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c-Myb and GATA-3 Cooperatively Regulate IL-13 Expression via Conserved GATA-3 Response Element and Recruit Mixed Lineage Leukemia (MLL) for Histone Modification of the IL-13 Locus

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The c-Myb and GATA-3 transcription factors play important roles in T cell development. We recently reported that c-Myb, GATA-3, and Menin form a core transcription complex that regulates GATA-3 expression and ultimately Th2 cell development in human peripheral blood T cells. However, c-Myb roles for Th2 cytokine expression were not demonstrated. In this article, we report that c-Myb and GATA-3 cooperatively play an essential role in IL-13 expression through direct binding to a conserved GATA-3 response element (CGRE), an enhancer for IL-13 expression. c-Myb and GATA-3 were shown to activate the CGRE–IL-13 promoter by ~160-fold, and mutation of the canonical Myb binding site completely abrogated CGRE enhancer activity. In contrast, mutation of the GATA binding site partially decreased CGRE enhancer activity. GATA-3 did not bind to CGRE when c-Myb expression was silenced. c-Myb, GATA-3, Menin, and mixed lineage leukemia (MLL) bound to CGRE in human primary CD4+ effector/memory cells. Moreover, c-myb silencing significantly decreased both methylation of histone H3K4 and acetylation of histone H3K9 at the IL-13 locus in CD4+ effector/memory cells. Therefore, in addition to the stronger enhancer effect for the transcription of IL-13, the c-Myb/GATA-3 complex recruits MLL to the CGRE for histone modification of the IL-13 locus during the differentiation of memory Th2 cells. The Journal of Immunology, 2011, 187: 5974–5982.
GATA-3, or may have been a consequence of affecting the c-Myb/ GATA-3 complex. In this article, we address c-Myb roles in regulating Th2 cytokine gene expression and demonstrate that c-Myb critically regulates IL-13 expression via direct binding to the conserved GATA-3 response element (CGRE).

Materials and Methods
Lymphocyte preparation and cell culture
Normal human peripheral blood CD4+ T lymphocytes were obtained from consenting donors via the Human Immunology Core Facility at the University of Pennsylvania School of Medicine. Naïve and effector/memory cells were sorted by CD45RO or CD45RA MicroBeads (Miltenyi Biotec) or PE-anti-human CD45RA Ab (BD Biosciences). Freshly isolated cells were cultured in RPMI 1640 medium supplemented with 10% FCS. Naïve CD4+ T cells were cultured with either IL-12 (10 ng/ml), IL-2 (20 ng/ml), and anti–IL-4 Ab (10 μg/ml) for Th1 cell development or with IL-4 (25–40 ng/ml), IL-2 (20 ng/ml), and anti–IL-12 Ab (5–10 μg/ml) to promote Th2 cell formation in 1–10 d. CD3/CD28 Abs (Dynabeads CD3/CD28 T cell expander; Invitrogen) were used for primary T cell stimulation. Jurkat and 293T cells were cultured in RPMI 1640 medium or DMEM, respectively, and supplemented with 10% FCS. PMA (200 ng/ml) and ionomycin (300 ng/ml) were used for the stimulation of Jurkat cells.

RNA interference for c-Myb silencing
To generate lentiviruses constructs for c-Myb RNA interference, c-Myb-1 (5'-tgttattgccaagcactta-3'), c-Myb-3 (5'-ctgcctggacgaactgata-3'), or control c-Myb-1 scramble (5'-ctttatacgtagtcataag-3') small interference RNA (siRNA) sequence was inserted into the H1UG lentivirus vector (a gift from Dr. E. J. Brown, University of Pennsylvania, Philadelphia, PA). The mixed lentiviruses with c-Myb-1 and c-Myb-3 short hairpin RNA (shRNA) were transduced into human primary CD4+ T cells as described previously (19). Forty-eight hours posttransduction, GFP+ cells were sorted using a FACSAria cell sorter (BD Biosciences). Jurkat cells were infected with lentivirus expressing GFP and c-Myb-3 shRNA. GFP+ cells were sorted and used to establish stable cell lines expressing c-Myb or control siRNA. c-Myb-1 and -3 siRNA were also used for transient c-Myb silencing in human T cells using Amnax nucleofection system (Lonza).

