. *JUN* overexpression caused a >10-fold increase in the luciferase signal. The increase in luciferase activity was expected, because Jurkat cells express endogenously both ATF3 and JUN (data not shown) as well as other AP-1 transcription factors that are able to form heterodimers with ATF3 and JUN. Most importantly, cotransfecting both *ATF3* and *JUN* overexpression vectors with the promoter constructs resulted in all cases in higher luciferase activity than transfecting *JUN* alone. When evaluated with the Student *t* test, these differences were also statistically significant ($p < 0.02$) in all cases but one (pIFNG940-GL4.10, $p = 0.14$). The results also showed that most likely there is more than one functional binding site for ATF3 and JUN in the *IFNG* promoter because the average signal obtained with cotransfection of the longest promoter construct with *ATF3* and *JUN* was 1.4-fold when compared with the average signal obtained with the shortest promoter construct ($p = 0.025$).

ATF3 and JUN binding sites on *IFNG* promoter were analyzed, and five putative sites were found: an AP-1 site from −978 to −968 (5′-*tcctgcctcagc*cct-3′, del1), an AP-1 site from −320 to −311 (5′-*agagtcaaca*-3′, del2), a JUN site from −200 to −191 (5′-*gtctgtctca*t-3′, del3), an ATF site from −62 to −52 (5′-*aaatacgtaat*-3′, del4), and a CREB site from −256 to −249 (5′-*tgaagtaaa*-3′, del5) (Fig. 7*A*). Deletion mutations were made to the pIFNG1147-GL4.10 plasmid to study the functionality of these sites. As indicated above, the putative binding sites are in italics, and the deleted regions are underlined. Each of the mutated constructs had one intact putative binding site and deletions in the four other sites. One construct had deletions in all five putative sites. The mutation constructs were cotransfected to Jtag cells with ATF3 and JUN plasmid constructs as above. The results (Fig. 7*C*, 7*D*) indicated that deletion of all five sites was needed to abolish binding of ATF3 to the promoter, and, thus, the binding was shown to be cooperative. However, JUN was still able to bind and transactivate transcription, suggesting that additional binding sites exist for other JUN complexes. Also, when all five binding sites were deleted, ATF3 inhibited JUN binding, possibly by sequestering JUN away from the promoter.

Discussion

To find potential new players involved in the initiation of human Th1 differentiation, we used transcriptomics and identified a panel of 26 genes that are regulated in a similar manner by IL-12, IFN-α, and the combination of IL-12 plus IL-18 in human umbilical cord blood $CD4^+$ cells. The set of the genes regulated by Th1-inducing cytokines included genes known and new in this context. *ATF3* was significantly upregulated by all the cytokines studied. Differential expression of ATF3 in human Th1-inducing conditions has previously not been reported. The expression of ATF3 was also increased at the protein level in Th1-promoting conditions. Furthermore, its expression was found to be downregulated by IL-4, a Th2-promoting cytokine.

ATF3 is a member of the mammalian ATF/CREB protein family of transcription factors. *ATF3* is a stress-inducible gene. However, little is known about the signaling pathways involved in the induction of *ATF3* as well as the role of ATF3 in stress responses. Previous work from our group demonstrated that *ATF3* is regulated via the IL-4R-induced STAT6 signaling pathway in murine $CD4^+$ cells and that its transcript is more abundant in Th2 cells

