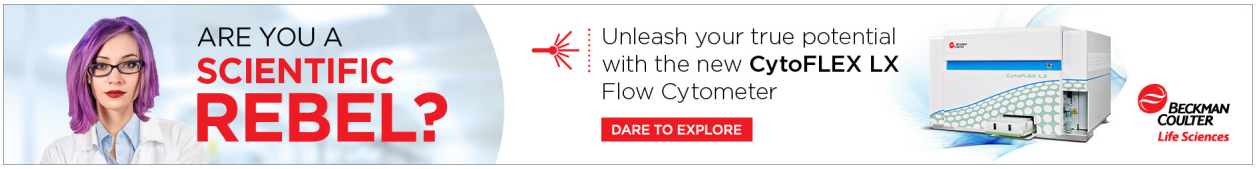

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Regulation of GATA-3 Expression during CD4 Lineage Differentiation

Idoia Gimferrer,*,[1] Taishan Hu,* Amie Simmons,* Chi Wang,[†] Abdallah Souabni,[‡] Meinrad Busslinger,[‡] Timothy P. Bender,[§] Gabriela Hernandez-Hoyos,[†] and José Alberola-Ila*,[¶]

GATA-3 is necessary for the development of MHC class II-restricted CD4 T cells, and its expression is increased during positive selection of these cells. TCR signals drive this upregulation, but the signaling pathways that control this process are not well understood. Using genetic and pharmacological approaches, we show that GATA-3 upregulation during thymocyte-positive selection is the result of additive inputs from the Ras/MAPK and calcineurin pathways. This upregulation requires the presence of the transcription factor c-Myb. Furthermore, we show that TH-POK can also upregulate GATA-3 in double-positive thymocytes, suggesting the existence of a positive feedback loop that contributes to lock in the initial commitment to the CD4 lineage during differentiation. ***The Journal of Immunology*****, 2011, 186: 3892–3898.**

During T cell development, the organism generates a T cell population with an extended repertoire of Ag specificities. This repertoire is molded in the thymus through signals derived from the interaction of TCRs on thymocytes with their ligands, MHC molecules with bound peptides. The majority of thymocytes bear TCRs that do not recognize the MHC molecules present in the thymus, and these cells die relatively rapidly (3–4 d). Those cells bearing a TCR able to interact with self-MHC can receive signals that induce either their differentiation into mature T cells (positive selection) or apoptosis (negative selection). Furthermore, those cells that are positively selected develop into two different lineages, CD4 or CD8, depending on the ability of their TCRs to bind MHC class II or I, respectively. The mechanisms that underlie this lineage commitment process have been extensively studied (reviewed in Refs. 1, 2), and although there is not yet a complete understanding of this process, a number of critical players have been identified in the last few years. These include signal transduction molecules such as Lck (3) and transcription factors, including GATA-3, Th-POK, Runx-1, and Tox (4–9).

GATA-3 is a member of the GATA family of transcription factors expressed by immune cells, from hematopoietic stem cells to mature T cells (10, 11). GATA-3 is required at multiple stages during T cell development and differentiation, from T cell commitment (12–14) to Th2 differentiation (15–17). During the process of positive selection and CD4/CD8 lineage commitment, GATA-3 is upregulated by TCR-mediated signals (4) and is required for the generation of CD4 T cells (4, 5).

The molecular mechanisms that control GATA-3 upregulation during positive selection are unknown, although our previous results showed that it was correlated to the strength of the TCR signal and the contribution of Lck to this signal (4). The only other known factor that contributes to the regulation of GATA-3 expression at this stage is the transcription factor c-Myb (18). In this article, we explore the contributions of different signal transduction pathways to TCR-mediated GATA-3 upregulation, as well as the role of c-Myb and Th-POK in this process. Our results show that: 1) the upregulation of GATA-3 in double-positive (DP) thymocytes is mediated by Ras/MAPK and calcineurin (CaN), and these pathways act in an additive fashion; 2) c-Myb is required for TCR-mediated induction of GATA-3, although signaling is normal in c-Myb-deficient DP thymocytes; and 3) Th-POK can upregulate GATA-3 in DP thymocytes.

Materials and Methods

Mice

C57BL/6 (B6), MHC$^{-/-}$ (C57BL/6 β2-microglobulin$^{-/-}$, I-Aβ$^{-/-}$ I-E–null), dnRas (19), and dLGF (3) mice were bred and maintained in the mouse facility at Oklahoma Medical Research Foundation. GATA-3–GFP knock-in mice have been described previously (20). They were bred into the MHC$^{-/-}$ background (G3/MHC°). c-Myb$^{f/f}$*cd4*Cre were obtained from Dr. Bender (21, 22) Adult mice used were between the ages of 6 and 12 wk.

Retroviral constructs

The MIG (MSCV-IRES-GFP) bicistronic retroviral vector has been previously described (23, 24). MIR (MSCV-IRES-RFP) is a variant generated in our laboratory where GFP has been replaced by DsRed (25). The LckF505 cDNA was provided by Dr. D. Straus (Virginia Commonwealth University, Richmond VA), RasV12 by Dr. J. Downward (Cancer Research UK, London, U.K.), GST-VIVIT and constitutively active (CA)-NFAT by Dr. F. Macian (Albert Einstein College of Medicine, Bronx, NY), CA Akt

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Abbreviations used in this article: CA, constitutively active; CaN, calcineurin; CsA, cyclosporin A; DP, double-positive; PdBu, phorbol dibutyrate; RT, reverse transcription; SP, single-positive.