Dual-Luciferase reporter assay
Seven reporter constructs were prepared by inserting the human IL-13 and IL-4 promoter with or without the regulatory element(s), which includes CGRE, conserved noncoding sequence 1 (CNS-1), intronic enhancer (IE), and hypersensitive site V (HSV) into a pGL3-basic vector (Promega). The constructs contain the following regions: IL13P, −254 to +60 bp; IL4P, −766 to +60 bp; CGRE, a 65-bp fragment from −1,740 to −1,676 bp upstream of the IL-13 transcription start site; CNS-1, a 371-bp fragment from −9,652 to −9,282 bp upstream of the IL-4 transcription start site; IE, a 127-bp fragment from +1,485 to +1,611 bp downstream of the IL-4 transcription start site; and HSV, a 364-bp fragment from +15,089 to +15,452 bp downstream of the IL-4 transcription start site. CGRE-IL-13P has the region from −1,740 to +60. The pcDNA3 vectors containing full-length c-Myb (1 μg) and/or full-length GATA-3 (1 μg) were cotransfected with phRL (0.01 μg) and the appropriate promoter and the regulatory element up/downstream of luciferase in pGL3 (0.5 μg) into human primary T cells using Human T cell Nucleofector kit (Lonza) or into Jurkat cells using Cell line Nucleofector kit (Lonza) or into 293T cells using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured from the pGL3 and phRL reporter constructs, respectively, according to instructions in the Dual-Luciferase reporter assay kit (Promega) using a luminometer (Orius Microplate Luminometer, Berthold Detection Systems).

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assays were completed using ChIP Assay kit (Millipore) according to the instruction manual. CD4+ naïve or effector/memory T cells were cultured under Th2 conditions for 3–5 d, except where noted (see below). The cells were restimulated with IL-4 and IL-2 at 1–6 h before cross-linking with formaldehyde. Immunoprecipita-

FIGURE 1. c-Myb expression levels are concordant with the production of Th2 cytokine. A. Naïve (CD45RA+), CD4+ T cells were cultured with Th2 cell promoting stimulatory conditions, and the cells and supernatants were collected before stimulation (0 d) and 1, 2, 3, and 5 d after stimulation. Left graph shows the amount of c-Myb mRNA, as measured by QRT-PCR. Middle graph shows the secreted IL-13 and IL-5 in each supernatant. Right graph shows the IL-4 mRNA expression in the cells. B. Human primary T cells were stimulated with IL-2 and CD3/CD28 Abs for 3 d, and then, the siRNA against c-Myb or control siRNA were transfected into the cells. The transfected cells were stimulated with IL-4, IL-2, and CD3/CD28 Abs or with IL-2 and CD3/CD28 Abs for 3 d to measure IL-13 and IL-5 or IL-4, respectively, and then, the concentration of IL-13, IL-5, and IL-4 in supernatants was measured by ELISA. Gene expression of c-Myb was measured by QRT-PCR in the same cells. T cells = no transfection with siRNA.*p < 0.05 compared control siRNA.
tion was performed with anti-c-Myb (a mixture of clone 1-1, Millipore; and H-141, Santa Cruz Biotechnology), anti-GATA-3 (a mixture of H-48, Santa Cruz Biotechnology; and HG3-31, Santa Cruz Biotechnology), anti-mixed lineage leukemia (MLL)1 (Bethyl), or anti-Menin (Bethyl). The precipitated DNA fraction was then amplified by quantitative real-time PCR (QRT-PCR) using standard protocols with Sybr green and specific primers for each regulatory element (CGRE, 5'-CCCCATCTCCCGTATAAG-3' (forward) and 5'-CCCGCTACCAAGCGAGCG-3' (reverse), 5'-CCTCGCACGCTCACG-3' (forward) and 5'-GGTGCGCTATGTAAACGGGA-3' (reverse); CNS-1, 5'-ACTATGCCACGACCAGACCGCT-3' (forward) and 5'-TTCTTGCAAACTCTGCTCCCT-3' (reverse); IE, 5'-CTCTTGCTCTGCTGCTG-3' (forward) and 5'-TTCTT GGCGCTTCTCCTA-3' (reverse); and HSV, 5'-TGCTGGTTATGGTAACGGGA-3' (forward) and 5'-CGATATTCCTCCACCTGCT-3' (reverse)). In ChIP assays for histone modification, CD4+ naive and effector/memory T cells were stimulated under Th2 promoting conditions for 7 and 4 d, respectively. Abs against di- and trimethylated H3K4 (ab6000), acetyl K9 (ab4441), and dimethyl K9 (ab1220) (Abcam, Cambridge, MA) were used for the ChIP assays probing the IL-13 and IL-4 locus in primary CD4+ T cells. The 22 primer pairs are listed in Supplemental Table I.

