

T Helper Cell-specific Regulation of Inducible Costimulator Expression via Distinct Mechanisms Mediated by T-bet and GATA-3*

Received for publication, September 13, 2007, and in revised form, October 22, 2007 Published, JBC Papers in Press, November 6, 2007, DOI 10.1074/jbc.M707693200

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Inducible costimulator (ICOS) expression is critical for T cell-mediated immunity. We showed previously that T cell receptor and CD28 coengagement up-regulate ICOS expression in activated T cells via the induction of NFATc2. Here, we examined the regulation of ICOS expression by Th-specific transcription factors T-bet and GATA-3. Overexpression of T-bet or GATA-3 alone could enhance, and NFATc2 could further synergize with either of them to increase, *icos* transcription. Although T-bet acted on the *icos* promoter, GATA-3 operated via an *icos* 3'-untranslated region element. Interestingly, NFATc2 was found to bind promiscuously the *icos* promoter in developing Th0, Th1, and Th2 cells but became selectively associated with T-bet at the promoter and with GATA-3 at the 3'-untranslated region in fully differentiated Th1 and Th2 cells, respectively. Collectively, our results reveal a temporally evolving circuit in which the non-selectively expressed NFATc2 cooperates with Th-restricted T-bet or GATA-3 to direct transcription of a costimulatory gene via distinct regulatory elements in different Th cells undergoing differentiation.

The inducible costimulator (ICOS)² is a member of the CD28 superfamily of costimulatory molecules that also includes the inhibitory CTLA-4 (cytotoxic T lymphocyte-associated protein 4). Unlike the constitutively expressed CD28, ICOS is lowly expressed or absent on naïve T cells but becomes inducibly up-regulated in activated T cells (1, 2). ICOS engages the cognate ligand B7-H2 (also known as B7RP-1, B7h, GL-50, LICOS, or ICOSL), which is expressed at low levels on B cells, macrophages, and dendritic cells (3) but can be induced on nonimmune cells by inflammatory agents such as lipopolysaccharide and tumor necrosis fac-

tor- α (4). Gene inactivation studies in mice (5–9) had indicated that ICOS and its ligand play a critical role in T cell-mediated immunity. In the absence of either, T-B cell interactions were compromised, resulting in impaired germinal center formation and humoral immunity.

After T cell receptor engagement, naïve T cells could polarize into T helper type 1 (Th1) or 2 (Th2) cells, depending on the antigenic stimulus and cytokine milieu (10). ICOS-deficient T cells exhibited defects in Th2 cytokine secretion, being selectively impaired in IL-4 but not IFN- γ expression after *in vitro* differentiation or *in vivo* priming by protein antigen (5). In agreement with a role for ICOS in Th2 responses, IL-4-dependent IgE production was defective in asthma-contracting mice lacking ICOS (5) or in which ICOS signaling was neutralized (11, 12). Recently, *in vitro* Th polarization experiments suggested that ICOS costimulated Th2 cell differentiation by an IL-4-driven mechanism involving NFATc1 and c-Maf (13). However, ICOS participated not only in Th2 costimulation but was also involved in Th1-mediated pathologies (14). For example, anti-ICOS therapy was shown to profoundly reduce both chronic and Th1-dependent acute allograft rejection (15). ICOS blockade increased susceptibility of CD28-deficient mice to Th1-type *Toxoplasma gondii* infection (16) and produced reciprocal disease outcomes in a murine model of experimental autoimmune encephalomyelitis, depending on the timing of blockade (17). Thus, ICOS regulates both Th1- and Th2-associated immune responses.

The cytokines and transcriptional machinery responsible for Th1 and Th2 cell differentiation have been well studied (18–20). The signature cytokines IL-12 and IL-4 are known to promote respectively, the development of Th1 and Th2 cells by inducing STAT4 and STAT6 activation and leading to lineage-specific gene expression (21). Similarly, the T-box transcription factor T-bet has been shown to direct Th1 lineage commitment (22), inducing both transcriptional proficiency of the *ifn- γ* locus and responsiveness to IL-12-transduced growth signal (23). On the other hand, the zinc finger transcription factor GATA-3 is a master regulator of Th2 differentiation (24), although the basic region leucine zipper transcription factor c-Maf plays an early role in this process by skewing naïve CD4⁺ T cells toward a Th2 phenotype via the initial induction of IL-4 (25). In addition, GATA-3 also specifies the transcriptional competence of the Th2 cytokine gene cluster, which encodes IL-4, IL-5, and IL-13 (26, 27).

* This work was supported by grants from the Biomedical Research Council of the Agency for Science, Technology and Research, Singapore. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: ICOS, inducible costimulator; NFAT, nuclear factor of activated T cells; luc, luciferase; PMA, phorbol-12-myristate-13-acetate; UTR, untranslated region; IL, interleukin; IFN, interferon; STAT, signal transducers and activators of transcription; Ab, antibody; RT, reverse transcription; RLU, relative light unit(s); siRNA, small interfering RNA; GFP, green fluorescent protein; PE, phycoerythrin.

Despite the extensively characterized role of ICOS in Th-specific immune responses, it is currently unclear how *icos* expression is being regulated during Th cell development. In particular, it is not known whether T-bet and GATA-3 play direct roles in regulating ICOS expression and whether ICOS regulation is subjected to different transcriptional mechanisms in different Th cells.

We previously reported that the Fyn-calcineurin-NFATc2 and MEK2-ERK1/2 signaling axes acted cooperatively to drive *icos* transcription in recently activated CD4⁺ T cells (28). It has been established that upon T cell receptor stimulation, NFAT proteins undergo calcineurin-mediated dephosphorylation and translocate to the nucleus where they cooperate with members of the AP-1 complex to activate target genes such as IL-2. Without their transcriptional partners, the binding of NFAT to gene promoters alone results in T cell anergy (29). Of relevance, NFAT proteins had been shown to partner T-bet or GATA-3 to drive IFN- γ (30) or IL-4 expression (31) respectively. Hence, we sought to determine whether the same partnership is invoked to regulate ICOS expression in Th cells.

In this study, we examined the roles of T-bet, GATA-3, and NFATc2 in regulating ICOS expression in Th1 and Th2 cells. We present evidence that the lineage-restricted T-bet and GATA-3 cooperated with the more broadly expressed NFATc2 to regulate *icos* transcription in a temporally dynamic and Th-specific manner through binding of distinct *icos* regulatory regions during Th cell development.