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by Dr. D. Cantrell (University of Dundee, Dundee, U.K.), CA PDK by Dr. A. Weiss (University of California, San Francisco, San Francisco, CA), and CA Ikkβ by Dr. J. Pomerantz (The Johns Hopkins University School of Medicine, Baltimore, MD). All cDNAs were cloned in MIR using conventional molecular biology techniques. ThPOK and ThPOK-HD retroviruses were provided by Dr. D. Kappes (Fox Chase Cancer Center, Philadelphia, PA).

Retroviral production and thymocyte infection

Retroviral constructs in the MIR vector were cotransfected together with the pCL/Eco plasmid into 293 cells as described (26). For viral transduction, 1–2 × 10^6 thymocytes were mixed with 2 ml retroviral supernatant plus 20 μg/ml polybrene per well in 24-well plates and centrifuged at room temperature for 1 h, at 210 × *g*, as described previously (26). Supernatant was replaced with fresh media, and the cells were used for experiments 24–48 h later. In some experiments, thymocytes were cultured over OP9Δ cells (27). The efficiency of the infection varied with the construct, oscillating between 3 and 20%. Survival at the time of analysis was generally around 50%.

DP thymocyte enrichment

Petri dishes were coated with anti-rat IgG + IgM Ab (Jackson ImmunoResearch) in PBS pH 8 (10 mg/ml) for 2 h at 37°C or overnight at 4°C. Thymocytes were incubated for 30 min on ice with rat anti-CD53 (clone OX-79; BD Pharmingen) at saturating concentrations (10 mg/ml). Cells were rinsed once, resuspended in RPMI 1640, and incubated in the coated plates for 1 h at 4°C. The negative fraction ($CD53^-$) was recovered by aspiration without disturbing the adherent cells. More than 90% of the recovered cells were DP population, as determined by FACS using CD4 and CD8 markers.

In vitro stimulation of thymocytes

Thymocytes from $MHC^{-/-}$ mice, or enriched DP thymocytes from other strains, were stimulated on anti-CD3 (clone 2C11; 10 μg/ml; BD Biosciences or eBiosciences) coated plates or with soluble anti-CD4/CD3 $F(ab')_2$ Abs (0.2–1 μg/ml) (4, 28). Cells were harvested at the times indicated and counted. In some experiments, thymocytes were stimulated overnight with 5 or 10 ng/ml phorbol dibutyrate (PdBu) and/or 200 or 500 ng/ml ionomycin.

Intracellular staining

Cells were fixed with 2% paraformaldehyde (Pella) for 10 min at 37°C and washed in P4F (1× PBS, 4% FCS, 0.1% sodium azide). Then cells were permeabilized with 1% Triton X-100 (Calbiochem) in PBS for 15 min at room temperature, washed twice with 1 ml P4F and stained with an Alexa Fluor 647-conjugated anti–GATA-3 Ab (eBiosciences) for 1 h at room temperature. Cells were washed and analyzed by flow cytometry.

Real-time RT-PCR

DP thymocytes from $Myb^{f/w}$*cd4*Cre and $Myb^{f/f}$ *cd4*Cre were stimulated overnight and lysed. Total RNA was extracted using Qiagen RNeasy mini kit according to the manufacturer's protocol. Reverse transcription (RT) was performed on 2 μg RNA using TaqMan RT reagents (Applied Biosystems). Ten nanograms cDNA was used for each assay. SYBR Green real-time PCR was performed using a BioRad CFX96 real-time PCR detection system. Gene-specific forward and reverse primers were designed across exon-exon junction using Primer Express software (Applied Biosystems) with equivalent efficiency to the β-actin primers. Data were analyzed using the comparative CT method using CFX software (BioRad). The primers used were as follows: EGR1: forward, 5′-AGT GGG AGC CGG CTG GAG AT-3′; reverse: 5′-GGG CGA TGT AGC AGG TGC GG-3′; EGR2, forward: 5′-TTG ACC AGA TGA ACG GAG TGG-3′; reverse: 5′-TGC CCA TGT AGG TGA AGG TCT-3′; NUR77, forward: 5′-AGT GGG AGC CGG CTG GAG AT-3′; reverse: 5′-GGG CAG TGT AGC AGG TGC GG-3′; β-actin, forward: 5′-CAA CGA GCG GTT CCG ATG -3′, reverse: 5′-GCC ACA GGA TTC CAT ACC CA-3′.

Results

Use of a GATA-3–GFP "knock-in" allele to follow GATA-3 expression

As a way to follow GATA-3 upregulation at a single cell level, we used a genetically modified strain of mice that have GFP knocked into the GATA-3 locus, so that GFP is driven by the endogenous GATA-3 regulatory regions (20). As shown in Fig. 1*A*, the expression levels of GFP in different thymocyte subpopulations correlate perfectly with the expression pattern of GATA-3, as analyzed by RT-PCR, Western blot (4), or a *lacZ* knock-in (29). For most of the experiments described in this article, the GATA-3–GFP mice were bred into an MHC null background (G3/MHC°) to ensure that DP thymocytes have not received any signals through their TCR in vivo.