**EMSA**

These experiments used 5 μl in vitro-translated c-Myb or GATA-3 proteins bound to 50 ng dsDNA oligonucleotide. The c-Myb and GATA-3 proteins were in vitro translated from their respective pcDNA expression vector using TNT Quick Coupled Transcription/Translation Systems (Promega). The binding reactions proceeded for 20 min in a buffer containing 50 μg/ml of poly(dI-dC), 10 mM HEPES (pH 7.9), 1 mM EDTA, 5 mM MgCl₂, 30 mM KCl, 10% glycerol, and protease inhibitors. Samples were separated by electrophoresis on a native 5% polyacrylamide gel (0.5× Tris-boric acid-EDTA buffer and 2% glycerol) for 2 h at 200 V. Gels were exposed to X-ray film. For competition assays, a 10-fold excess of a cold competitor was included. The sequences of the two strands of the oligonucleotides used were as follows (consensus c-Myb and GATA3 binding site sequences in underlined and italic, respectively): CGRE, 5'-CTTA-TCGGGCCCATCTCCGTTACATAAGGCCACCCCCCTATCTCCGCG-3'. The sequences of the two strands of the oligonucleotides with the mutated consensus c-Myb binding sites were as follows (mutated nucleotides in bold): CGRE mut Myb, 5'-CTTATCGGGCCCACTTCCGTTACATAAGGCCACCCCCCTATCTCCGCGGGCCATCGCG-3'.

**FIGURE 2.** c-Myb AND GATA-3 COOPERATIVELY REGULATE IL-13 and IL-4 expression through regulatory elements in the Th2 cytokine gene in cooperation with GATA-3. A, Schematic representation of the IL-13 and IL-4 gene locus. Exons (black rectangles) and regulatory elements (blank rectangles) of IL-13 and IL-4 are depicted in the locus. The schemas below each arrow show the Myb (black rectangles) and GATA binding sites (blank rectangles) in the regulatory elements. B, ChIP assays were performed with anti-c-Myb and anti-GATA-3 Abs using primary human CD4+ T cells after stimulation with IL-4, IL-2, and Abs against CD3/CD28. Fold enrichments were measured by QRT-PCR with specific primers for CGRE, CNS-1, IE, and HSV regions. All results shown (all gray bars) are statistically significant (p < 0.05) compared with control IgG. C, Upper schema shows reporter constructs in the pGL3 vector for promoter analysis. The order of promoter and regulatory elements in the constructs is the same as the arrangement in the human gene as shown in the upper schema of Fig. IA. Graphs show the results of reporter assays for IL-13 (right) and IL-4 (left). Reporter assays were carried out in 293T cells 24 h after transfection with each construct in the presence of c-myb and/or GATA-3 expression constructs or control empty vectors. *p < 0.05 compared with the activity of the promoter alone.
Statistical analyses

Statistical comparisons of the data were completed using the two-tailed Student t test. The level of significance was set at $p < 0.05$ in all cases.

Results

c-Myb expression levels are concordant with the production of Th2 cytokine

To initially test whether c-Myb expression levels affect the production of Th2 cytokines in human primary T cells, we first measured the amounts of c-myb and IL-4 mRNA and concentration of IL-13 and IL-5 using QRT-PCR and ELISA in primary human CD4+CD45RA+ (naive) T cells that were stimulated under Th2 cell-promoting conditions. The expression of c-myb and IL-4 and the secretion of IL-13 were the greatest after 5 d of stimulation, indicating that the expression levels were concordant (Fig. 1A). We next silenced c-myb expression by siRNA in human primary T cells and measured the secretion of Th2 cytokines using ELISA after stimulation. Silencing c-myb in the cells significantly decreased the concentration of IL-5, IL-13, and IL-4 in the cell culture media (Fig. 1B), suggesting that the expression level of c-Myb directly affects the production of Th2 cytokines.

c-Myb regulates IL-13 and IL-4 expression through regulatory elements in the Th2 cytokine gene in cooperation with GATA-3

