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TCR-Dependent Translational Control of GATA-3 Enhances Th2 Differentiation

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The differentiation of CD4+ T cells into the Th2 subset is controlled by the transcription factor GATA-3. GATA-3 is both necessary and sufficient for Th2 differentiation and works through the induction of chromatin remodeling at the Th2 effector cytokine loci. We show in this study that IL-4 stimulation induces GATA-3 mRNA upregulation, but the level of GATA-3 protein induced is insufficient for Th2 differentiation. The levels of GATA-3 protein and Th2 differentiation are enhanced by concomitant TCR signaling through the PI3K/mammalian target of rapamycin pathway. The PI3K-mediated increase in GATA-3 protein occurs without increasing the GATA-3 mRNA level. Rather, TCR signaling through PI3K specifically enhances the translation rate of GATA-3 without affecting the protein stability. Importantly, this role of TCR signaling is independent of the effects of TCR without increasing the GATA-3 mRNA level. Rather, TCR signaling through PI3K specifically enhances the translation rate of GATA-3 mRNA levels.

Additional effects of TCR signaling on Th2 differentiation have also been implicated in GATA-3 transcriptional regulation. Changing the affinity of the TCR for peptide MHC complexes using altered peptide ligands or changing the peptide dose impacts the GATA-3 mRNA level. Genetic disruption, or inhibition, of the downstream signaling molecules NF-kB and ERK also alters GATA-3 transcription. In addition, it has been difficult to elucidate specific effects of TCR signaling on GATA-3 expression and Th2 differentiation, because TCR signaling is required for initial T cell activation, survival, and expansion.

To bypass the requirement for TCR signaling in T cell expansion, we have used activated, undifferentiated T cells to determine specific contributions of IL-4R and TCR signaling toward GATA-3 expression and Th2 differentiation. We show in this study that expansion of activated, undifferentiated T cells is not dependent on Ag stimulation, but TCR signaling is absolutely required for Th2 differentiation. The impact of TCR signaling is not mediated through upregulation of GATA-3 transcription, as IL-4 induces GATA-3 mRNA equivalently in the presence or absence of Ag stimulation instead.

T cell stimulation

CD4+ T cells were purified by negative selection from DO11.10 TCR transgenic mice. All mouse experiments were approved by the University Committee on Animal Resources at the University of Rochester (Rochester, NY). Magnetic column separation was used to enrich for >90% CD62Lhi cells. T cells were stimulated with 0.2 μM OVA peptide presented by irradiated BALB/c spleencytes plus 10 U/ml human IL-2 (hIL-2). For activated, undifferentiated T cells, 10 μg/ml anti-IL-4 was added to the cultures; for Th1 cells, 10 μg/ml anti-IL-4 and 10 ng/ml IL-12 was added; and for Th2 cells, 20 ng/ml IL-4 was added. Activated, undifferentiated cells were restimulated on day 5 with mitomycin C-treated 6132 Pro cell

Materials and Methods

T cell stimulation

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transfectants expressing class II (I-A<sup>b</sup>) and B7-1 (ProAd-B7) (21) ≥ 2 μM OVA peptide and ≥ 20 ng/ml IL-4. A total of 10 U/ml hIL-2 was added to all restimulation cultures to provide for equivalent T cell expansion. Where indicated, cells were labeled with 1 μM CFSE at 37°C for 5 min prior to restimulation. Where indicated, 10 μM LY294002 or 10 nM rapamycin was added during the restimulation. To assay for cytokine secretion, T cells were stimulated overnight with 1 μg/ml plate-bound anti-CD3 (2C11), and supernatants were assayed by capture ELISA for IL-2, IL-4, and IFN-γ. For intracellular cytokine staining, brefeldin A was added 16–20 h post-stimulation. Four hours later, cells were collected, fixed with paraformaldehyde, and permeabilized with 0.5% saponin. Cytokine staining was performed in 0.5% saponin with PE or allophycocyanin-labeled anti-IL-4 (BD Biosciences, San Jose, CA/Ebioscience, San Diego, CA).