MATERIALS AND METHODS

Mouse Strains and Cell Lines—8–12-week-old C57BL/6 mice were bred and maintained in the Biopolis animal facilities according to Institutional Animal Care and Use Committee regulations. The EL4 thymoma T cell line was maintained as described (28). The AE7 Th1 and CDC35 Th2 cell clones were kind gifts of Dr. I.-C. Ho (Harvard Medical School, Brigham and Women's Hospital, Boston, MA) and cultured as described (32, 33) but with some modifications. Briefly, AKR/J splenocytes were treated with 50 μ g/ml of mitomycin C (Sigma) at 37 °C for 45 min and washed before incubation with $1\text{--}2 \times 10^6$ AE7 cells and 5 μ M of pigeon cytochrome C in complete medium for 48 h. The cells were then passaged 1:5 into medium with 10% rat concanavalin A and expanded every 3–4 days. The CDC35 cells were cultured similarly with Balb/c splenocytes and 100 μ g/ml of rabbit γ -globulin.

CD4⁺ T Cell Purification and in Vitro Differentiation of Th Cells—CD4⁺ T cells were isolated as described (28) to >95% purity as assessed by flow cytometry. Purified T cells (2×10^6 /ml) were stimulated with 1 μ g/ml plate-bound anti-CD3 and 2 μ g/ml soluble anti-CD28 Abs under Th0 (3 μ g/ml anti-IL-12, 5 μ g/ml anti-IFN- γ , and 10 μ g/ml anti-IL-4), Th1 (5 ng/ml IL-12 and 10 μ g/ml anti-IL-4), or Th2 (10 ng/ml IL-4, 3 μ g/ml anti-IL-12, and 5 μ g/ml anti-IFN- γ) skewing conditions. 10 ng/ml recombinant mouse IL-2 was added after 24 h, and the cells were expanded in complete medium containing IL-2 for 2 or 8 days. Recombinant mouse IL-2, IL-4, and IL-12 were purchased from PeproTech Inc. (Rocky Hill, NJ). NA/LE-grade anti-CD3 (145–2C11), anti-CD28 (37.51), anti-IL-12 (C17.8),

anti-IFN- γ (XMG1.2), and anti-IL-4 (11B11) Abs were from BD Biosciences.

Western Blot Analyses—Equal amounts of whole cell lysates from $2\text{--}5 \times 10^6$ cells, quantified using Bradford assay, were electrophoresed in a 7–10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane, and immunoblotted with Abs against T-bet (4B10), GATA-3 (HG3–31), and ERK2 (C-14), all obtained from Santa Cruz Biotechnology.

RNA Isolation and Real Time RT-PCR Analyses—Total RNA was isolated and cDNA was prepared as described (28). Each reaction was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in a Mx3005P QPCR system (Stratagene, Agilent Technologies, Santa Clara, CA). The primers used for real time RT-PCR were: ICOS, 5'-TGA CCC ACC TCC TTT TCA AG-3' and 5'-TTA GGG TCA TGC ACA CTG GA-3'; β -actin, 5'-GAT CTG GCA CCA CAC CTT CT-3' and 5'-ACC AGA GGC ATA CAG GGA CA-3'. The mRNA levels of ICOS were normalized to those of β -actin, and those in activated CD4⁺ T or differentiated Th cells were expressed as a fold change relative to the levels in naïve cells.

Plasmid Constructs, Transient Transfections, and Luciferase Reporter Assays—The *icos* promoter, encompassing –288 to –1 bp relative to the first nucleotide of the mouse cDNA, was cloned upstream of a luc reporter as described (28). The *icos* 259-bp 3'-UTR fragment (NCBI GenBank™ accession number AK030827) was generated by PCR from C57BL/6 mouse genomic DNA using primers 5'-GAT GTT CCC ATA TTC TCC-3' and 5'-CCA GGA GAA TGT TTG CCC-3' and cloned into BamHI/Sall sites downstream of a luc reporter driven by the minimal *icos* promoter spanning –52 to –1 bp. Transient transfection experiments were performed as described previously (28). Relative light units (RLU) from firefly luciferase was normalized for transfection efficiency to the *Renilla* luciferase RLU in each lysate (normalized RLU = $\text{RLU}_{\text{firefly luciferase}}/\text{RLU}_{\text{Renilla luciferase}}$). For effect of overexpressed proteins on *icos* promoter and 3'-UTR activities, the fold induction was calculated as Fold Induction = $\text{normalized RLU}_{\text{protein expressing vector, unstimulated or stimulated}}/\text{normalized RLU}_{\text{null vector, unstimulated}}$. The pBJ5-NFATc2 (pSH210) vector (34) was a gift of Dr. G. R. Crabtree (Stanford University, Stanford, CA). The T-bet vector pcDNAHis-T-bet (22) was a gift from Dr. L. H. Glimcher (Harvard School of Public Health, Boston, MA). The pcDNAHis-GATA-3 vector was constructed by cloning full-length murine GATA-3 cDNA, generated using primers 5'-GGA ATT CGA CAT GGA GGT GAC TGC G-3' and 5'-GCT CTA GAC TAA CCC ATG GCG GTG A-3', into the EcoRI and XbaI sites of the pcDNAHis vector. All of the DNA constructs were confirmed by sequencing.

siRNA Knockdown of T-bet and GATA-3 in Th Cell Lines—AE7 cells were transfected using Lipofectamine 2000 with 100 nM of *tbx21* siRNA identification number 181595 (T-bet siRNA 1), siRNA identification number 181596 (T-bet siRNA 2), or *Silencer*® GFP siRNA (catalog number AM4626) as negative control. CDC35 cells were transfected with 1 μ M of *gata-3* siRNA identification number 61725 (GATA-3 siRNA 1), siRNA ID identification number 61780 (GATA-3 siRNA 2) or GFP siRNA as negative control. Following transfection, the cells were rested for 24 h before being stimulated with PMA and ionomycin for 16 h, and RNA and cell lysates were prepared