Because we previously reported that c-myb silencing decreases GATA-3 expression in Th2 cells (19), we next examined whether the observed decrease in Th2 cytokine expression is a direct effect by c-Myb or an indirect effect by the decrease of GATA-3. The Th2 cytokine locus has numerous DNase I hypersensitive sites (DHSs), which are known to act as regulatory elements for Th2 cytokine gene expression. Among the DHSs known as regulatory elements activated by GATA-3 directly in the Th2 cytokine gene locus are the conserved GATA-3 response element (CGRE) (20, 21), the CNS-1 (22, 23), the IE (22) and the HSV (Fig. 2A) (22, 24). Sequence comparison revealed that all four regulatory elements are highly conserved between human and mouse, and further sequence analysis revealed that all four elements contain strong GATA binding sites and surprisingly contain c-Myb binding sites in close proximity to the identified GATA binding sites within all four regulatory elements (Fig. 2A). Because of this rare arrangement of c-Myb and GATA binding sites in the four regulatory elements, we hypothesized that c-Myb might play an important role with GATA-3 in the function of the regulatory elements.

To explore the function of c-Myb and the relationship between c-Myb and GATA-3 in these four elements, ChIP assays were performed to test whether c-Myb and GATA-3 bound to the four elements in primary human CD4+ T cells with stimulation under Th2 promoting conditions for 3–5 d. ChIP assays revealed that in the stimulated CD4+ T cells, both c-Myb and GATA-3 bound to all four of the elements (Fig. 2B). Strikingly, c-Myb binding to the CGRE locus was increased $\sim 18$-fold compared with control IgG.

To develop an understanding of the role of c-Myb and GATA-3 in Th2 cytokine gene expression, we carried out Dual-Luciferase reporter assays using IL-4 and IL-13 promoter constructs that include the well-conserved promoter region, with or without the four regulatory elements in 293T cells that express neither c-Myb nor GATA-3. The canonical Myb binding site in the CGRE is essential for the strong enhancer activity of the CGRE for IL-13 expression by c-Myb and GATA-3. A, Left schema shows CGRE and IL-13 promoter locus and Myb and GATA binding sites in CGRE. Dual-luciferase reporter assays were performed in 293T cells 24 h after cotransfection with IL-13 promoter (IL13P), wild-type CGRE-IL13 promoter (CGRE-IL13P) and CGRE-IL13 promoter containing a mutated canonical Myb binding site within the CGRE (mut Myb). B, Upper schema shows GATA binding sites and the generated mutation sites in CGRE. Reporter assays carried out in 293T cells 24 h after cotransfection with CGRE-IL13P, CGRE-IL13 promoter containing a mutated canonical Myb binding site within the CGRE (mut Myb), CGRE-IL13P containing a mutated (shown with X in upper schema) high-affinity GATA-3 binding site (mut GATA#3), or CGRE-IL13P containing four mutated GATA binding sites (mut GATA#1–#4). C, Dual-luciferase reporter assays were performed with Jurkat cells with CGRE-IL13P, or CGRE-IL13P containing a mutated Myb binding site (CGRE-IL13P mut Myb). Cells were stimulated 24 h posttransfection with PMA (200 ng/ml) and ionomycin (300 ng/ml) for 24 h. *$p < 0.05$, **$p < 0.01$. 

The Journal of Immunology 5977

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nor GATA-3 (Fig. 2C, upper schema). Using the IL-4 promoter construct either with or without the regulatory elements, the effects of c-Myb or GATA-3 expression alone were minimal (Fig. 2C, left graph). However, the combined expression of c-Myb and GATA-3 significantly increased luciferase activity for the CGRE-IL-4 promoter and CNS1-IL-4 promoter 5.6- and 3.2-fold, respectively (Fig. 2C, left graph). When we performed the luciferase reporter assays using IL-13 promoter constructs without/with regulatory elements, we, surprisingly, observed that combined c-Myb and GATA-3 expression increased CGRE-IL-13 promoter activity by \(~160\)-fold and increased IL-13 promoter–CNS-1 element activity by \(~22\)-fold (Fig. 2C, right graph). These results suggest that CGRE is an enhancer for IL-13 and that the enhancer is regulated by c-Myb and GATA-3.