**Real-time PCR**

Total RNA was isolated from 10⁶ T cells using TRizol (Invitrogen, Carlsbad, CA), and cDNA was made with Superscript II Reverse Transcriptase (Invitrogen). The GATA-3 real-time PCR primers that spanned the exon 2–3 junction or the exon 4–5 junction and the CD3δ and Lck primers were all obtained from Applied Biosystems (Carlsbad, CA). The custom exon 1a- and exon 1b-specific primers were also ordered from Applied Biosystems. The sequences for exon 1a primers are: forward 5′-AGCTGCGCCCTAGAGAGGA-3′ and reverse 5′-CCCTAGACAGCTGTGCT-3′ with probe 5′-TTCGCCGATCACCTCAGTAC-3′; and for exon 1b are: forward 5′-GCTCTCTCTCTCTCTCTAC-3′ and reverse 5′-CGCTCAGAGCTGTTGCT-3′ with probe 5′-TTCGCCGATCACCTCAGTAC-3′. Plates were run on the 7900HT RT-PCR System (Applied Biosystems). GATA-3 level was normalized to CD3δ (except where indicated) relative to activated, undifferentiated T cells prior to restimulation.

**Protein analysis**

Nuclear and cytosolic fractions were isolated from 3 × 10⁶ T cells using NE-PER reagents (Pierce, Rockford, IL), and Western blots were probed with anti–GATA-3 mAb or anti-proliferating cell nuclear Ag (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA). For all analyses, a 2-fold dilution curve of Th2 cell lysates was included on each blot and used to calculate fold differences between experimental samples. GATA-3 values were normalized to PCNA relative to activated, undifferentiated T cells prior to restimulation. For eukaryotic initiation factor (eIF) 2α experiments, cytosolic lysates were isolated using a Nonidet P-40 lysis buffer [1% Nonidet P-40, 50 mM Tris (pH 8), 150 mM NaCl, leupeptin, aprotinin, PMSF, and phosphatase inhibitor mixture I (Sigma-Aldrich, St. Louis, MO)] and probed with anti–phospho-eIF2 (Ser51), anti-eIF2α (Cell Signaling Technology, Danvers, MA), and anti-Lck (Santa Cruz Biotechnology). For biosynthetic labeling, T cells were pulse-labeled with 300 Ci/ml [35S]methionine/cysteine into newly synthesized proteins to determine the overall translational efficiency, the percent incorporation of [35S]methionine/cysteine into newly synthesized proteins was determined following precipitation of the pulse-labeled proteins overnight at −20°C with 100% ethanol.

**Results**

**Th2 differentiation is specifically dependent on TCR signaling**

To study the specific effects of IL-4R and TCR signaling on Th2 differentiation, we have generated activated, undifferentiated Th cells. Naïve T cells from DO11.10 TCR transgenic mice are primed in the absence of polarizing cytokines and in the presence of anti–IL-4 to prevent Th2 differentiation. At day 5, these activated, undifferentiated cells were then restimulated with IL-4, Ag, or Ag plus IL-4 for 6 d. The number of cells recovered at the end of the restimulation is similar in all stimulation conditions (Fig. 1B). This indicates that TCR signaling of activated, undifferentiated cells is not required for T cell expansion. The ability of T cells to expand via cytokines allows us to determine if TCR signaling is specifically required for Th2 differentiation. Restimulation with either IL-4 or Ag on its own is not sufficient to induce Th2 differentiation, as measured by IL-4 (Fig. 1C) and IL-5 (data not shown) production. Th2 differentiation only occurs poststimulation with both Ag and IL-4 (Fig. 1C). To show that the dependence on TCR signaling for Th2 differentiation is not linked to cell division, we labeled activated, undifferentiated T cells with CFSE prior to restimulation. T cells efficiently enter the cell cycle under all stimulation conditions, although restimulation with IL-4 in the absence of Ag results in slightly delayed kinetics of CFSE dilution (Fig. 2A). Over time, T cells undergo similar rounds of cell division (Fig. 2A), corresponding to the equivalent level of T cell expansion (Fig. 1B). However, little or no IL-4–producing cells are detected poststimulation with IL-4 or Ag alone (Fig. 2B). Only cells that had been stimulated with Ag plus IL-4 are capable of producing IL-4 (Fig. 2B). Thus, the defect in Th2 differentiation by cells stimulated with IL-4 or Ag is clearly not a secondary effect caused by a failure in cell division. Taken together, these data show that TCR signaling has a specific role in Th2 differentiation that is independent of the requirement for TCR signaling in T cell survival and expansion.