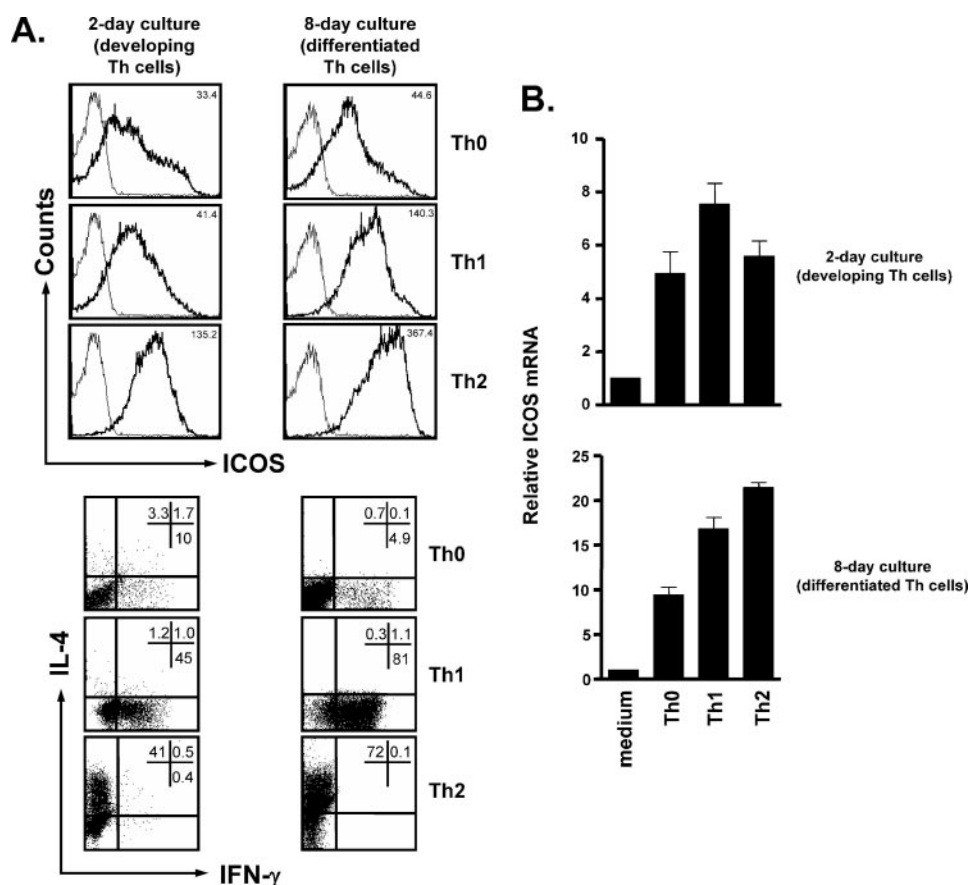


FIGURE 1. ICOS is differentially expressed in different Th cell subsets. Purified CD4⁺ T cells were differentiated for 8 days under Th0-, Th1-, and Th2-polarizing conditions and examined for cell surface expression of ICOS and, after restimulation with PMA and ionomycin for 6 h, for intracellular expression of IL-4 and IFN- γ by flow cytometry (A) and examined for ICOS mRNA levels by real time RT-PCR (B). The data shown are representative of three independent experiments, and the value at top right of each histogram plot in A indicates the mean fluorescence intensity of ICOS expression.

from them. All of the siRNA duplex oligonucleotides were from Ambion, Inc. (Applied Biosystems, Austin, TX).

Retroviral Constructs and Transduction—The bicistronic vectors GFP-RV and T-bet-RV (22) were gifts of Dr. Glimcher (Harvard). The Phoenix-Eco packaging cell line (35) was obtained from Dr. G. P. Nolan (Stanford). Generation of the GATA-3-RV vector, transfection of packaging cell line and retroviral transduction of primary CD4⁺ T cells were as described (36). Flow cytometric analysis of surface CD4 and ICOS expression on GFP⁺ cells was performed 48 h after infection. For RNA or protein extraction, the cells were first sorted to >98% GFP⁺ on a FACS-Vantage Flow Cytometer/Cell Sorter (Becton Dickinson, Mountain View, CA).

Intracellular Cytokine Staining—Intracellular cytokine staining was performed with the BD Cytofix/CytopermTM Plus fixation/permeabilization solution kit following the manufacturer's instructions. Briefly, $\sim 2 \times 10^6$ Th cells were treated with BD GolgiStopTM, PMA (50 ng/ml), and ionomycin (0.5 μ g/ml) for 6 h at 37 °C. After harvesting and staining with anti-CD4 Ab, they were washed and permeabilized before being incubated with anti-IFN- γ fluorescein isothiocyanate and anti-IL-4 PE Abs or their isotype controls and finally visualized on a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest Pro software. The following antibodies were obtained from BD

Pharmingen (San Diego, CA): fluorescein isothiocyanate and biotin-conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD8 (53-6.7), PE-conjugated anti-ICOS (7E.17G9), PE-Cy5-conjugated streptavidin, anti-IFN- γ fluorescein isothiocyanate (XMGI.2), and anti-IL-4 PE (11B11).

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed according to the manufacturer's protocol (Upstate Biotechnology, Inc., Lake Placid, NY). The Abs used were anti-NFATc2 (G1-D10), anti-T-bet (4B10), and anti-GATA3 (HG3-31) from Santa Cruz Biotechnology (Lake Placid, NY). The following primers were used for PCR: ICOS P, 5'-CCG CTC GAG CAT GCA TGC ATC CAT C-3' and 5'-CCC AAG CTT AGT GCT CAA AAG TGT CAG-3' (288-bp product); ICOS 3'-UTR, 5'-GAT GTT CCC ATA TTC TCC-3' and 5'-CCA GGA GAA TGT TTG CCC-3' (259-bp product).

RESULTS

ICOS Is Differentially Expressed in Different Th Cell Subsets—To assess whether ICOS is differentially expressed in various Th cells, we activated primary CD4⁺ T cells with anti-CD3 and anti-CD28 Abs and differentiated them for 8 days under

three types of polarizing conditions: Th0, Th1, or Th2 (37). These treatment regimes yielded substantial fractions of IFN- γ - and IL-4-producing cells, indicating that the Th1- and Th2-polarizing conditions were optimal (Fig. 1A, bottom right panels). Interestingly, we found the level of surface ICOS expression to vary in the different Th cell subsets: Th2 cells, consistent with earlier reports (1, 38), expressed the highest level of ICOS, followed by Th1 and Th0 cells (Fig. 1A, left panels). To determine whether this variation in surface expression of ICOS could in part be attributed to variation in *icos* transcript levels, we quantified ICOS mRNA levels in the Th subsets and found that these indeed correlated with levels of surface expression, with the highest amount of ICOS mRNA found in Th2, followed by Th1 and Th0 cells (Fig. 1B). The data suggest that the expression of ICOS may be under transcriptional control in different Th cells.