The canonical Myb binding site in the CGRE is essential for the strong enhancer activity of the CGRE for IL-13 expression by c-Myb and GATA-3

Having determined that the CGRE markedly enhanced IL-13 promoter activity by c-Myb and GATA-3 (Fig. 2C, right graph), we next performed mutational analysis of the Myb and GATA binding sites within the CGRE locus to more precisely define regulation of IL-13 gene expression by c-Myb and GATA-3. The CGRE locus has one canonical Myb binding site, which was mutated (5'-TAACGG-3' to 5'-TGACGG-3') in the CGRE-IL13 promoter construct and was tested in dual-luciferase reporter assays in 293T cells. The IL-13 promoter construct containing the CGRE enhancer was activated \(~70\)-fold by GATA-3 alone and \(~190\)-fold by the addition of c-Myb (Fig. 3A), with similar results shown in Fig. 1C. Surprisingly, this strong enhancer effect on IL-13 expression by c-Myb and GATA-3 was completely lost when the Myb binding site in the CGRE was mutated (Fig. 3A). The levels of luciferase activity of the CGRE-IL-13 promoter containing a mutated Myb binding site was similar to the activity of the minimal IL-3 promoter (25) with expression of c-Myb and GATA-3 or GATA-3 alone. These results suggest that c-Myb and GATA-3 cooperatively regulate IL-13 expression through CGRE directly. The Myb binding site is essential for the enhancer activity in CGRE.

Next, GATA binding sites in CGRE were mutated and tested in the dual-luciferase reporter assay. Mutation of a consensus GATA binding site, 5'-AGATAG-3', with high affinity for GATA-3 (26), within the CGRE locus decreased IL-13 promoter activity by \(~60\%\) (Fig. 3B). Mutation of this site still resulted in higher enhancer activity compared with the IL-13 minimal promoter alone. It was possible that the three remaining GATA binding sites contributed to the enhancer activity. On the basis of sequence analysis, the three remaining GATA binding sites should have high affinity for GATA-1 (26). Dual-luciferase reporter assays in the absence and presence of c-Myb and/or GATA-3 in 293T cells showed that mutation of all four GATA binding sites in CGRE (mut GATA #1−#4) resulted in the same level of enhancer activity as mutation of the consensus GATA-3 binding site (mut GATA#3) (Fig. 3B).

The IL-13 promoter reporter constructs with or without a mutated c-Myb binding site within the CGRE locus were tested in Jurkat cells, which are a human T leukemic cell line that expresses IL-13 upon stimulation with PMA and ionomycin (27). Mutation of the c-Myb binding site resulted in loss of the CGRE enhancer effect in Jurkat cells (Fig. 3C). This confirms the observations in 293T cells.

c-Myb and GATA-3 bind to CGRE directly

CGRE is positioned at a locus \(~1.7\) Kb downstream from the IL-13 transcription start site. Three potential GATA-3 binding sites and four possible sites for Myb binding lie between human CGRE and the IL-13 promoter containing a mutated Myb binding site was similar to the activity of the minimal IL-3 promoter (25) with expression of c-Myb and GATA-3 or GATA-3 alone. These results suggest that c-Myb and GATA-3 cooperatively regulate IL-13 expression through CGRE directly. The Myb binding site is essential for the enhancer activity in CGRE.

FIGURE 4. c-Myb and GATA-3 bind to CGRE directly. A, ChIP assays were performed with anti-c-Myb and anti-GATA-3 Abs using primary human CD4+ naive and effector/memory T cells after stimulation with IL-4, IL-2, and anti-CD3/CD28 Abs for 3–4 d. The fold enrichments were measured by QRT-PCR with eight specific primers upstream of the IL-3 locus including CGRE as shown in the bottom schema. The transcription start site is indicated. B, EMSA for binding of c-Myb and GATA-3 to CGRE. Proteins were in vitro translated from their respective pcDNA expression vector using TNT Quick Coupled Transcription/Translation Systems (Promega) and subjected to EMSA with a radiolabeled CGRE oligonucleotide (CGRE) and CGRE oligonucleotide containing Myb’s mutant binding sequence (CGRE mut Myb). Oligonucleotide competition was carried out with 10-fold excess of unlabeled CGRE oligonucleotide (CGRE).
the transcription start site of IL-13. ChIP was used to detect c-Myb binding to the locus using eight primers approximately −360 to −2900 bp from the transcription start site of the IL-13 gene. ChIP assays were performed with c-Myb, GATA-3, and control Abs in human primary CD4+ naïve and effector/memory T cells with stimulation under Th2 promoting conditions. The peak fold enrichment by c-Myb and GATA-3 Abs for the locus was shown with the #4 primers that amplify the region including the Myb binding site in CGRE in both stimulated CD4+ naïve and effector/memory T cells (Fig. 4A). This result strongly demonstrated both c-Myb and GATA-3 bound to Myb and GATA binding sites in the CGRE locus in human primary CD4+ T cells.