**TCR signaling increases GATA-3 protein levels without affecting GATA-3 mRNA levels**

Previous reports have suggested that TCR signaling components may play a role in the transcriptional regulation of GATA-3 (17–20, 22). We measured the induction of GATA-3 mRNA to determine if TCR signaling enhances GATA-3 expression during the restimulation of activated, undifferentiated T cells. Notably, we find that IL-4 stimulation drives GATA-3 mRNA induction in the presence or absence of Ag stimulation (Fig. 3A). TCR signaling is unable to drive GATA-3 mRNA on its own or, more importantly, amplify the level of GATA-3 mRNA when added to IL-4 stimulation (Fig. 3A). We believe that this induction of mRNA is reflective of trans-
GATA-3 protein without increasing the level of GATA-3 mRNA (Fig. 3).

IL-4 stimulation, despite equivalent induction of GATA-3 mRNA.

IL-4 stimulation induces a greater level of GATA-3 protein than protein levels. IL-4 stimulation induces a small increase in GATA-3 induced GATA-3 mRNA, TCR signaling does increase GATA-3

Fig. 2. (A) Activated, undifferentiated T cells were CFSE labeled and then restimulated with IL-4, OVA peptide presented by ProAd-B7 cells (Ag), or with both Ag and IL-4; hIL-2 was added to all conditions. CFSE dilution was measured by flow cytometry 48 (dashed line), 96 (thin line), and 144 (thick line) h postrestimulation. Labeled T cells without any restimulation are shown (shaded) in each panel. One experiment, representative of five, is shown. B, Six days postrestimulation, T cells were stimulated overnight on plate-bound anti-CD3 and stained for intracellular IL-4 (bottom panels); isotype control is shown for comparison (top panels). One experiment, representative of six, is shown.

scripational activation, as there is no change in GATA-3 mRNA stability in the presence or absence of Ag stimulation (data not shown). The relative amount of GATA-3 mRNA in cells primed under Th2 conditions during initial priming is similar to the level of IL-4–induced GATA-3 mRNA, whereas Th1 cells have greatly reduced GATA-3 mRNA (Fig. 3B). Similar results were obtained for GATA-3 mRNA induction using primers that span both the exon 2–3 (Fig. 3A, 3B) and the exon 4–5 junctions (data not shown). GATA-3 transcripts can be initiated by two different promoters, resulting in mRNA molecules that differ only in the exon 1 portion of their 5′ untranslated region (UTR) (8). Transcription from the downstream promoter represents the majority of the overall GATA-3 mRNA level in resting T cells [data not shown (23)]. To determine if IL-4 signaling preferentially induces one of these promoters, we designed real-time PCR primers that span the exon 1A and exon 1B–2 junctions. We find that IL-4 stimulation induces GATA-3 mRNA from both the upstream and downstream promoters Lck (Fig. 4A) and MHC class I (data not shown). Furthermore, the percent incorporation of [35S]methionine/cysteine into newly synthesized proteins is equivalent between IL-4– and Ag plus IL-4–stimulated cells, indicating that the overall translational efficiency is not increased by Ag stimulation (Fig. 4B).