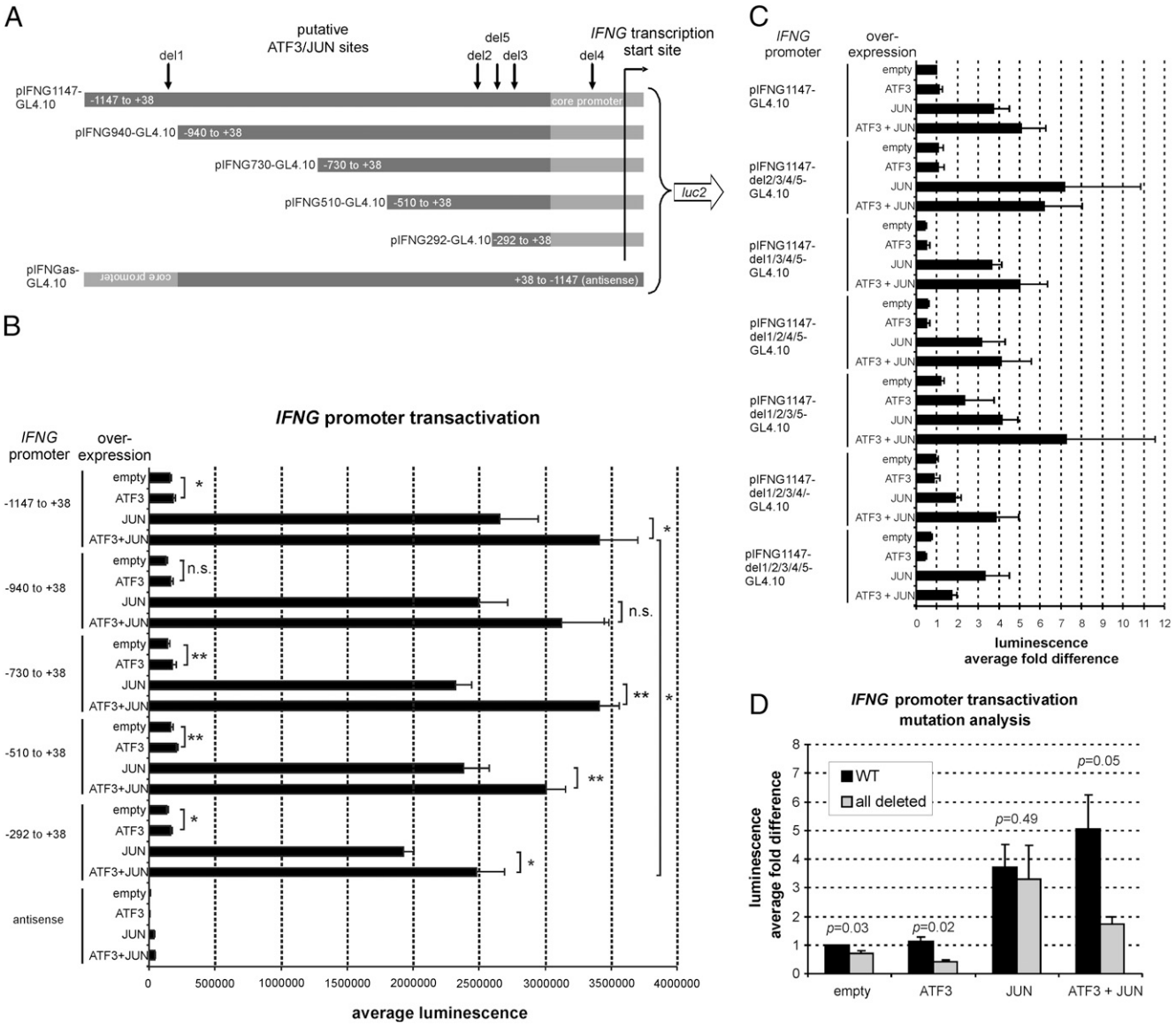

FIGURE 7. ATF3 and JUN transactivate the *IFNG* promoter. *A*, Up to ~1.1 kb fragment of the human *IFNG* promoter was cloned in front of the *luc2* gene in pGL4.10 vector. Sequence analysis revealed five putative ATF3/JUN binding sites, which are marked with arrows. *B*, The *IFNG* promoter constructs were cotransfected to Jurkat Jtag cells with either pATF3-IRES2-H-2K^k or pJUN-IRES2-H-2K^k or both. The cells were activated with PMA plus PHA the next day, and the luminescence signals were read 24 h postactivation. The figure shows average values from two to seven independent cultures. *C*, The mutated *IFNG* promoter constructs were cotransfected to Jurkat Jtag cells with pATF3-IRES2-H-2K^k or pJUN-IRES2-H-2K^k or both. Each promoter construct had only one intact binding site left. One construct had deletions in all putative binding sites. *D*, Deletion of all five putative ATF3/JUN binding sites abolished the ability of ATF3 to transactivate the *IFNG* promoter. Statistical significance was determined with Student *t* test. Error bars indicate SEM. $*p < 0.05$; $**p < 0.01$.