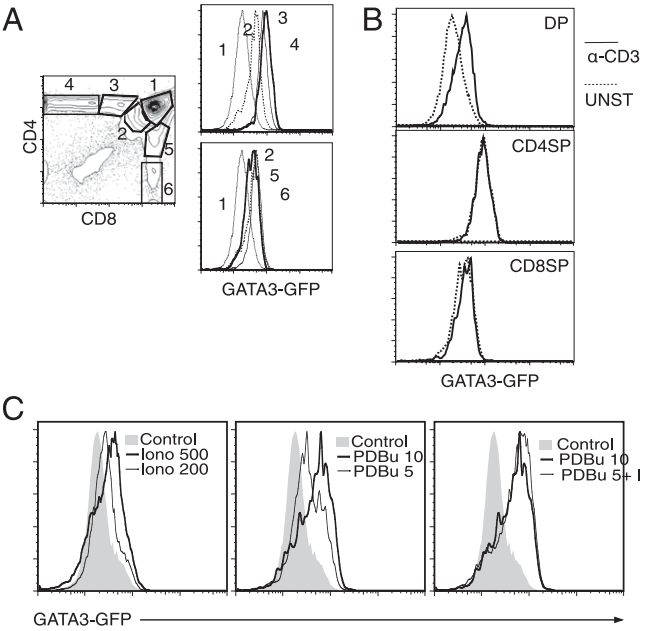

FIGURE 1. *A*, Expression pattern of the GATA-3–GFP reporter in different thymocyte subpopulations. Thymocytes from a GATA-3–GFP mouse were stained with CD4 and CD8, and GATA-3–GFP expression analyzed in different subpopulations (1–6). The two-color histogram shows the different gates, and the histograms show expression within the CD4 development series (1–4) (*top panel*) or CD8 development series (1, 2, 5, 6) (*bottom panel*). *B*, Induction of GATA-3 by TCR signals in different thymic subpopulations. GATA-3 GFP mice were stimulated with plate-bound anti-CD3 (10 μg/ml) and 18 h later stained with CD4 and CD8. Shown are histograms of GATA-3–GFP expression in gated DP, CD4 SD, and CD8 SD thymocytes, unstimulated (dotted lines) and stimulated (solid lines). *C*, Pharmacological upregulation of GATA-3. DP G3/MHC° thymocytes were stimulated overnight with different doses of PdBu or ionomycin, alone or in combination, as indicated. After overnight incubation, GATA-3 levels (GFP) were analyzed by flow cytometry. Shown is one representative experiment out of three.

TCR regulation of GATA-3 is specific for DP thymocytes

Our previous results had shown that, in DP thymocytes, TCR stimulation was sufficient to induce maximal GATA-3 upregulation. In contrast, in naive CD4 T cells, TCR stimulation alone can only weakly induce GATA-3 transcription, and additional signals mediated by IL-4 are required for maximal induction (30). Using the GATA-3–GFP mice, we decided to compare the responsiveness of GATA-3 with TCR cross-linking in different thymocyte subpopulations. Total thymocytes were stimulated on plate-bound anti-CD3 for 18 h and GATA-3 induction in different subpopulations followed using GFP as a readout. As shown in Fig. 1*B*, DP thymocytes are the only subpopulation that responds significantly to this stimulus, suggesting that GATA-3 regulation is different in DP versus more mature T cell populations.

GATA-3 can be upregulated in DP thymocytes by phorbol esters and calcium ionophores

As a first approximation to the signal transduction pathways involved in this process, we decided to use pharmacological agents

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that are known to activate two of the main signal transduction pathways in T cells, the Ras/MAPK cascade (the phorbol ester PdBu) and intracellular calcium (ionomycin, a calcium ionophore). GATA-3–GFP/MHC° thymocytes were treated with different concentrations of PdBu or ionomycin, and GATA-3 upregulation was detected 18 h later by monitoring GFP by flow cytometry. Our results (Fig. 1*C*) show that both PdBu and ionomycin upregulate GATA-3 expression in a dose-dependent manner. Furthermore, a combination of a low dose of both pharmacological products acts additively. These experiments suggested that both Ras/MAPK and calcium are involved in the TCR-induced upregulation of GATA-3 in DP thymocytes.

Activated Lck maximally induces GATA-3 in DP thymocytes

Our previous work showed that, in DP thymocytes, GATA-3 is upregulated in vitro by TCR signals (4), and suggested that signals that have a stronger Lck component were able to induce a stronger and more sustained upregulation. To test directly the involvement of Lck in GATA-3 upregulation, we used a CA form of Lck, Lck_{Y505F}, cloned in a modified version of the MIG retroviral vector that expresses DsRed instead of GFP (MIR). Thymocytes from GATA-3–GPF/MHC° mouse were infected in vitro, cultured over OP9Δ cells, and analyzed 48 h later by flow cytometry. Culture over OP9Δ improves the viability of the cells but does not affect the levels of GATA-3–GFP in the cultures (data not shown). Analysis was performed by gating on $DsRed^+$ (infected cells expressing the Lck_{Y505F}) and $DsRed^-$ cells (uninfected cells), and comparing the GFP levels. Expression of CD69 was analyzed as a control of the activation. As a negative control, the cells were infected with empty MIR virus. As shown in Fig. 2*A*, $DsRed^+$ cells have greater levels of GFP than the $DsRed^-$. Furthermore, the induction level was almost as high as that induced by plate-bound CD3 after 24 h. Because activation of Lck is a direct consequence of the binding of CD4/CD8 coreceptors with the MHC molecules, and CD4 coreceptor binds a greater amount of Lck, this result is consistent with the idea that CD4 engagement by class II MHC molecules induces greater levels of GATA-3.