Fig. 3. IL-4 stimulation is sufficient to drive maximal GATA-3 mRNA, but coengagement of TCR is required to induce maximal GATA-3 protein levels. A, Activated, undifferentiated T cells were restimulated with IL-4, OVA peptide presented by ProAd-B7 cells (Ag), or with both Ag and IL-4; hIL-2 was added to all conditions. GATA-3 mRNA was measured by real-time PCR at different times following restimulation. The fold induction of GATA-3 mRNA, relative to activated, undifferentiated cells, is depicted as mean ± SEM. B, Freshly isolated CD62L-enriched DO11.10 CD4+ T cells were stimulated with OVA peptide presented by irradiated BALB/c splenocytes in the presence of Th1- or Th2-polarizing cytokines. On day 5, T cells were harvested, and GATA-3 mRNA was measured by real-time PCR and quantified relative to activated, undifferentiated cells. Data are depicted as mean ± SEM. C and D, T cells were stimulated as in A, and GATA-3 mRNA containing exon 1A (C) and exon 1B (D) was measured by real-time PCR. The fold induction of GATA-3 1A and 1B mRNA, relative to activated, undifferentiated cells, is depicted as mean ± SEM. E and F, T cells were stimulated as in A, and GATA-3 protein was measured 48 h postrestimulation by Western blot of nuclear lysates, which were subsequently stripped and reprobed for PCNA. GATA-3 protein level at 48 h postrestimulation, relative to unstimulated cells. Data are depicted as mean ± SEM. p = 0.0003 for IL-4 versus Ag + IL-4 by paired t test; n = 8.

implicates a mechanism of TCR-dependent posttranscriptional control of GATA-3 expression.

GATA-3 translation rate is enhanced by Ag stimulation

To determine if the posttranscriptional increase is mediated by an increase in GATA-3 protein synthesis, we measured the rate of GATA-3 translation by pulse labeling with [35S]methionine/cysteine. A significant increase in the level of de novo synthesis of GATA-3 is detected in Ag plus IL-4–stimulated cells when compared with IL-4–stimulated cells (Fig. 4A). The increase in Ag-stimulated cells is specific for GATA-3, as we detect similar levels of the control proteins Lck (Fig. 4A) and MHC class I (data not shown). Furthermore, the percent incorporation of [35S]methionine/cysteine into newly synthesized proteins is equivalent between IL-4– and Ag plus IL-4–stimulated cells, indicating that the overall translational efficiency is not increased by Ag stimulation (Fig. 4B). To determine the GATA-3 translation rate, we measured the amount of protein produced during the pulse label (Fig. 4A) and divided by the amount of mRNA (Fig. 4C), both relative to Lck. We see a consistent 4-fold increase in the GATA-3 translation rate in the presence of TCR signaling (Fig. 4D). Thus, these data show that TCR...
TCR signaling does not affect phosphorylation of eIF2α. A and C, Activated, undifferentiated T cells were restimulated with IL-4 or with both IL-4 and OVA peptide presented by ProAd-B7 cells (Ag), or with both Ag and IL-4; hIL-2 was added to both stimulation conditions. A, Forty-eight hours postrestimulation, T cells were pulsed with [35S]methionine/cysteine for 10 min. Nuclear and cytosolic lysates were immunoprecipitated for GATA-3 and Lck and analyzed on SDS gels. One experiment, representative of three, is shown. B. The overall translational efficiency was determined by measuring the percent incorporation of [35S]methionine/cysteine into ethanol-precipitable proteins from the pulse-labeled lysates. Data are depicted as mean + SEM; n = 3. The fold induction of GATA-3 mRNA, relative to Lck, is shown for the experiment in A. D. The GATA-3 translation rate was calculated as the amount of detectable radioactivity from the pulse period divided by the amount of mRNA at the time of the pulse label, both normalized to Lck. Data are depicted as mean + SD. p < 0.0001 for IL-4 versus Ag + IL-4 by unpaired t test; n = 3.

FIGURE 5. TCR signaling does not affect phosphorylation of eIF2α. A and C, Activated, undifferentiated T cells were restimulated with IL-4, OVA peptide presented by ProAd-B7 cells (Ag), or with both Ag and IL-4; hIL-2 was added to all conditions (left). B and D. Fibroblast cells were treated with 5 μg/ml Tun for 2 h (right). Phosphorylation of eIF2α at Ser 51 was measured 48 h postrestimulation by Western blot of cytosolic lysates, which were subsequently stripped and reprobed for total eIF2α and Lck. The ratio of phosphorylated to total eIF2α, relative to unstimulated T cells (C) and vehicle-treated fibroblast cells (D). Data are depicted as mean + SEM. n = 5 for T cells; n = 4 for fibroblasts. Tun, tunicamycin.