T-bet or GATA-3 Enhances ICOS Expression in T Cells—Because *icos* transcripts were expressed differently in different Th cell subsets and, in particular, at higher levels in Th1 and Th2 cells compared with Th0 cells, we asked whether there was a role for Th1-specific T-bet and Th2-specific GATA-3 transcription factors in driving ICOS expression. It was shown previously that the Th1-polarizing cytokines IL-12 and/or IL-23

could enhance ICOS expression in activated human T cells independent of their predefined differentiation status (39). Similarly, the Th2-polarizing cytokine IL-4 and growth factor IL-2 were shown to regulate ICOS expression in activated CD4⁺ T cells. Although retroviral transduction of GATA-3 was shown to increase surface ICOS expression in stimulated murine CD4⁺ T cells (40), it was not clear whether this was a direct effect of GATA-3 on *icos* gene regulation. We therefore ascertained whether T-bet or GATA-3 *per se* may directly enhance ICOS expression in the respective Th cells.

To test the individual roles of T-bet and GATA-3 in regulating ICOS expression, we first overexpressed these transcription factors in EL4 T cells and measured the level of *icos* transcripts after overnight culture. Real time RT-PCR analyses indicated that the ectopic expression of T-bet (Fig. 2A, left panel) or GATA-3 (right panel) could increase the level of *icos* transcripts in EL4 cells in a dose-dependent manner. To control for the functionality of T-bet and GATA-3, we also observed concomitant increases in *ifn-γ* and *il-5* transcripts, respectively, in the transfected EL4 cells (data not shown and Refs. 22 and 41). Next, we determined whether the up-regulation of ICOS by T-bet or GATA-3 in EL4 cells was reproducible in primary CD4⁺ T cells. We infected CD4⁺ T cells activated by anti-CD3/CD28 Abs with retroviruses that expressed GFP alone or together with T-bet or GATA-3 and examined ICOS expression in these cells 48 h after infection. Retroviral transduction of T-bet (Fig. 2B, left panel) or GATA-3 (right panel) in activated, nonpolarized CD4⁺ T cells also resulted in the higher expression of ICOS protein as well as mRNA in these cells when compared with control cells transduced with vector expressing GFP alone.

Finally, to establish that T-bet and GATA-3 play a direct role in driving *icos* transcription in committed Th1 and Th2 cells, respectively, we used siRNA against T-bet and GATA-3 to knock down their protein expression in AE7 Th1 and CDC35 Th2 cell lines, respectively. Knockdown of *tbx21* and *gata-3* transcripts led to the efficient abrogation of their protein expression in AE7 (Fig. 3A) and CDC35 (Fig. 3B) cells, whereas knockdown of the irrelevant *gfp* gene hardly affected T-bet expression in AE7 or GATA-3 expression in CDC35 cells. More importantly and in agreement with data obtained so far, the knockdown of T-bet significantly reduced the amount of *icos* transcripts in activated AE7 cells (Fig. 3A), whereas knockdown of GATA-3 profoundly diminished ICOS mRNA in CDC35 cells (Fig. 3B). However, the reduction in ICOS mRNA was not absolute, suggesting that other transcription factors are likely involved in regulating ICOS expression. Taken together, our overexpression and siRNA knockdown data indicate that T-bet and GATA-3 play enhancing roles in driving *icos* transcription in Th cells.

T-bet Enhances NFATc2-induced Transactivation of the *icos* Promoter—Because T-bet and GATA-3 are transcription factors shown to transactivate the promoters of *ifn-γ* (22) and *il-5* (42) genes, respectively, we were interested to determine whether they would also regulate *icos* gene expression via binding to its promoter region. Initial algorithmic searches using the TRANSFAC data base did not reveal any putative binding sites for T-bet or GATA-3 in the *icos* promoter. We had previously