Both Ras and CaN can partially induce GATA-3 in DP thymocytes

To directly test whether the Ras/MAPK pathway could drive GATA-3 upregulation in DP thymocytes, we used a CA Ras mutant (RasV12) cloned into the MIR vector in experiments similar to those described earlier. As shown in Fig. 2*A*, expression of RasV12 can upregulate GATA-3 in DP thymocytes, although contrary to what we observed with the active Lck, the magnitude of this effect was clearly inferior to that induced by direct TCR cross-linking.

Because the best characterized calcium effector during T cell activation is the CaN–NFAT pathway, we also tested whether a CA-NFAT was sufficient to induce GATA-3 in DP thymocytes using a similar approach. As shown in Fig. 2*A*, overexpression of CA-NFAT results also in the upregulation of GATA-3, and as observed with RasV12, this effect is less pronounced than the upregulation induced by CD3. These data suggest that both pathways are implicated in the GATA-3 upregulation after TCR stimulation, and also that both pathways have an additive effect on that upregulation. It must be noted that both mutants can induce maximal levels of other activation markers, such as CD69.

In contrast with the effects of Ras and CaN, constitutive expression of activated mutants in other signal transduction pathways (Akt, PDK, Stat5, IKKβ) did not induce major changes in GATA-3 expression (Fig. 2*B*).

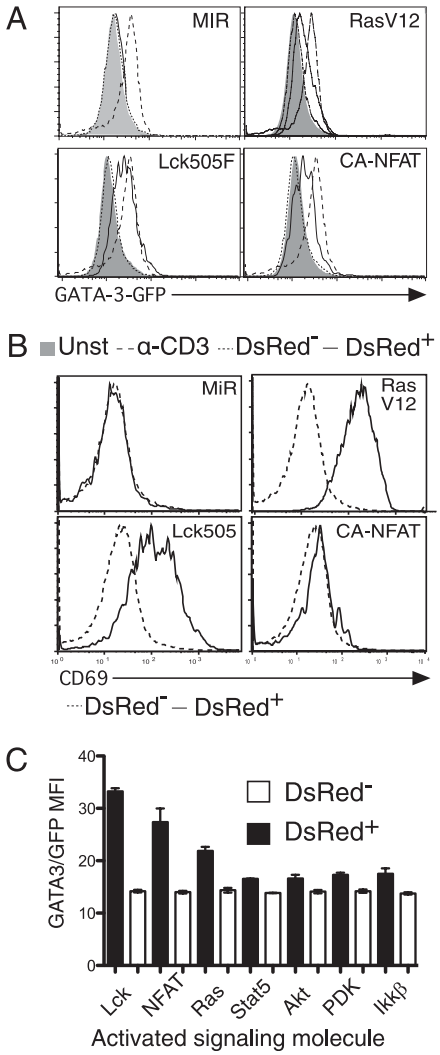

FIGURE 2. *A*, Upregulation of GATA-3 with CA forms of Lck (Lck_{F505}), Ras (RasV12), and NFAT (CA-NFAT). DP thymocytes from G3/MHC° mice were infected with the indicated constructs cloned in MIR vector or with empty MIR. Forty-eight hours later, the thymocytes were analyzed by flow cytometry, gating on $DsRed^+$ and $DsRed^-$. As a positive control of GATA-3 induction, an aliquot of DP thymocytes was cultured with plate-bound anti-CD3 (10 μg/ml) overnight. The histograms are representative of three to five independent experiments for each construct. *B*, The same samples were analyzed for CD69 expression. *C*, Comparison of the effects of CA Lck with other activated mutants in different signal transduction pathways. The experiments were performed as described earlier, at least three independent times for each construct.

Blockade of the Ras/MAPK pathway and CaN can partially inhibit TCR-induced GATA-3 upregulation in DP thymocytes

To confirm the implication of Ras/MAPK and CaN in TCR-induced GATA-3 upregulation, we used a loss-of- function approach. To study the Ras/MAPK pathway, we crossed the GATA-3–GFP/MHC° mice to a transgenic line that expresses high levels of a dominant negative form of Ras (RasN17) (19). We stimulated thymocytes from these mice in vitro with increasing doses of anti-CD3/CD4 bispecific $F(ab')_2$ and analyzed GFP expression by flow cytometry 18 h later. As shown in Fig. 3*A* and 3*B*, GATA-3 expression (GFP expression) was less upregulated in the cells expressing dnRas, and this blockade could not be overcome by increasing doses of the Ab (Fig. 3*C*). This partial blocking of GATA-3 upregulation in the dnRas cells, together with the result with the RasV12 protein,