To confirm binding to CGRE and to test for direct binding an EMSA was used. A specific c-Myb and CGRE complex was detected using the radiolabeled CGRE oligonucleotide probe (CGRE) and the complex formation was inhibited by an excess of the oligonucleotide containing the consensus binding site for c-Myb (Fig. 4B). The specific complex was not detected using the CGRE oligonucleotide containing Myb’s mutant binding sequence (CGRE mut Myb) as probe. A specific GATA-3 and CGRE complex was detected and the complex formation was inhibited by an excess of the oligonucleotide containing the consensus binding site for GATA-3 (Fig. 4B). These results indicate that c-Myb and GATA-3 directly bind the CGRE locus.

**GATA-3 associates with c-Myb to bind CGRE**

We next determined the importance of c-Myb associating with GATA-3 within CGRE and affecting IL-13 expression. To identify a GATA-3/c-Myb complex in Jurkat cells, we used Flag-tagged GATA-3 expression vectors. Immunoprecipitation assays with the cells showed that Flag-GATA-3 proteins bound to c-Myb (Fig. 5A). We next silenced c-myb expression in Jurkat cells using a lentivirus c-myb shRNA expressing system as we previously reported (19). Forty-eight hours after transfection of the Flag-GATA-3 vector into Jurkat cells, ChIP assays performed with anti-Flag Abs revealed a dramatic enrichment the CGRE upstream of IL-13 compared with when the ChIP assays were completed with mouse IgG. When Jurkat cells, with silenced c-myb expression, were transfected with Flag-GATA-3, we observed a marked decrease in the enrichment of CGRE by ChIP assays compared with when Jurkat cells express c-Myb (Fig. 5B), suggesting that GATA-3 requires the association with c-Myb to bind to the CGRE. To determine the importance of the c-Myb/GATA-3 complex on GATA-3 and IL-13 expression, we treated Jurkat cells with control or c-myb siRNA and used QRT-PCR to determine gene expression. The c-myb siRNA suppressed c-myb gene expression 70% and decreased GATA-3 and IL-13 gene expression by ∼75 and 70%, respectively, in Jurkat cells after stimulation with PMA and ionomycin compared with control siRNA treated cells (Fig. 5C).

We also examined whether c-Myb binds to CGRE differentially in Th0, Th1, or Th2 promoting conditions using ChIP assays. CD45RA+ CD4 (naive) T cells were cultured under Th1 or Th2 promoting conditions for 4 d, and then, the cells were restimulated with IL-12 and IL-2 or IL-4 and IL-2, respectively, for 1 h before cross-linking with 1% formaldehyde. The ChIP assays showed that c-Myb bound to CGRE under Th2 promoting conditions but did not bind to CGRE under Th1 promoting conditions or in Th0 cells (Fig. 5D).

**FIGURE 5.** GATA-3 associates with c-Myb to bind CGRE. A. Immunoprecipitation (IP) was performed with Jurkat cells 48 h posttransfection of pcDNA GATA-3-Flag tag expression vector or control pcDNA Flag-tag vector using anti-Flag tag Abs. The Western blot was performed with anti-c-Myb Ab. H chain = the IgH. B. The upper panel shows c-Myb and β-actin protein expression as determined by Western blot in the lentivirus c-myb shRNA expressing or control Jurkat cells that were used for ChIP assays. The graph in the lower panel shows the relative enrichment of CGRE gene fragments by ChIP assays that were carried out with anti-Flag tag Ab (Flag ab) or normal mouse IgG (mlgG). The precipitated DNA fractions were analyzed by QRT-PCR with primers specific to the CGRE region. C. Jurkat cells transfected with c-myb siRNA or control scrambled siRNA (19) were cultured with PMA and ionomycin for 24 h, and QRT-PCR was then performed with primers specific to measure c-myb, GATA-3, and IL-13 gene expression. D. Purified nucleoprotein complex was obtained from CD4+ CD45RA+ cells cultured under Th1 or Th2 promoting conditions and then used for ChIP assays using either anti-c-Myb (c-Myb) or mouse IgG. The precipitated DNA fractions were analyzed by QRT-PCR with primers specific to the CGRE. *p < 0.05.
MLL and Menin bind to CGRE in CD4+ effector/memory T cells while MLL does not bind to CGRE in CD4+ naive T cells under Th2 cell promoting conditions