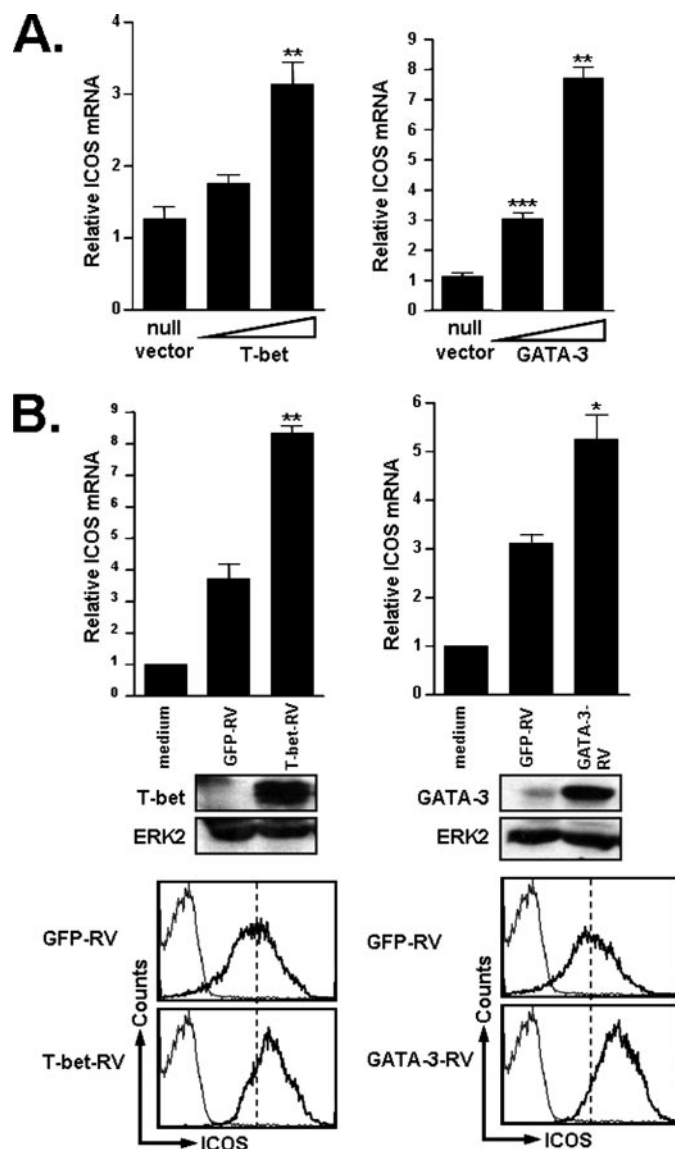


FIGURE 2. Ectopic expression of T-bet or GATA-3 enhances ICOS expression in T cells. A, real time RT-PCR analyses of ICOS mRNA levels in murine EL4 T cells transfected with a null vector or increasing amounts of vectors encoding for either T-bet or GATA-3. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$. B, flow cytometry and real time RT-PCR analyses of ICOS expression in purified CD4⁺ T cells transduced with retroviruses encoding GFP (GFP-RV), GFP, and T-bet (T-bet-RV), or GFP and GATA-3 (GATA-3-RV). Only GFP⁺ cells were examined for ICOS expression by flow cytometry and FACS-sorted for the analysis of *icos* transcript levels. *, $p < 0.05$; **, $p < 0.001$. Western blots were included to show overexpression of T-bet or GATA-3 in the retrovirally transduced samples. The anti-ERK-2 blot served as control for equal loading of cell lysates.

shown that NFATc2 enhanced ICOS expression by acting within a 288-bp promoter region upstream of the *icos* transcription start site (Fig. 4A and Ref. 28). Hence, to find out whether this region could respond to T-bet and/or GATA-3, we introduced a luc reporter plasmid driven by the 288-bp *icos* promoter, with or without accompanying plasmids encoding T-bet, NFATc2, or both, into EL4 cells by transient transfection.

Ectopic expression of NFATc2 led to an increase in *icos* promoter activity as reflected by the fold induction of luc activity in PMA/ionomycin-activated EL4 cells (Fig. 4B), concordant with our earlier work demonstrating that

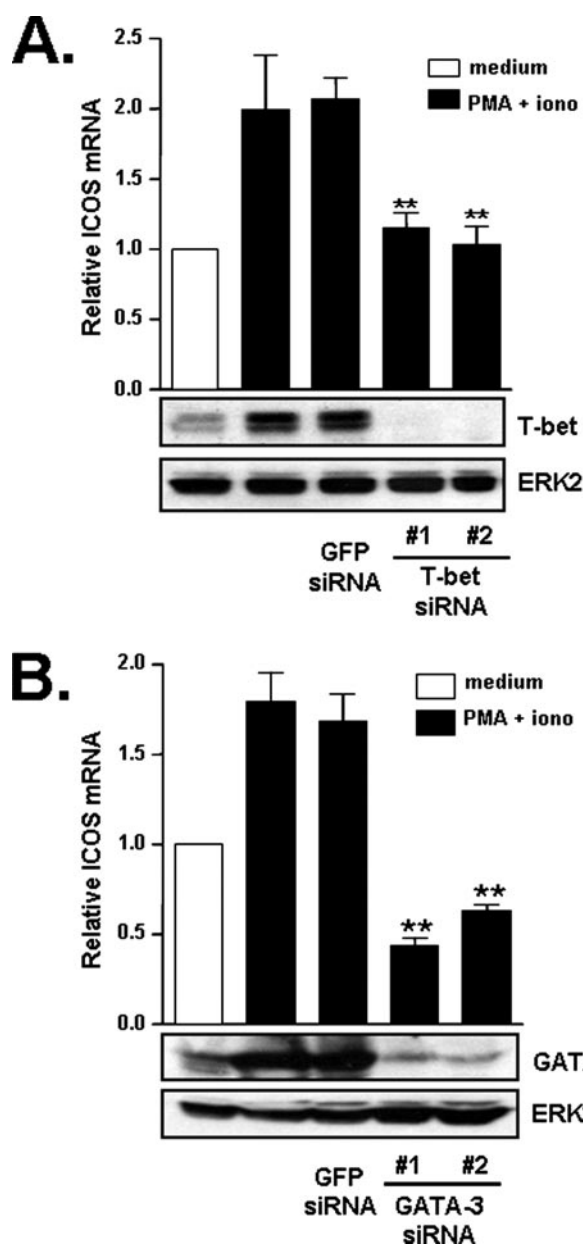


FIGURE 3. Knockdown of T-bet or GATA-3 reduces *icos* transcripts in AE7 Th1 and CDC35 Th2 cell lines. AE7 Th1 (A) or CDC35 Th2 (B) cells were transfected with siRNA against GFP (as control), T-bet, or GATA-3, rested for 24 h, and stimulated with PMA and ionomycin for 16 h. The amount of *icos* transcripts present was assessed by real time RT-PCR. The knockdown efficiencies in these cells were assessed by Western blotting as shown. The anti-ERK2 blot serves as loading control. **, $p < 0.001$. PMA + iono, PMA and ionomycin.

NFATc2 regulates ICOS expression. The introduction of T-bet also resulted in the up-regulation of luc activity in stimulated EL4 cells, suggesting that T-bet acted via the *icos* promoter to activate gene expression. Interestingly, the cointroduction of T-bet and NFATc2 could induce promoter activity exceeding that produced by introduction of either alone, implying that T-bet enhances NFATc2-induced transcription at the promoter. In contrast, overexpressing GATA-3 did not increase promoter activity beyond what was observed with null vector control (Fig. 4D, left panel), suggesting the *icos* promoter does not possess GATA-3-responsive elements.