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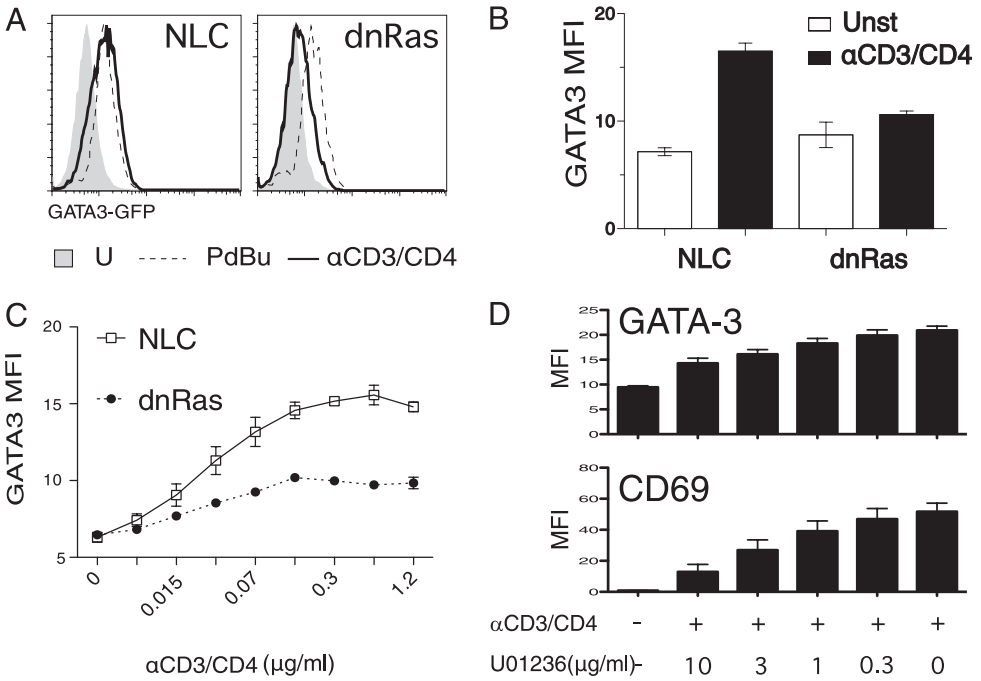

FIGURE 3. Blocking of the Ras pathway partially inhibits TCR-induced GATA-3 upregulation. *A*, DP thymocytes from dnRas/G3/MHC° mice or G3/MHC° littermates (NLC) were stimulated overnight with 0.5 μg/ml anti-CD3/CD4 F(ab′)$_2$ or 10 ng/ml PdBu, and changes in GATA-3 transcription monitored by flow cytometry. *B*, Quantification of the changes in GFP mean fluorescence intensity (MFI) in three independent experiments. *C*, DP thymocytes from dnRas/G3/MHC° mice or G3/MHC° littermates (NLC) were stimulated overnight with increasing concentrations of anti-CD3/CD4 F(ab′)$_2$, and changes in GATA-3 transcription monitored by flow cytometry (n = 3). *D*, DP thymocytes from G3/MHC° were stimulated overnight with 0.5 μg/ml anti-CD3/CD4 F(ab′)$_2$ in the presence of decreasing concentrations of U0126 (10, 3, 1, 0.3, and 0 μM). GFP and CD69 expression was monitored by flow cytometry. Shown is the average and SEM of three experiments.

confirms the implication of this Ras pathway in the GATA-3 upregulation after TCR stimuli.

One of the molecules activated downstream of Ras is the Erk MAPK kinase, MEK. We used U0126, a MEK-specific inhibitor, to confirm that the Ras–MEK–ERK pathway is implicated in the upregulation of GATA-3 after TCR stimuli. Thymocytes from GATA-3–GFP/MHC° mice were stimulated overnight with 0.5 μg/ml anti-CD3/CD4 F(ab′)$_2$ plus increasing concentrations of U0126 inhibitor. CD69 was used as a control of cell activation. GATA-3 upregulation was only partially inhibited by U0126, even at supraoptimal concentrations (Fig. 3*D*), confirming that the Ras/MAPK pathway is implicated in GATA-3 upregulation downstream the TCR, but also that it is responsible for only part of the response.