than in Th1 cells (32). *ATF3* gene expression has also been reported to be induced by both IFN-γ and IL-4 in murine macrophages (53), by pegylated IFN-α in PBMCs (54) and by IL-12 in murine NK cells (55). A study using systems biology approaches identified ATF3 as a negative regulator of TLR-stimulated murine macrophage responses (56). In that study, ATF3, as a part of a transcriptional complex also containing members of the NF-κB transcription factors, was shown to act as a transcriptional repressor of *Il6* and *Il12b* upon macrophage activation. Furthermore, Whitmore et al. (57) showed that *ATF3* gene expression was upregulated by TLR signaling in mouse and human APCs and that ATF3 acted as a negative regulator of IL-12 and IL-6 production by macrophages. ATF3 has also been shown to be a negative regulator of *IFNG* gene expression in mouse NK cells (55). Recently, the role of ATF3 was studied in a mouse asthma model (58). Gilchrist et al. (58) showed that ATF3 was induced in the lungs of OVA-challenged mice and that ATF3-deficient mice showed more severe symptoms of asthma than wild-type mice. Consistent with our previous studies in mouse cells (32), they demonstrated that the expression of ATF3 in CD4$^+$ mouse T cells was upregulated by Th2 cytokines but not by Th1 cytokines. They further showed that ATF3 bound the Th2 cytokine locus in the chromatin of mouse Th2 cells and that ATF3 inhibited IL-4, IL-5, and IL-13 secretion.

Importantly, there are no previous data on ATF3 expression or function in human CD4$^+$ T cells in response to Th1/Th2-inducing cytokines. We demonstrated in this study that, in contrast to mouse CD4$^+$ T cells, in human CD4$^+$ cells, ATF3 protein levels were clearly higher in cells induced to differentiate along a Th1 lineage than along a Th2 lineage and that ATF3 positively regulated

human *IFNG* gene expression and Th1 differentiation. Thus, using a different mechanism, ATF3 plays a similar role in human and mouse $CD4^+$ Th cell differentiation: to drive the Th1/Th2 balance toward the Th1 lineage.

AP-1 transcription factors consist of JUN and FOS family proteins that can form homo- or heterodimers not only with each other, but also with proteins of the ATF/CREB or MAF protein families (59). AP-1 transcription factors are induced upon activation of naive Thp cells, and the different complexes have selective functions in the regulation of effector Th cell differentiation (60, 61). In general, the ATF3 homodimer is known to act as a repressor of transcription. However, the function of ATF3 is dependent on its binding partner because selective heterodimers ATF3/JUN and ATF3/JUND have been shown to result in transcriptional activation (51, 52). JUN is a known positive regulator of *IFNG* gene expression (62, 63). JUN is able to form heterodimers with ATF2 that have been shown to bind and transactivate the *IFNG* promoter (49, 62, 63). As JUN was also upregulated by IL-12 and IL-12 plus IL-18 in our microarray analysis, we hypothesized that ATF3 may form an activating complex with JUN and thereby induce IFN-γ production. To study this, we performed coimmunoprecipitation assays of primary human $CD4^+$ T cells induced to differentiate along Th1 or Th2 lineages and showed that ATF3/JUN complexes were formed in Th1-inducing conditions. ATF3 and JUN showed an additive effect on *IFNG* gene expression when transfected to Jurkat cells as compared with transfecting with either one of them alone. Recruitment of both ATF3 and JUN to a human *IFNG* promoter was verified by ChIP analysis, which showed specific binding of both in Th1-promoting conditions. Luciferase reporter assays further showed that both ATF3 and JUN were able to transactivate the *IFNG* promoter, and the assays also suggested that multiple binding sites for ATF3 and JUN exist. Sequence analysis revealed five putative binding sites, and their functionality was studied by making deletion mutations of the sites. The recruitment of ATF3 to the promoter was cooperative, as the assays showed that all five sites needed to be deleted to abolish ATF3 binding. However, when all putative sites were deleted, JUN was still able to bind and transactivate the promoter. Highest luciferase activity was obtained when ATF3 and JUN were cotransfected with the promoter constructs. This, and the finding that ATF3 inhibited binding of JUN when all five putative binding sites were deleted, suggests complex formation. Additional studies, such as sequential ChIP, could be performed to verify ATF3 and JUN corecruitment to the *IFNG* locus.