FIGURE 6. TCR signaling does not increase GATA-3 protein stability. Activated, undifferentiated T cells were restimulated with IL-4 or with both IL-4 and OVA peptide presented by ProAd-B7 cells (Ag + IL-4); hIL-2 was added to both stimulation conditions. A and B. Forty-eight hours postrestimulation, T cells were pulsed with [35S]methionine/cysteine for 10 min, then chased with unlabeled media for various times. Nuclear and cytosolic lysates were immunoprecipitated for GATA-3 and Lck and analyzed on SDS gels. The cell number was adjusted so that the IL-4 and Ag + IL-4 stimulated samples have similar starting levels of GATA-3. We label to compensate for the decreased GATA-3 translation in the absence of TCR signaling, so the IL-4– and Ag plus IL-4–stimulated samples have similar starting levels of GATA-3. We find that the degradation of biosynthetically labeled GATA-3 protein is not affected by Ag stimulation (Fig. 6A, 6B). This result is confirmed when GATA-3 protein stability is measured after blocking new protein synthesis with cycloheximide (Fig. 6C, 6D). GATA-3 has a similar rate of degradation in cells stimulated with IL-4 in the presence or absence of TCR signaling. Thus, the

signaling specifically increases the translation of GATA-3, resulting in higher GATA-3 protein levels and Th2 differentiation.

The priming of CD4+ T cells induces components of the integrated stress response, resulting in phosphorylation of eIF2α at Ser 51 (24). This phosphorylation prevents exchange of GDP for GTP by eIF2B and renders initiation complexes ineffective, resulting in translational arrest of key proteins, including IL-4. Acute restimulation through the TCR relieves the stress response, resulting in dephosphorylation of eIF2α and an increase in global translational efficiency. To determine if the stress response is contributing to the TCR-dependent increase in GATA-3 translation, we measured the phosphorylation state of eIF2α at Ser 51 in activated, undifferentiated cells stimulated with IL-4, Ag, or Ag plus IL-4. At 48 h postrestimulation, the time we detect an increase in the GATA-3 translation rate, there is no difference in the amount of eIF2α phosphorylation (Fig. 5A, 5C). As a control, we measured induction of eIF2α phosphorylation in fibroblast cells treated with tunicamycin, a potent inducer of the stress response, which results in a 5-fold increase in phosphorylation (Fig. 5B, 5D). Taken together, these data further show that the TCR-dependent translational increase is specific for GATA-3 and not due to a global enhancement of translation.

TCR signaling does not enhance GATA-3 protein stability

It has been shown that signaling through ERK can increase GATA-3 protein stability (25). To determine if an additional TCR-mediated effect on GATA-3 protein stability also contributes to the posttranscriptional increase in GATA-3 protein, we calculated the GATA-3 protein t1/2 with a pulse-chase assay (Fig. 6A, 6B). The number of IL-4–stimulated cells was adjusted during the pulse

label to compensate for the decreased GATA-3 translation in the absence of TCR signaling, so the IL-4– and Ag plus IL-4–stimulated samples have similar starting levels of GATA-3. We find that the degradation of biosynthetically labeled GATA-3 protein is not affected by Ag stimulation (Fig. 6A, 6B). This result is confirmed when GATA-3 protein stability is measured after blocking new protein synthesis with cycloheximide (Fig. 6C, 6D). GATA-3 has a similar rate of degradation in cells stimulated with IL-4 in the presence or absence of TCR signaling. Thus, the
posttranscriptional increase in GATA-3 protein in Ag-stimulated cells is a direct effect of an increased GATA-3 translation rate and not due to an increase in GATA-3 protein stability.