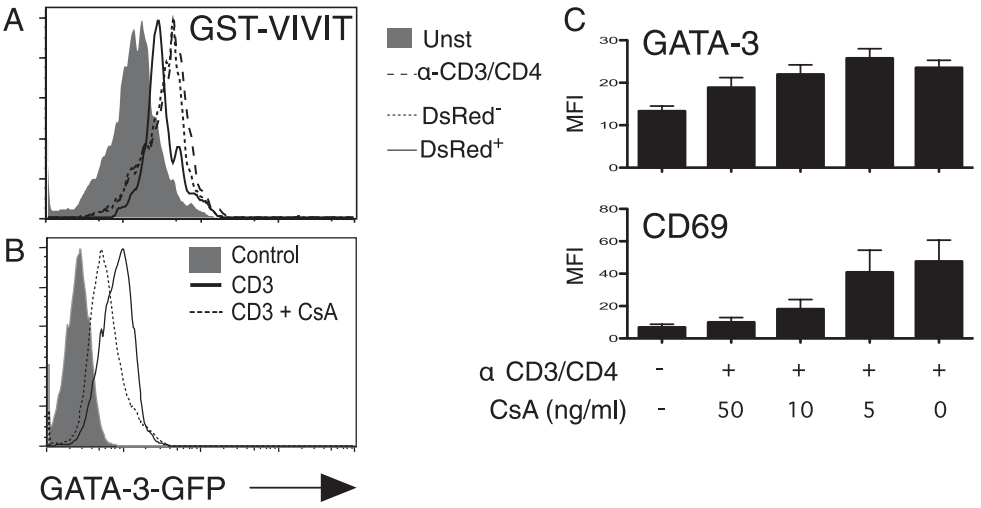

FIGURE 4. Blocking of the CaN–NFAT pathway partially inhibits TCR-induced GATA-3 upregulation. *A*, DP thymocytes from G3/MHC° mice were infected with GST-VIVIT cloned into MIR. Thirty-six hours later, the thymocytes were stimulated overnight with plate-bound anti-CD3, collected, and analyzed by flow cytometry. The GFP expression levels of DsRed$^+$ or DsRed$^-$ were compared. Uninfected G3/MHC°, stimulated or not overnight, was used as a positive and negative control, respectively. Shown is one representative experiment out of three. *B*, DP G3/MHC° thymocytes were stimulated overnight with 0.5 μg/ml anti-CD3/CD4 F(ab′)$_2$ in the presence of CsA (50 ng/ml). Some cells were left unstimulated. Cells were harvested and analyzed by flow cytometry. *C*, DP G3/MHC° thymocytes were stimulated overnight with 0.5 μg/ml anti-CD3/CD4 F(ab′)$_2$ in the presence of different concentrations of CsA (50, 10, 3, and 0 ng/ml). Some cells were left unstimulated. Cells were harvested, stained with CD69, and analyzed by flow cytometry. Shown is the average and SEM of three experiments.

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The other pathway implicated in GATA-3 upregulation in our gain-of-function experiments is CaN and NFAT. To study the effects of loss of function of this pathway on GATA-3 response to TCR stimuli, we first used a genetic approach, overexpressing the GST fusion protein GST-VIVIT, which competes with CaN for NFAT (31), using our MIR vector. Thymocytes from GATA-3–GFP/MHC° were infected with MIR-GST-VIVIT, cultured overnight over OP9Δ, and then stimulated for 18 h with anti-CD3/CD4 $F(ab')_2$. Analysis was performed by gating on $DsRed^+$ and $DsRed^-$ cells, and comparing the GFP levels in unstimulated and stimulated samples. As shown in Fig. 4*A*, thymocytes expressing GST-VIVIT ($DsRed^+$) had a partial block in GATA-3 upregulation. These results were confirmed using a pharmacological approach with the CaN inhibitor cyclosporin A (CsA), which also partially blocked this upregulation in a dose-dependent manner (Fig. 4*B*, 4*C*). As observed with the blockade of the Ras/MAPK pathway, we could not completely block TCR-induced GATA-3 upregulation with VIVIT, or with supraoptimal doses of CsA, even though they were sufficient to block other activation markers, such as CD69 (Fig. 4*C*).

Because these results, and those presented in Fig. 1*C*, suggest that both Ras and CaN partially control TCR-mediated GATA-3 upregulation in DP thymocytes, we tested whether simultaneously blocking both pathways would be sufficient to completely block it. For these experiments, we used DP thymocytes from dnRas/MHC°/GATA-3GFP and MHC°/GATA-3GFP mice, and stimulated them in vitro with anti-CD3 in the presence or absence of CsA (50 ng/ml). As shown in Fig. 5, the combination of the dnRas transgene plus CsA almost completely abrogated CD3-induced GATA-3 upregulation, suggesting that the combined input of these two pathways is responsible for most of the upregulation.

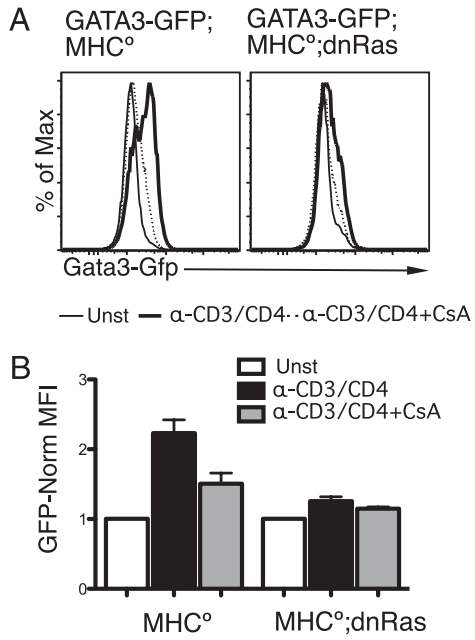

FIGURE 5. Simultaneous blocking of the CaN–NFAT and Ras pathways blocks TCR-induced GATA-3 upregulation. DP thymocyte from G3/MHC° mice or G3/MHC°/dnRas mice were stimulated overnight with 0.5 μg/ml anti-CD3/CD4 $F(ab')_2$ in the presence or absence of CsA (50 ng/ml), collected, and analyzed by flow cytometry. The GFP expression levels were compared. Shown is the average and SEM of three experiments. *A*, Representative histogram of the induction; (*B*) quantification of three independent experiments with a pair of G3/MHC° and G3/MHC°/dnRas mice per experiment.