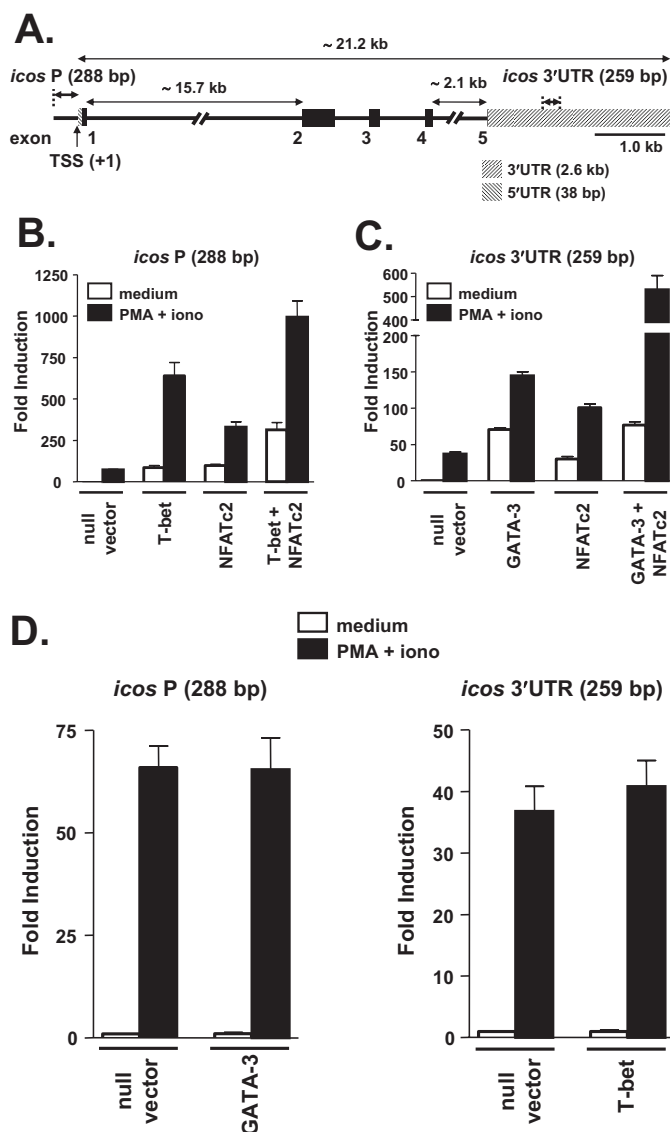


FIGURE 4. T-bet and GATA-3 act through distinct *icos* regulatory regions to regulate gene expression. A, schematic diagram showing putative regulatory regions within the *icos* gene locus. The promoter (P), transcription start site, and 5'- and 3'-UTRs are depicted. B, T-bet or NFATc2 acts alone and with each other to transactivate the *icos* promoter. EL4 cells were transiently transfected in triplicate with a luciferase reporter driven by the 288-bp *icos* promoter together with either a null vector or vector encoding T-bet, NFATc2, or both and subsequently left in medium alone (white bars) or stimulated with PMA and ionomycin (black bars) for 16 h. Fold induced luc activity was calculated as under "Materials and Methods." C, GATA-3 and NFATc2 act synergistically via a 3'-UTR element to transactivate *icos* expression. EL4 cells were transfected in triplicate with a 52-bp minimal *icos* promoter-driven luciferase reporter and a 259-bp 3'-UTR segment together with either a null vector or vector encoding GATA-3, NFATc2 or both. Fold induced activity was quantified as in B. D, the *icos* promoter and 3'-UTR do not respond to, respectively, GATA-3 and T-bet overexpression. EL4 cells were transfected in triplicate as above, but without vector encoding NFATc2, and fold induction was quantified.

GATA-3 Synergizes with NFATc2 to Regulate Gene Expression via *icos* 3'-UTR—To delineate where GATA-3 might act in the *icos* genomic locus, we searched for potential GATA-3-binding motifs and identified a cluster of conserved GATA-3- and NFATc2-binding sites within a 259-bp stretch in the 3'-UTR of *icos* (Fig. 4A). To test the functional relevance of this 3'-UTR site, we cloned the 259-bp fragment downstream of a luciferase reporter driven by a previously described 52-bp minimal *icos* promoter and

introduced this construct into EL4 cells. Stimulation of EL4 cells harboring this construct resulted in a 40-fold induction of luc activity (Fig. 4C, *null vector*), suggesting that this 259-bp segment was sufficient to confer PMA and ionomycin-mediated inducibility on *icos* transcription. To determine whether this element could respond to T-bet and/or GATA-3, we cotransfected, together with the 259-bp 3'-UTR reporter, vectors expressing NFATc2, GATA-3, and T-bet in EL4 cells. Overexpression of either GATA-3 or NFATc2 could augment the 3'-UTR-mediated transcriptional activity by ~150- and 100-fold, respectively. Strikingly, the coexpression of GATA-3 and NFATc2 resulted in a >500-fold increase in luc activity, suggesting that GATA-3 synergizes with NFATc2 to enhance *icos* transcription via this 3'-UTR site. On the other hand, overexpressing T-bet failed to yield an increase in 3'-UTR-mediated activity (Fig. 4D, *right panel*), indicating that this region was probably nonresponsive to T-bet. Hence, GATA-3 and NFATc2 appear to act on the 259-bp 3'-UTR element of *icos*.

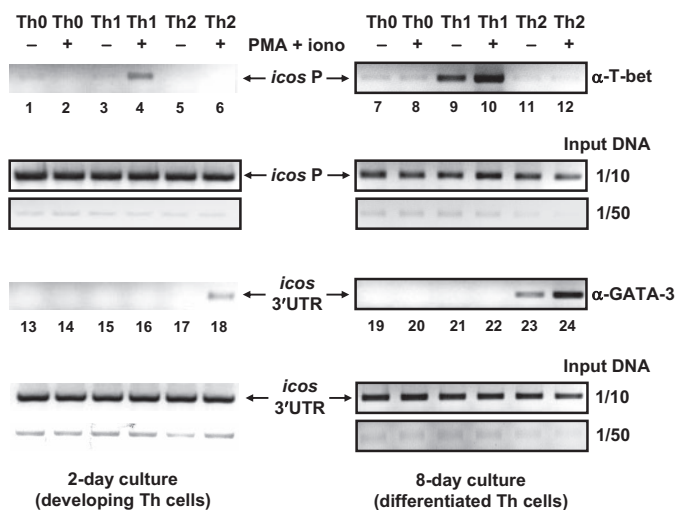
Differential Association of T-bet/NFATc2 with *icos* Promoter and GATA-3/NFATc2 with *icos* 3'-UTR during Th1 and Th2 Differentiation, Respectively—Data from the luc assays suggest that ICOS may be regulated distinctly in different Th cell subsets, likely with T-bet acting at the promoter and GATA-3 at the 3'-UTR in Th1 and Th2 cells, respectively, and with NFATc2 acting as the common coactivator in both Th subsets. Indeed, NFATc2 has been reported to cooperate with T-bet or GATA-3 in binding the regulatory regions of the *ifn- γ* gene in stimulated Th1 (30) or the *il-4* gene in stimulated Th2 cells (31), respectively. We therefore asked whether T-bet, GATA-3, and NFATc2 could differentially associate with the different *icos* regulatory regions during Th cell development. To address this, we assessed the binding dynamics of T-bet, GATA-3, and NFATc2 to *icos* regulatory regions in developing and fully differentiated Th1 and Th2 cells.