Our recent work showed that c-Myb bound to GATA-3 via an adaptor protein, Menin, and cooperatively regulated GATA-3 expression (19). In addition, we demonstrated that c-Myb bound MLL through Menin in human leukemic cells and primary T cells (28). c-Myb silencing decreases methylation of histone H3K4 and acetylation of histone H3K9 at the GATA-3 locus in primary human CD4+ effector/memory T cells stimulated under Th2 promoting conditions (19). Therefore, we tested our hypothesis that c-Myb, GATA-3, and Menin form a transcriptional complex, which recruits MLL for histone modification of the Th2 cytokine gene locus. ChIP was used to test whether MLL and Menin bind to the CGRE locus in human primary CD4+ T cells. Under Th2 promoting conditions, Menin strongly bound to CGRE in CD4+ naive T cells stimulated for 3–4 d, whereas MLL binding was not detected (Fig. 6, upper graph). However, both MLL and Menin bound to CGRE in CD4+ effector/memory T cells under the same stimulation conditions (Fig. 6, lower graph).

c-Myb silencing decreases methylation of histone H3K4 and acetylation of histone H3K9 at the IL-13 locus in stimulated primary human CD4+ effector/memory T cells

Histone modification of the IL-13 locus was examined and extended to the IL-4 locus due to the potential for coordinate regulation. Abs directed to Histone H3 di- and trimethylated K4, Histone H3 acetyl K9, and Histone H3 dimethylated K9 were used in ChIP assays utilizing at least 22 primer sets from within the ∼35 kb Th2 cytokine gene locus including the IL-13 and IL-4 loci.

Because MLL bound CGRE only in CD4+ effector/memory T cells, we tested histone modification in CD4+ effector/memory T cells with and without silenced c-Myb. Compared with control siRNA-treated cells, silencing c-Myb led to considerably decreased levels of H3K4 di- and trimethylation, as well as decreased H3K9 acetylation in the loci in the cells (Fig. 7A). Although the levels of H3K4 di- and trimethylation and H3K9 acetylation were very low in CD4+ naive T cells stimulated under Th2 promoting conditions compared with CD4+ effector/memory T cells, the levels of histone methylation and acetylation were decreased overall following c-Myb silencing in the naive T cells (Fig. 7B). Interestingly, silencing c-Myb did not affect the level of histone H3K9 dimethylation in both naive and CD4+ effector/memory T cells. Moreover, the methylation status of the IL-13 and IL-4 loci in naive and effector/memory T cells are similar, suggesting that histone H3K9 dimethylation is not important for regulation of Th2 cytokine gene locus (Fig. 7A, 7B, bottom graphs). These results demonstrate that c-Myb significantly contributes to chromatin remodeling at the Th2 cytokine gene locus in primary human memory Th2 cells. The data suggest that c-Myb forms a complex with GATA-3 and/or Menin and recruits MLL in the transition from CD4+ effector to memory T cells and/or maintenance of memory Th2 cells.

Discussion

Despite the high level of c-Myb expression in primary peripheral T cells following activation, little is known regarding the function of c-Myb in this population of cells. It is reported in this article that c-Myb has a direct role in regulating Th2 cytokine expression in primary CD4+ T cells. The importance of the c-Myb binding site found within the CGRE for enhanced transcription of IL-13 was shown (Fig. 3). In primary human naive and effector/memory CD4+ T cells under Th2 promoting conditions, both c-Myb and GATA-3 bound to CGRE (Fig. 4). GATA-3 required c-Myb to bind CGRE (Fig. 5). Moreover, MLL bound to CGRE in primary CD4+ effector/memory T cells (Fig. 6), and silencing c-Myb considerably decreased levels of H3K4 di- and trimethylation as well as H3K9 acetylation in the IL-13 and IL-4 loci in the cells (Fig. 7).