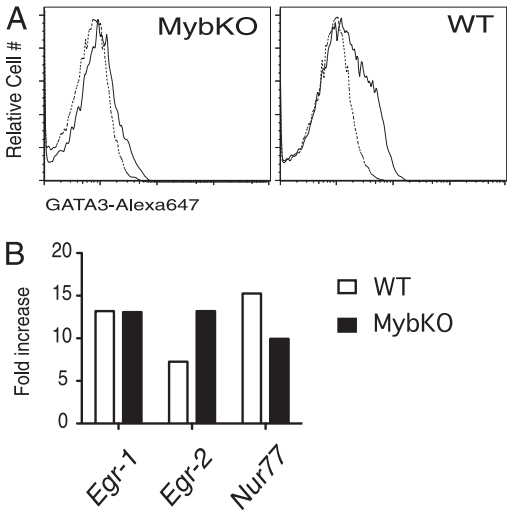

FIGURE 6. c-Myb is required for TCR-induced GATA-3 upregulation. *A*, Enriched DP thymocytes from c-$Myb^{f/f}$*cd4*Cre (MybKO) or c-$Myb^{f/w}$*cd4*Cre (wild-type [WT]) thymocytes were stimulated overnight with anti-CD3/CD4 $F(ab')_2$ (1 μg/ml) and analyzed for GATA-3 expression by flow cytometry. The histogram shows GFP expression in unstimulated (dotted lines) versus stimulated (solid lines) thymocytes. Shown is one representative experiment out of three. *B*, Enriched DP thymocytes from c-$Myb^{f/f}$*cd4*Cre (MybKO) or c-$Myb^{f/w}$*cd4*Cre (WT) mice were stimulated for 3 h with plate-bound anti-CD3 mAb (10 μg/ml). Induction of Egr-1, Egr-2, and Nur77 was assessed by quantitative RT-PCR, and fold increase versus the unstimulated cells calculated by normalizing to β-actin. Shown are the results of one representative experiment out of two.

The effect of TCR on GATA-3 expression requires the presence of c-Myb

c-Myb is important for CD4 generation, although the loss of CD4 single-positive (SP) cells is less dramatic in c-$Myb^{f/f}$*cd4*Cre mice than in GATA-$3^{f/f}$*cd4*Cre mice (21, 22). It has been suggested that this is due to a direct regulation of GATA-3 by c-Myb (18), and in our hands, overexpression of GATA-3 can rescue the CD4 defect in c-$Myb^{f/f}$*cd4*Cre mice (T. Hu and J. Alberola-Ila, unpublished observations). Therefore, we decided to test whether TCR stimulation could upregulate GATA-3 in c-$Myb^{f/f}$*cd4*Cre thymocytes. For these experiments, we bred our GATA-3–GFP mice to c-$Myb^{f/f}$*cd4*Cre mice and used enriched $CD53^-$ DP thymocytes for the stimulations. Overnight stimulation of GATA-3GFP/c-$Myb^{f/f}$*cd4*Cre thymocytes with anti-CD3/CD4 $F(ab')_2$ did not induce any increase in GFP expression (Fig. 6*A*), suggesting that in the absence of c-Myb, the GATA-3 locus is refractory to stimulation. This is not due to effects of c-Myb on the signal transduction cascades downstream the TCR, because well-characterized downstream effectors of Ras/MAPK (Egr-1) (32), calcium (Nur77)

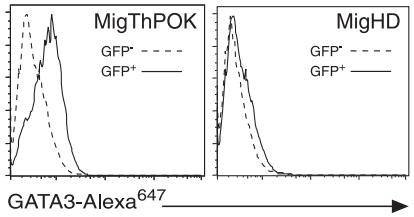

FIGURE 7. Th-POK upregulates GATA-3 in DP cells. Fetal DP cells, 15.5 d old, from MHC° mice were infected with MigThPOK or Mig-HD. Twenty-four hours later, cells were permeabilized and stained against GATA-3. Histogram shows GATA-3 protein expression in uninfected (dashed lines) or infected (solid lines) thymocytes. Shown is one representative experiment out of three.

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(33), or MAPK and CaN (Egr-2) (32) are upregulated normally in c-Myb–deficient DP thymocytes (Fig. 6*B*). These results support the idea that c-Myb is an important component of the circuit that regulates GATA-3 expression during positive selection, and that its presence is required to render the GATA-3 locus permissive to TCR induction (18).

Overexpression of Th-POK in DP thymocytes can drive GATA-3 expression

GATA-3 upregulation during CD4 lineage differentiation is sustained, and mature CD4 SP cells express significantly greater levels of GATA-3 than DP or CD8 SP. It is therefore likely that additional factors besides the TCR signals contribute to the regulation of GATA-3 expression during this process. We were interested in a possible role for Th-POK because, like GATA-3, this transcription factor is needed for CD4 commitment and is upregulated by TCR signals at later stages than GATA-3 (34). To test whether Th-POK expression could affect GATA-3, we infected 15.5-d-old fetal thymocytes from MHC$^{\circ}$ mice with a vector expressing Th-POK or with empty vector. After 72 h of culturing the cells over OP9Δ, they were permeabilized, stained for GATA-3 and analyzed by flow cytometry. Cells infected with Th-POK increased the level of GATA-3 in comparison with uninfected cells, indicating that Th-POK can upregulate GATA-3 by itself in DP thymocytes. In contrast, we did not observe GATA-3 upregulation in cells infected with the natural point mutation in Th-POK observed in HD mice (6, 35) (Fig. 7). Similar results were obtained using adult DP cells from MHC$^{\circ}$ mice.