**PI3K signaling through mammalian target of rapamycin is required for enhanced GATA-3 translation and Th2 differentiation**

The PI3K-mediated conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate serves as a membrane-recruitment site for pleckstrin homology domain-containing proteins including Akt, PDK1, Ikt, and Vav (26). Signaling through Akt leads to activation of the mammalian target of rapamycin (mTOR) kinase, a protein central to cell growth and the regulation of both gene-specific and global translation. To determine if the PI3K–mTOR pathway is involved in GATA-3 translation, we measured GATA-3 mRNA and protein levels in the absence of PI3K signaling. Inhibition of PI3K with the inhibitor LY294002 has no effect on GATA-3 mRNA levels, but specifically decreases the level of GATA-3 protein (Fig. 7A–C). These data suggest that PI3K signaling is required for the TCR-dependent increase in GATA-3 translation. To determine whether the decrease in GATA-3 protein is due to a specific translational defect or reflective of a global effect of PI3K, we measured de novo protein synthesis by $[^{15}S]$methionine/cysteine pulse labeling. Consistent with the role of PI3K signaling in global translational efficiency, there is a slight decrease in the overall translation rate in the presence of the PI3K inhibitor (Fig. 7D). However, in addition to this global effect, there is a specific decrease in GATA-3 translation (Fig. 7E, 7F). The reduction in GATA-3 translation is greater than the reduction in global translation, and when GATA-3 is normalized, for both mRNA and protein, to Lck, we detect a specific decrease in the GATA-3 translation rate (Fig. 7E, 7F). Thus, the addition of LY294002 specifically decreases the GATA-3 translation rate, indicating that the TCR-dependent specific increase in GATA-3 translation is mediated by signaling through PI3K. To determine if this pathway is dependent on mTOR, T cells were treated with rapamycin. Addition of rapamycin to Ag plus IL-4–stimulated cells does not affect the level of GATA-3 mRNA (Fig. 7G). Rather, the level of GATA-3 protein is decreased in the absence of mTOR signaling (Fig. 7H, 7I), indicating that mTOR signaling is required for the TCR-mediated translational upregulation of GATA-3. Taken together, these data indicate that the TCR-mediated induction of GATA-3 translation is dependent on PI3K signaling through mTOR.

The induction of Th2 responses in vivo has been shown to be dependent on PI3K signaling. However, these effects may be T cell independent, as alteration of PI3K signaling has effects on dendritic cell IL-12 production, mast cell and eosinophil recruitment, and airway hyperresponsiveness in asthma models (27–30). In vitro, the effect of PI3K inhibition on Th2 differentiation is concomitant with a decrease in cell division, making it unclear if the effect is specific for the Th2 program or a secondary consequence of the block in T cell expansion (31). Consistent with this, we detect major decreases in cell expansion poststimulation of naive T cells in the presence of LY294002 or rapamycin (data not shown). However, using activated, undifferentiated T cells allows us to measure Th2 differentiation while bypassing this effect of PI3K inhibition on cell expansion. Although there is a slight decrease in cell division, addition of LY294002 or rapamycin has no effect on T cell expansion of previously activated cells (Fig. 8A, 8C). In contrast to the minimal

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**FIGURE 7.** PI3K signaling through mTOR is required for enhanced GATA-3 translation. Activated, undifferentiated T cells were restimulated with both IL-4 and OVA peptide presented by ProAd-B7 cells in the presence of 10μM LY294002 (LY294), 10 nM Rap, or vehicle control DMSO; hkl = 2 was added to all stimulation conditions. A, GATA-3 mRNA was measured by real-time PCR at different times following restimulation. The fold induction of GATA-3 mRNA, relative to activated, undifferentiated cells, is depicted as mean ± SEM; n = 5 for each time point. B, GATA-3 protein was measured 48 h postrestimulation by Western blot of nuclear lysates. C, The amount of GATA-3 protein relative to unstimulated cells is depicted as mean + SEM; p = 0.0018 for DMSO- versus LY294-treated samples by paired t test; n = 4. D and E, Forty-eight hours postrestimulation, T cells were pulsed with $[^{15}S]$methionine/cysteine for 10 min. D, The overall translational efficiency was determined by measuring the percent incorporation of $[^{15}S]$methionine/cysteine into ethanol-precipitable proteins from the pulse-labeled lysates. Data are depicted as mean ± SEM; n = 2. E, Nuclear and cytosolic lysates were immunoprecipitated for GATA-3 and Lck and analyzed on SDS gels. F, The GATA-3 translation rate was calculated as the amount of detectable radioactivity from the pulse period divided by the amount of mRNA at the time of the pulse label, both normalized to Lck. Data are depicted as mean + SEM; n = 2. G, GATA-3 mRNA was measured as in A and is depicted as mean ± SEM; n = 3. H and I, GATA-3 protein was measured as in B and is depicted as mean + SEM, p = 0.0041 for DMSO- versus Rap-treated samples by paired t test; n = 5. Rap, rapamycin.
effects on cell expansion, the amount of Th2 differentiation is greatly reduced with LY294002 or rapamycin treatment (Fig. 8B, 8C). These data indicate that there is a specific defect in Th2 differentiation in the absence of PI3K/mTOR signaling and that this defect correlates with a failure to upregulate GATA-3 translation.