We also aimed to clarify the role of IFN-α in human Th1 differentiation. Our results on transient *IFNG* gene induction by IFN-α in cord blood $CD4^+$ cells (Fig. 1) are in concordance with the finding that IFN-α is able to only transiently phosphorylate STAT4 (21, 64). Furthermore, with flow cytometric analysis, we cannot detect any increase in the number of IFN-γ–producing cord blood $CD4^+$ cells following activation and IFN-α treatment for either 48 h (28) or 7 d (data not shown) when compared with activation only. Thus, IFN-α was not able to drive Th1 differentiation.

Significantly, however, we show in this study that IFN-α regulates a number of genes in a similar manner to IL-12 or the combination of IL-12 plus IL-18. Among these are also genes undoubtedly involved in Th1 differentiation, namely *TBX21*, *IL12RB2*, *IL18RAP*, and *IFNG*, which all were significantly upregulated. Based on our results, we propose the following model whereby IFN-α promotes human Th1 differentiation. IFN-α, IL-12, and IL-18 are produced by activated macrophages during microbial infections. Our findings suggest that by increasing the expression of *IL12RB2*, *IL18RAP*, *TBX21*, and *IFNG*, IFN-α increases the ability of Thp cells to respond to IL-12 and IL-18. This in turn efficiently drives Thp cells toward the Th1 lineage.

Acknowledgments

We thank Paula Suominen, Arja Reinikainen, Miina Miller, Outi Melin, Sarita Heinonen, Matti Raitio, and the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. We also thank Robert Moulder for revising the language, the Turku University hospital staff for collecting the umbilical cord blood samples, and Dr. Anjana Rao for a careful review and comments on the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

1. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136: 2348–2357.
2. Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L. A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203: 2271–2279.
3. Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jäger, T. B. Strom, M. Oukka, and V. K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448: 484–487.
4. Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten, and C. Dong. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* 448: 480–483.
5. Infante-Duarte, C., H. F. Horton, M. C. Byrne, and T. Kamradt. 2000. Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* 165: 6107–6115.
6. Zhou, L., I. I. Ivanov, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard, and D. R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8: 967–974.
7. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
8. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 4: 330–336.
9. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
10. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat. Immunol.* 4: 337–342.
11. Zheng, Y., and A. Y. Rudensky. 2007. Foxp3 in control of the regulatory T cell lineage. *Nat. Immunol.* 8: 457–462.
12. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2: 933–944.
13. Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty, and M. K. Gately. 1996. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* 4: 471–481.
14. Wu, C., X. Wang, M. Gadina, J. J. O'Shea, D. H. Presky, and J. Magram. 2000. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J. Immunol.* 165: 6221–6228.
15. Kaplan, M. H., Y. L. Sun, T. Hoey, and M. J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382: 174–177.
16. Robinson, D., K. Shibuya, A. Mui, F. Zonin, E. Murphy, T. Sana, S. B. Hartley, S. Menon, R. Kastelein, F. Bazan, and A. O'Garra. 1997. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity* 7: 571–581.
17. Brinkmann, V., T. Geiger, S. Alkan, and C. H. Heusser. 1993. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. *J. Exp. Med.* 178: 1655–1663.
18. Sareneva, T., S. Matikainen, M. Kurimoto, and I. Julkunen. 1998. Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. *J. Immunol.* 160: 6032–6038.
19. Cho, S. S., C. M. Bacon, C. Sudarshan, R. C. Rees, D. Finbloom, R. Pine, and J. J. O'Shea. 1996. Activation of STAT4 by IL-12 and IFN-alpha: evidence for the involvement of ligand-induced tyrosine and serine phosphorylation. *J. Immunol.* 157: 4781–4789.
20. Rogge, L., D. D'Ambrosio, M. Biffi, G. Penna, L. J. Minetti, D. H. Presky, L. Adorini, and F. Sinigaglia. 1998. The role of Stat4 in species-specific regulation of Th cell development by type I IFNs. *J. Immunol.* 161: 6567–6574.
21. Athie-Morales, V., H. H. Smits, D. A. Cantrell, and C. M. Hilkens. 2004. Sustained IL-12 signaling is required for Th1 development. *J. Immunol.* 172: 61–69.