Discussion

Our data show that GATA-3 expression during CD4/CD8 lineage differentiation is tightly regulated and controlled at different levels. First, even though GATA-3 is expressed at low levels in preselection DP thymocytes, and this basal expression is mostly independent of c-Myb, its induction by TCR signals requires the presence of c-Myb. The mechanism that underlies this requirement is unknown, although our results suggest that it is not related to effects of c-Myb on TCR signaling. It is possible that c-Myb controls accessibility of the GATA-3 locus, because it has been shown by chromatin immunoprecipitation that c-Myb is associated to the GATA-3 promoter in DP thymocytes (18). Second, induction of GATA-3 by TCR signals is mediated by at least two different signal transduction pathways downstream of the TCR and Lck, the Ras/MAPK pathway and CaN. Interestingly, these inputs seem to act additively and independently. Neither pathway is absolutely required for induction and neither is able by itself to achieve maximal induction, but when both are simultaneously inhibited, TCR-induced GATA-3 upregulation is almost completely blocked. Therefore, GATA-3 transcription functions as an integrative readout of the total signaling output of the TCR and coreceptor–Lck complex, so that stronger and/or more sustained signals result in higher expression of GATA-3, driving those cells toward the CD4 fate. Third, our data show that Th-POK is able to induce GATA-3 expression in DP thymocytes by itself. This observation could have important implications for our understanding of the molecular mechanisms that control CD4 lineage differentiation. Normally, GATA-3 upregulation precedes Th-POK induction, and in GATA-3–deficient mice, thymocytes are blocked before the stage where Th-POK can be induced (5, 36, 37), suggesting that one role of GATA-3 is to drive development up to the stage when Th-POK becomes inducible (2). However, GATA-3 has additional roles in this process, as shown by the fact that overexpression of Th-POK fails to rescue development of CD4 cells in GATA-3–deficient animals (37). This suggests that GATA-3 expression needs to be sustained at high levels during CD4 lineage differentiation. Our results indicate that Th-POK may be important for this maintenance, and that this positive feedback loop may contribute to the irreversibility of the commitment decision, because high levels of GATA-3 expression are incompatible with development into the CD8 lineage (4). However, Th-POK is not required for sustained GATA-3 expression in mature CD4 T cells (38), and the population where our experiments have been performed (preselection DP thymocytes) is not the exact population where Th-POK effects on GATA-3 expression would occur (CD69^{+} postselection CD4^{+}CD8^{lo} thymocytes). Additional experiments will be necessary to confirm that in vivo Th-POK plays a role in the regulation of GATA-3 during the differentiation of CD4 T cells in the thymus.

It is also interesting to contrast what we have learned about the control of GATA-3 expression during CD4/CD8 lineage differentiation with its regulation during differentiation of naive CD4 T cells into Th2 cells. The *Gata3* gene encodes two transcripts with alternative untranslated first exons, 1a and 1b (30), and the transcription of each one is driven by independent promoters. The proximal promoter drives expression of the transcript containing the exon 1b and is selectively active in thymus, whereas the distal promoter, which drives the expression of exon 1a, is active in the brain and during Th2 differentiation. Stimulation of naive CD4^{+} T cells with anti-CD3 and anti-CD28 Abs weakly induces exon 1b transcripts, whereas costimulation with IL-4 strongly induces both 1b and 1a transcripts, and is critical for Th2 differentiation (30). In contrast, in DP thymocytes, TCR stimulation by itself maximally activates GATA-3 transcription (4). Furthermore, the distal promoter has binding sites for, and is responsive to Notch, whereas the proximal promoter is not (39, 40). Accordingly, overexpression of Notch does not alter GATA-3 expression in DP thymocytes (G. Hernandez-Hoyos and J. Alberola-Ila, unpublished observations). Furthermore, control of GATA-3 levels in Th2 cells is also regulated by posttranscriptional mechanisms, such as phosphorylation by p38 (41, 42) or Erk (43), and ubiquitination (43), whereas in thymocytes undergoing positive selection and lineage commitment, protein expression seems to correlate with transcriptional activity (4). Additional studies will be necessary to discard the possibility that these posttranslational mechanisms play a role in the regulation of GATA-3 levels during CD4 lineage differentiation in the thymus, because our current study was mostly focused on transcriptional readouts. However, it is interesting to point out that, in Th2 cells, inhibition of activation of Ras/MAPK cascade by PD98059 or a dnRas transgene had no blocking effect on GATA-3 mRNA expression, but altered protein stability by increasing its ubiquitination (43). In contrast, our results demonstrate that, in DP thymocytes, the Ras/MAPK directly controls transcription of the *Gata3* gene.

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Disclosures

The authors have no financial conflicts of interest.

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