Discussion

The transcription factor GATA-3 is a tightly regulated protein that is essential for Th2 responses (1). To understand the signaling pathways that lead to GATA-3 upregulation, we have developed stimulation conditions that allow us to separately study the contributions of IL-4R and TCR signaling. We have identified a role of TCR signaling in GATA-3 translation that is independent of the role of TCR signaling in initial T cell activation, survival, and expansion. This translational increase is specific for GATA-3 and responsible for the TCR-dependent increase in GATA-3 protein that is associated with increased Th2 differentiation.

The majority of data investigating GATA-3 expression has focused on transcriptional upregulation. It was initially shown that addition of IL-4 to activated T cells promotes Th2 differentiation and GATA-3 transcription through the activation of the transcription factor STAT6 (7, 32, 33). Although the role of STAT6 in promoting GATA-3 transcription is well established, the previous experiments have been done in the presence of concomitant TCR signaling, making it difficult to determine if STAT6 is working independently or in concert with molecules downstream of the TCR. A role for TCR signaling in regulating GATA-3 induction and subsequent Th2 differentiation was first implicated by altering TCR signaling through the use of altered peptide ligands or changing the Ag dose (17, 18). Additionally, T cells from p50- or Bcl-3-deficient mice have a defect in GATA-3 induction and Th2 differentiation in the presence of exogenous IL-4, suggesting a role for NF-κB signaling in conjunction with STAT6 for GATA-3 upregulation (19, 34). However, the NF-κB pathway is activated downstream of many different receptors, and genetic disruption of this pathway results in developmental defects both within and outside the T cell compartment. Furthermore, inhibition of TCR signaling using knockout animals or by altering the peptide dose or affinity may simply reduce the early TCR-dependent production of IL-4 rather than directly affect GATA-3 induction. It was recently shown that TCR activation can induce GATA-3 mRNA in the absence of IL-4 through the transcription factor T cell-specific factor-1 (22). Although our data in both naive (data not shown) and previously activated cells, as well as previous reports, have shown a dependence on IL-4 and STAT6, this recent finding suggests that under some conditions, signaling through the TCR may be sufficient to induce GATA-3 mRNA upregulation. Thus, TCR signaling can impact GATA-3 mRNA upregulation in multiple ways: direct induction independent of STAT6, in conjunction with IL-4R signaling, or indirectly through induction of IL-4. We have designed our experiments to restrict the effects of TCR signaling to modulation of Th2 differentiation, and under these conditions, we find that TCR signaling does not have any impact on GATA-3 mRNA upregulation. Despite the many ways in which TCR signaling can potentially impact GATA-3 mRNA, these results highlight that none of these pathways are required, as IL-4 is capable of driving GATA-3 mRNA independently of Ag stimulation. Although we cannot rule out any long-lasting effect of the initial stimulation, we show that in the absence of acute TCR stimulation, IL-4 stimulation is sufficient to drive GATA-3 mRNA induction and that addition of TCR signaling does not increase the level of IL-4-driven GATA-3 mRNA.

Priming of naive CD4+ T cells both in vivo and in vitro can lead to proliferating cells that produce IL-2, but neither IL-4 nor IFN-γ (35, 36). These cells maintain a flexible differentiation state and can be pushed into different subsets depending on the nature of the secondary challenge. This flexibility could be compromised if exposure to polarizing cytokines in the absence of cognate Ag resulted in GATA-3 protein upregulation. Our data establish a clear requirement for concomitant TCR and IL-4R signaling to direct Th2 responses for primed, undifferentiated T cells, with IL-4R signaling important for upregulation of GATA-3 mRNA and TCR signaling for enhancement of GATA-3 translation. This establishes the translational control of GATA-3 as a key point of signal integration associated with Th2 differentiation, providing a layer of protection against aberrant GATA-3 production, which could lead to Th2-biased immune responses and atopic diseases, such as asthma.