22. Yssel, H., J. E. De Vries, M. Koken, W. Van Blitterswijk, and H. Spits. 1984. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods* 72: 219–227.
23. Hamalainen, H., S. Meissner, and R. Lahesmaa. 2000. Signaling lymphocytic activation molecule (SLAM) is differentially expressed in human Th1 and Th2 cells. *J. Immunol. Methods* 242: 9–19.
24. Hamalainen, H. K., J. C. Tubman, S. Vikman, T. Kyrölä, E. Ylikoski, J. A. Warrington, and R. Lahesmaa. 2001. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal. Biochem.* 299: 63–70.
25. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. 1997. *Current Protocols in Molecular Biology.* John Wiley & Sons, New York.
26. Tahvanainen, J., M. Pykäläinen, T. Kallonen, H. Lähteenmäki, O. Rasool, and R. Lahesmaa. 2006. Enrichment of nucleofected primary human CD4+ T cells: a novel and efficient method for studying gene function and role in human primary T helper cell differentiation. *J. Immunol. Methods* 310: 30–39.
27. Northrop, J. P., K. S. Ullman, and G. R. Crabtree. 1993. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. *J. Biol. Chem.* 268: 2917–2923.
28. Ylikoski, E., R. Lund, M. Kyläniemi, S. Filén, M. Kilpeläinen, J. Savolainen, and R. Lahesmaa. 2005. IL-12 up-regulates T-bet independently of IFN-gamma in human CD4+ T cells. *Eur. J. Immunol.* 35: 3297–3306.
29. Lund, R., H. Ahlfors, E. Kainonen, A. M. Lahesmaa, C. Dixon, and R. Lahesmaa. 2005. Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur. J. Immunol.* 35: 3307–3319.
30. Lund, R., T. Aittokallio, O. Nevalainen, and R. Lahesmaa. 2003. Identification of novel genes regulated by IL-12, IL-4, or TGF-beta during the early polarization of CD4+ lymphocytes. *J. Immunol.* 171: 5328–5336.
31. Egwuagu, C. E., C. R. Yu, M. Zhang, R. M. Mahdi, S. J. Kim, and I. Gery. 2002. Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J. Immunol.* 168: 3181–3187.
32. Chen, Z., R. Lund, T. Aittokallio, M. Kosonen, O. Nevalainen, and R. Lahesmaa. 2003. Identification of novel IL-4/Stat6-regulated genes in T lymphocytes. *J. Immunol.* 171: 3627–3635.
33. Rogge, L., E. Bianchi, M. Biffi, E. Bono, S. Y. Chang, H. Alexander, C. Santini, G. Ferrari, L. Sinigaglia, M. Seiler, et al. 2000. Transcript imaging of the development of human T helper cells using oligonucleotide arrays. *Nat. Genet.* 25: 96–101.
34. Rogge, L., L. Barberis-Maino, M. Biffi, N. Passini, D. H. Presky, U. Gubler, and F. Sinigaglia. 1997. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J. Exp. Med.* 185: 825–831.
35. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
36. Lund, R. J., E. K. Ylikoski, T. Aittokallio, O. Nevalainen, and R. Lahesmaa. 2003. Kinetics and STAT4- or STAT6-mediated regulation of genes involved in lymphocyte polarization to Th1 and Th2 cells. *Eur. J. Immunol.* 33: 1105–1116.
