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) 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 weakly expressed or absent on naive 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 factor-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, naive 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-induced 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 naive 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).
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 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 μg/ml of mitomycin C (Sigma) at 37 °C for 45 min and washed before incubation with 1–2 × 10⁶ AE7 cells and 5 μM of pigeon cytochrome C in complete medium for 48 h. The cells were then passed 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 μg/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 × 10⁶/ml) were stimulated with 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 Abs under Th0 (3 μg/ml anti-IL-12, 5 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4), Th1 (5 ng/ml IL-12 and 10 μg/ml anti-IL-4), or Th2 (10 ng/ml IL-4, 3 μg/ml anti-IL-12, and 5 μg/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. (Rockey 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 2–5 × 10⁶ 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 naive 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 TTG TTC CCT TTC TCC-3′ and 5′-CCA GGA GAA TGT TTG CCC-3′ and cloned into BamHI/Sall sites downstream of a luciferase reporter. For the effect of overexpressed proteins on *icos* promoter and 3′-UTR activities, the fold induction was calculated as Fold Induction = normalized RLUprotein expressing vector / normalized RLUnull vector. 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 Xbal 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 μm 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.
Regulation of ICOS Expression by T-bet and GATA-3

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). 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). 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 cell 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 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
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.

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 luc 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 transcripational 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 acting as the common coactivator in both Th subsets. Strikingly, the coexpression of GATA-3 and NFATc2 resulted in synergism with NFATc2 to enhance transcription via this 3′-UTR in Th1 cells and could be further enhanced when the cells were stimulated (Fig. 5A, lanes 1–6). This binding was significantly elevated in fully differentiated (8-day culture) Th0 cells cultured for 2 days and can be further increased when the cells were stimulated (lanes 7–12), 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.

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

...ulating pathways are nascent. As T-bet 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 promote 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 (Treg), the follicular helper T (TFH), 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 pathogeneses 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-γt 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 Treg 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_{F11} 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.

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