Chromatin immunoprecipitation assays revealed detectable binding of T-bet to the *icos* promoter (*icos* P) in developing (2-day culture) Th1 cells after stimulation of these cells for 6 h with PMA and ionomycin (Fig. 5A, *lanes 3 and 4*). This binding was significantly elevated in fully differentiated (8-day culture) Th1 cells and could be further enhanced when the cells were stimulated (*lanes 9 and 10*). On the other hand, T-bet was not observed to bind the promoter in developing or differentiated Th0 (*lanes 1, 2, 7, and 8*) and Th2 (*lanes 5, 6, 11, and 12*) cells, consistent with the virtual absence of T-bet expression in these cells. Similarly, binding of GATA-3 to the 3'-UTR element of *icos* was evident after 2 days of culture in developing Th2 cells (*lanes 17 and 18*). This association was greatly augmented in Th2 cells that approached terminal differentiation after 8 days of culture and could be further enhanced upon activation of these cells (*lanes 23 and 24*). Again, GATA-3 binding to the 3'-UTR of *icos* was undetectable in developing or differentiated Th0 (*lanes 13, 14, 19, and 20*) or Th1 cells (*lanes 15, 16, 21, and 22*), in agreement with the preferential expression of GATA-3 in Th2 cells.

Binding of NFATc2 to the *icos* promoter was detectable in Th0 cells cultured for 2 days and can be further increased when these cells were stimulated (Fig. 5B, *lanes 1 and 2*), in accordance with our previous demonstration that NFATc2 was found

Regulation of ICOS Expression by T-bet and GATA-3

A.



B.

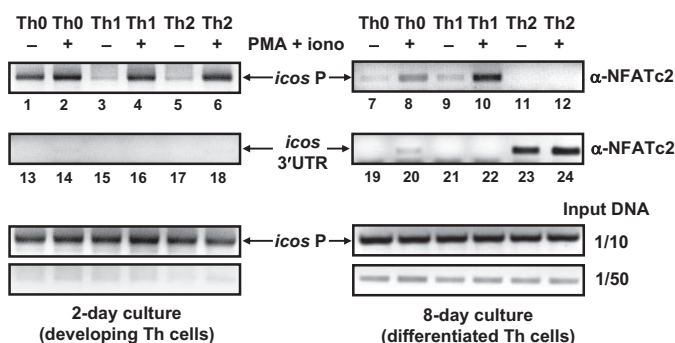


FIGURE 5. Differential association of T-bet, GATA-3, and NFATc2 with *icos* regulatory regions during *de novo* Th1 and Th2 cell differentiation. Chromatin immunoprecipitation assays using Abs against T-bet and GATA-3 (A) and NFATc2 (B) were performed on primary CD4+ T cells differentiated for 2 or 8 days under Th0-, Th1-, or Th2-polarizing conditions and left in medium or restimulated for further 6 h with PMA and ionomycin. Primers specific for the *icos* promoter (P) or 3'-UTR element were used in the PCR. As input controls, DNA extracted from the samples before immunoprecipitation was serially diluted (1:10 and 1:50) and quantified by the same PCR. Immunoprecipitation with isotype-matched control Abs did not yield visible PCR bands and was not shown for reason of brevity.

to be associated with the *icos* promoter during anti-CD3/CD28-mediated activation of naïve CD4+ T cells. Intriguingly, the association of NFATc2 with the *icos* promoter was found initially to be promiscuous across all developing Th cells (*lanes 1–6*) but became progressively Th1-restricted, Th0-diminished, and Th2-extinguished as cells became terminally differentiated (*lanes 7–12*). On the other hand, the binding of NFATc2 to the 3'-UTR of *icos* was largely undetectable in various developing Th cells at an early stage of differentiation (*lanes 13–18*) but became conspicuously and specifically accrued in terminally differentiated Th2 cells (*lanes 23 and 24*).

Taken together, the evolving patterns of T-bet, GATA-3, and NFATc2 binding to the promoter and 3'-UTR of *icos* support a model in which NFATc2 functions as the major common transcription factor driving ICOS expression via the promoter during early Th differentiation when T cell receptor-mediated sig-

naling is dominant and cytokine pathways are nascent. As Th differentiation progresses and the influence of polarizing cytokines increases, T-bet cooperates with NFATc2 at the *icos* promoter in Th1 cells, whereas GATA-3 synergizes with NFATc2 at the 3'-UTR element in Th2 cells to amplify the activity of the minimal *icos* promoter and hence direct gene expression.

DISCUSSION

ICOS plays a critical role in the costimulation of T cell responses in mice and humans. It was initially believed that ICOS could be more important for Th2-type immune responses because characterization of ICOS-deficient mice revealed defects in the ability of mutant T cells to mediate B-cell antibody class switching to IgG1 and IgE during the primary response to protein antigen *in vivo* and to produce IL-4 when restimulated by the same antigen *in vitro*, although they produced normal or enhanced levels of IFN- γ (5, 8). A report subsequently confirmed the tight correlation between ICOS expression in CD4+ T cells and their production of Th2-biased cytokines (43). Indeed, inhibition of the ICOS costimulatory pathway during infection by the gastrointestinal helminth *Trichinella spiralis* decreased tumor necrosis factor- α , IL-4, IL-5, and IgE production in mice (44). Nevertheless, ICOS signaling is also implicated in autoimmune disorders and graft *versus* host disease. For instance, ICOS blockade by treatment with anti-B7RP-1 monoclonal Ab or genetic inactivation substantially reduced clinical progression of murine experimental autoimmune uveoretinitis (45), whereas targeting of ICOS expressed on alloreactive donor T cells inhibited graft *versus* host disease and promoted bone marrow engraftment in recipient mice (46).