37. Chtanova, T., R. A. Kemp, A. P. Sutherland, F. Ronchese, and C. R. Mackay. 2001. Gene microarrays reveal extensive differential gene expression in both CD4(+) and CD8(+) type 1 and type 2 T cells. *J. Immunol.* 167: 3057–3063.
38. Nagai, S., S. Hashimoto, T. Yamashita, N. Toyoda, T. Satoh, T. Suzuki, and K. Matsushima. 2001. Comprehensive gene expression profile of human activated T(h)1- and T(h)2-polarized cells. *Int. Immunol.* 13: 367–376.
39. Sareneva, T., I. Julkunen, and S. Matikainen. 2000. IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J. Immunol.* 165: 1933–1938.
40. Filen, J. J., S. Filen, R. Moulder, S. Tuomela, H. Ahlfors, A. West, P. Kouvonen, S. Kantola, M. Bjorkman, M. Katajamaa, et al. 2009. Quantitative proteomics reveals GIMAP family proteins 1 and 4 to be differentially regulated during human T helper cell differentiation. *Mol. Cell. Proteomics* 8: 32–44.
41. Lund, R. J., M. Löytömäki, T. Naumanen, C. Dixon, Z. Chen, H. Ahlfors, S. Tuomela, J. Tahvanainen, J. Scheinin, T. Henttinen, et al. 2007. Genome-wide identification of novel genes involved in early Th1 and Th2 cell differentiation. *J. Immunol.* 178: 3648–3660.
42. Berenson, L. S., M. Gavrieli, J. D. Farrar, T. L. Murphy, and K. M. Murphy. 2006. Distinct characteristics of murine STAT4 activation in response to IL-12 and IFN-alpha. *J. Immunol.* 177: 5195–5203.
43. Usui, T., J. C. Preiss, Y. Kanno, Z. J. Yao, J. H. Bream, J. J. O'Shea, and W. Strober. 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *J. Exp. Med.* 203: 755–766.
44. Mei, Y., Z. Yuan, B. Song, D. Li, C. Ma, C. Hu, Y. P. Ching, and M. Li. 2008. Activating transcription factor 3 up-regulated by c-Jun NH(2)-terminal kinase/c-Jun contributes to apoptosis induced by potassium deprivation in cerebellar granule neurons. *Neuroscience* 151: 771–779.
45. Pearson, A. G., C. W. Gray, J. F. Pearson, J. M. Greenwood, M. J. During, and M. Dragunow. 2003. ATF3 enhances c-Jun-mediated neurite sprouting. *Brain Res. Mol. Brain Res.* 120: 38–45.
46. Cai, Y., C. Zhang, T. Nawa, T. Aso, M. Tanaka, S. Oshiro, H. Ichijo, and S. Kitajima. 2000. Homocysteine-responsive ATF3 gene expression in human vascular endothelial cells: activation of c-Jun NH(2)-terminal kinase and promoter response element. *Blood* 96: 2140–2148.
47. Liang, G., C. D. Wolfgang, B. P. Chen, T. H. Chen, and T. Hai. 1996. ATF3 gene. Genomic organization, promoter, and regulation. *J. Biol. Chem.* 271: 1695–1701.
48. Seijffers, R., C. D. Mills, and C. J. Woolf. 2007. ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J. Neurosci.* 27: 7911–7920.
49. Samten, B., J. C. Townsend, S. E. Weis, A. Bhoumik, P. Klucar, H. Shams, and P. F. Barnes. 2008. CREB, ATF, and AP-1 transcription factors regulate IFN-gamma secretion by human T cells in response to mycobacterial antigen. *J. Immunol.* 181: 2056–2064.
50. Chen, B. P., G. Liang, J. Whelan, and T. Hai. 1994. ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. *J. Biol. Chem.* 269: 15819–15826.