In addition to the signaling pathways that modulate transcriptional activation of GATA-3, it has been reported that ERK signaling can modulate GATA-3 protein stability (25). Targeted deletion of growth factor independent-1, for which induction is reduced by inhibition of ERK, results in decreased stability of GATA-3 protein and subsequent Th2 differentiation (37). However, we find that Ag stimulation has no effect on GATA-3 protein stability. Furthermore, inhibition of ERK activation did not alter the level of TCR-induced GATA-3 protein (data not shown). Although it remains possible that under some stimulation conditions TCR signaling may contribute to GATA-3 stability through ERK activation, this mechanism is not required for TCR signaling to enhance GATA-3 protein levels. The more significant contribution of TCR...
signaling toward GATA-3 protein levels is through the enhancement of the GATA-3 translation rate.

Translational control of gene expression can be mediated through effects on the global translational machinery or through gene-specific effects. TCR signaling has been shown to impact global translational efficiency following initial T cell activation through effects on the expression level and functional activity of translation initiation factors (24, 38, 39). Specifically, the dephosphorylation of eIF2α following acute TCR stimulation has been shown to enhance translational activity (24). The ability of TCR signaling to promote dephosphorylation of eIF2α is likely a time-restricted event that is dependent on the strength of the signaling. Accordingly, we find that there is no change in the phosphorylation state of eIF2α in the presence or absence of Ag stimulation at the time we detect an increase in the GATA-3 translation rate. Rather, we find that Ag stimulation specifically induces translation of GATA-3 without affecting phosphorylation of eIF2α, global translational efficiency, or the translation of control proteins.

In addition to the effects on eIF2α, TCR signaling can also impact both global and gene-specific translation through PI3K and mTOR. The best-characterized mechanism of mTOR-dependent control of translation is through phosphorylation of eIF4E-BP, an inhibitor of ribosome recruitment to the 5′ end of an mRNA molecule (40). This phosphorylation decreases affinity of eIF4E-BP for eIF4E, allowing eIF4G to bind eIF4E and recruit the ribosome to initiate translation. This process appears to be more complex than initially suggested, however, as overexpression of eIF4E selectively enhances translation of mRNA molecules that contain secondary structures within their 5′ UTR (41). GATA-3 transcripts contain unusually long 5′ UTRs, which contain many potential structural elements. The PI3K pathway effect on eIF4E-BP phosphorylation may have a larger effect on these structured 5′ UTR-containing mRNA molecules, including GATA-3. An additional step necessary only for translation of mRNAs with structured 5′ UTR is the eIF4A helicase-dependent unwinding of mRNA (42). The activity of eIF4A can be regulated by the PI3K–mTOR pathway through the phosphorylation-dependent binding of eIF4B to eIF4A (43). Reduction of this phosphorylation through PI3K inhibition could lead to decreased translation of GATA-3 and other mRNAs with structured 5′ UTR. Global translation is also enhanced by PI3K/mTOR signaling through enhanced generation of ribosome components. This enhanced translation is dependent on mTOR and the presence of a 5′ terminal oligopyrimidine tract, a polypyrimidine stretch adjacent to the 5′ end of mRNA molecule (44). Interestingly, GATA-3 mRNA molecules contain multiple polypyrimidine stretches within their 5′ UTR. Although these elements are not in the correct position to be classified as a terminal oligopyrimidine, these regions could confer mTOR-dependent translational control.

Further experiments are needed to determine if these potential mechanisms are responsible for the TCR-mediated enhancement of GATA-3 translation. Understanding how the different transcriptional and posttranscriptional regulatory elements cooperate to mediate the fine control of GATA-3 expression will be key to understanding how this pathway may be misregulated in atopic diseases or can be targeted for immune manipulation.

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Disclosures

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References