The complex function of ICOS in Th1- and Th2-mediated immunity prompted us to investigate whether ICOS is differentially regulated in Th1 and Th2 cells and, if so, to decipher the mechanisms involved. We first showed that ICOS is expressed highest in terminally differentiated Th2, lowest in Th0, and intermediate between the two in Th1 cells. In addition, ectopic expression of T-bet and GATA-3 could enhance ICOS expression both in EL4 as well as primary CD4+ T cells and coexpression of NFATc2 led to its synergy with either of them to enhance ICOS expression in the appropriate differentiating lineage. Hence, T-bet and GATA-3, along with NFATc2, play important roles in driving ICOS expression in Th1 and Th2 cells.

The critical roles for T-bet and GATA-3 in the regulation of Th1 and Th2 lineage-associated cytokine genes have been well documented. For instance, T-bet is known to activate the transcription of *ifn- γ* (30, 47), whereas GATA-3 is known to regulate the IL-4, -5, and -13 cytokine cluster (10, 48). In addition, NFATc2 has been shown to bind the *ifn- γ* 5' conserved non-coding sequences and promoter in Th1 cells and the *il-4* enhancer in Th2 cells (30, 31). In this study, we showed that a single costimulatory gene, *icos*, is regulated by both T-bet and GATA-3 via distinct mechanisms in Th cells, with the former acting on the promoter whereas the latter acted via a 3'-UTR element of *icos*.

Another interesting finding in our study is the dynamic manner in which the broadly expressed NFATc2 partners with Th-

restricted T-bet or GATA-3 to drive *icos* transcription. In the first 2 days of differentiation, NFATc2 activates *icos* transcription through its promoter in all Th lineages. As the Th cells approached their developmental fate (*i.e.* 8-day culture), NFATc2 cooperates with T-bet via the promoter and with GATA-3 via a 3'-UTR element to enhance ICOS expression in Th1 and Th2 cells, respectively. Why binding of NFATc2 to the promoter is diminished in terminally differentiated Th2 cells is presently unknown. It is highly likely that chromatin remodeling of the regulatory regions and the consequent DNA-association patterns of T-bet/NFATc2 and GATA-3/NFATc2 were conditioned by the combined action of T cell receptor-mediated signaling and polarizing cytokines responsible for molding Th identity.

Our data so far indicate that the cytokine networks and transcriptional apparatus initiating and reinforcing Th lineage commitment appear to instruct ICOS expression in a Th-specific manner, which, together with earlier work demonstrating roles for ICOS in Th subset polarization (49) and the transcriptional control of Th2 differentiation (13), provide a basis for feed forward amplification linking ICOS expression and Th cell differentiation. Of course, Th1 and Th2 cells are by no means the only Th cell subsets found in the adaptive immune system. A number of groups have recently uncovered the existence of other Th cell subsets including, among them, the regulatory T (T_{reg}), the follicular helper T (T_{FH}), as well as the IL-17-producing (Th17) T cells. All of these Th lineages likely express or can be induced to express ICOS. However, it is presently unclear how the level of ICOS expression compare among different Th lineages and whether T-bet, GATA-3 or other transcription factors regulate ICOS expression via different pathways in these other subsets. Consider the example of Th17 cells, which have been causally linked to several inflammatory and autoimmune diseases including collagen-induced arthritis and experimental autoimmune myasthenia gravis, the pathogenesis of which were reportedly dependent on ICOS (50, 51). Both CD28 and ICOS were found to be required for the generation of effector CD4+ T cells that produce IL-17 (52). However, data accumulated so far suggest that the Th17 developmental pathways are independent of STAT-1/4 and T-bet as well as STAT-6 and GATA-3, which are required respectively for Th1 and Th2 differentiation. Are T-bet and GATA-3 therefore dispensable for Th17-intrinsic regulation of ICOS? Combined actions of the pleiotropic transforming growth factor- β and pro-inflammatory cytokines such as IL-6 were shown to induce Th17 development (53–55), whereas the retinoic acid-related orphan receptor ROR- γ t was established as necessary and apparently sufficient for Th17 commitment (56). What is the extent of contribution, if any, by these factors to ICOS expression in the Th17 lineage? Other than Th17 cells, what possible mechanisms underlie ICOS regulation in T_{reg} cells, particularly in the population expressing high levels of ICOS and are high producers of IL-10?

Apart from regulation at the transcriptional level, post-transcriptional mechanisms may also govern ICOS expression. In *sanroque* mice carrying a mutated *Rc3h1* gene encoding for the roquin protein, T_{FH} cells hyperproliferated, and germinal center development was exacerbated, and these mice eventually

succumbed to autoimmune disease (57). Incidentally, *sanroque* CD4⁺ T cells expressed higher levels of ICOS compared with their wild type counterparts, suggesting that roquin may play a role in limiting ICOS expression. Roquin contains amino-terminal RING-1 and CCCH zinc finger domains, the former being shared by E3 ubiquitin ligases and the latter by RNA-binding proteins such as tristetraprolin. Tristetraprolin is known to bind to the 3'-UTR of target genes and facilitate the processing, stabilization, and translation of their mRNAs. It is thus conceivable that roquin may modulate ICOS expression post-transcriptionally through affecting mRNA and/or protein stability. Consistent with this idea, we found roquin mRNA to be expressed highest in Th0, followed by Th1 and Th2 cells (data not shown).

In summary, given the Th-differential expression of ICOS and its pivotal role in costimulating immune and autoimmune responses, elucidating the molecular pathways that control the levels of ICOS expression in different Th cell subsets is of immense interest. However, whether the regulatory elements determined in this study to control ICOS transcription in Th cells differentiated *in vitro* have functional significance *in vivo* remains an open question. One way to address this is to generate BAC transgenic mice that carry mutations or deletions in the T-bet-, GATA-3-, and/or NFAT-binding sites in the *icos* locus and ask whether such mice develop aberrant Th1 *versus* Th2 immune responses to pathogens. Such an understanding will enable intervention strategies that modulate ICOS expression in Th cells to be devised, favoring host immunity while avoiding autoimmunity.

Acknowledgments—We thank staff of the Singapore Biological Resource Center for care and maintenance of mice and members of the LAM laboratory for helpful discussions and critical review of the manuscript.

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