51. Chu, H. M., Y. Tan, L. A. Kobierski, L. B. Balsam, and M. J. Comb. 1994. Activating transcription factor-3 stimulates 3′,5′-cyclic adenosine monophosphate-dependent gene expression. *Mol. Endocrinol.* 8: 59–68.
52. Hsu, J. C., R. Bravo, and R. Taub. 1992. Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G1 phase of liver regeneration. *Mol. Cell. Biol.* 12: 4654–4665.
53. Drysdale, B. E., D. L. Howard, and R. J. Johnson. 1996. Identification of a lipopolysaccharide inducible transcription factor in murine macrophages. *Mol. Immunol.* 33: 989–998.
54. Taylor, M. W., W. M. Grosse, J. E. Schaley, C. Sanda, X. Wu, S. C. Chien, F. Smith, T. G. Wu, M. Stephens, M. W. Ferris, et al. 2004. Global effect of PEG-IFN-alpha and ribavirin on gene expression in PBMC in vitro. *J. Interferon Cytokine Res.* 24: 107–118.
55. Rosenberger, C. M., A. E. Clark, P. M. Treuting, C. D. Johnson, and A. Aderem. 2008. ATF3 regulates MCMV infection in mice by modulating IFN-gamma expression in natural killer cells. *Proc. Natl. Acad. Sci. USA* 105: 2544–2549.
56. Gilchrist, M., V. Thorsson, B. Li, A. G. Rust, M. Korb, J. C. Roach, K. Kennedy, T. Hai, H. Bolouri, and A. Aderem. 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 441: 173–178.
57. Whitmore, M. M., A. Iparraguirre, L. Kubelka, W. Weninger, T. Hai, and B. R. Williams. 2007. Negative regulation of TLR-signaling pathways by activating transcription factor-3. *J. Immunol.* 179: 3622–3630.
58. Gilchrist, M., W. R. Henderson, Jr., A. E. Clark, R. M. Simmons, X. Ye, K. D. Smith, and A. Aderem. 2008. Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation. *J. Exp. Med.* 205: 2349–2357.
59. Mechta-Grigoriou, F., D. Gerald, and M. Yaniv. 2001. The mammalian Jun proteins: redundancy and specificity. *Oncogene* 20: 2378–2389.
60. Rincón, M., B. Dérijard, C. W. Chow, R. J. Davis, and R. A. Flavell. 1997. Reprogramming the signalling requirement for AP-1 (activator protein-1) activation during differentiation of precursor CD4+ T-cells into effector Th1 and Th2 cells. *Genes Funct.* 1: 51–68.
61. Rincón, M., and R. A. Flavell. 1999. Reprogramming transcription during the differentiation of precursor CD4+ T cells into effector Th1 and Th2 cells. *Microbes Infect.* 1: 43–50.
62. Penix, L. A., M. T. Sweetser, W. M. Weaver, J. P. Hoeffler, T. K. Kerppola, and C. B. Wilson. 1996. The proximal regulatory element of the interferon-gamma promoter mediates selective expression in T cells. *J. Biol. Chem.* 271: 31964–31972.
63. Zhang, F., D. Z. Wang, M. Boothby, L. Penix, R. A. Flavell, and T. M. Aune. 1998. Regulation of the activity of IFN-gamma promoter elements during Th cell differentiation. *J. Immunol.* 161: 6105–6112.
64. Matikainen, S., A. Paananen, M. Miettinen, M. Kurimoto, T. Timonen, I. Julkunen, and T. Sareneva. 2001. IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK cells: differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. *Eur. J. Immunol.* 31: 2236–2245.