DNase I hypersensitivity is postulated to reflect a localized “open” chromatin configuration where the DNA is accessible to binding by many factors (29). It has been shown that regulatory elements like tissue-specific enhancers and locus control regions are located in DHSs (30), and many DHSs develop in the Th2 cytokine gene during Th2 lineage commitment. Among DHSs in the Th2 cytokine gene, CGRE is located 1.7 kb upstream of IL-13 and was described as a highly conserved Th2-specific hypersensitive site (20, 21). We clearly demonstrated that c-Myb and GATA-3 cooperatively increased the activity of CGRE for the IL-13 promoter, whereas no obvious regulation to IE and HSV were observed, and a small effect for CNS-1 was shown (Fig. 2C). Important studies suggested that CNS-1, a DHS located between the IL-13 and IL-4 loci, is a coordinate regulator of Th2 cytokine genes. Deletion of human CNS-1 resulted in a reduction of cells producing IL-4, IL-5, and IL-13 (31). Subsequent studies in transgenic reporter mice demonstrated that CNS-1 has strong enhancer activity for the IL-4 promoter (22), whereas other studies revealed CNS-1 to be responsive to GATA-3 enhancer activity as well as chromatin remodeling, which is consistent with the identification of potential GATA-3 sites by gel shift analyses (22, 23). The enhancer activity of CNS-1 with c-Myb and GATA-3 was strong for IL-13, but it was limited for the IL-4 promoter, whereas...
c-Myb and GATA-3 bound CNS-1. Many factors have roles for the IL-4 locus (12, 16, 17), and GATA-3 and c-Myb may require additional molecules or binding sites to activate CNS-1 for IL-4 expression in 293T cells. This concern also should apply to IE and HSV. With transgenic reporter assays, IE was determined to enhance IL-4 promoter activity, and when IE was combined with CNS-1, it conferred GATA-3–dependent enhancement of IL-4 promoter activity (22). In transient transfection and transgenic reporter assays, the combination of HSV and HSVa had strong enhancer activity (22, 24) when NFAT1 and GATA-3 bound to HSVa. HSV/CNS-2+HSVa knockout mice showed reduced expression of IL-4, IL-5, and IL-13 in Th2 cells (32). Because both c-Myb and GATA-3 bound to IE and HSV in stimulated human CD4+ cells in ChIP assays (Fig. 2B), c-Myb would have a role for regulation of those elements. However, significant activation of IE and HSV by c-Myb and GATA-3 was not observed (Fig. 2C).

Because the combination of IE and HSV regulatory elements showed enhancer activity for IL-4 promoter (22, 24), we hypothesize that a combination of regulatory elements might be required for the activation of IL-4 promoter by c-Myb.

c-Myb augmented the enhancer activity of GATA-3 for the IL-13 promoter. The mechanism by which c-Myb and GATA-3 regulate IL-13 expression was determined. Mutation of the canonical Myb binding site within the CGRE abrogated the strong enhancer activity of the CGRE; however, the enhancer activity was only partially abrogated when the GATA binding site was mutated (Fig. 3). GATA-3 activated the CGRE without c-Myb, whereas this activity was suppressed by the mutation of the Myb binding site in the CGRE. It is possible that B-Myb might contribute to this complex regulation because B-Myb and c-Myb bind to the same Myb binding site (33) and could partially compensate for their respective functions when c-Myb or B-Myb expression is individually silenced by RNA interference (34). B-Myb could bind to the CGRE in 293T cells and weakly assist GATA-3’s function. However, exogenous expression of B-Myb did not change CGRE-IL-13 promoter activity in our reporter assays (data not shown). Endogenously high B-Myb expression would suppress the affect of the promoter activity by the exogenous expression of B-Myb. Neither c-Myb nor B-Myb can bind to the mutated Myb binding site, and the mutation completely removed the activity of CGRE. However, the mutated GATA binding sites partially decreased CGRE activity. We previously showed that c-Myb bound to GATA-3 through Menin or when c-Myb was in an activated truncated form (19). GATA-3 formed a complex with c-Myb to bind to CGRE and the complex might partially bind to the mutated GATA binding sites with the assistance of c-Myb or another protein. GATA binding sites in the IL-13 promoter might compensate for the mutated binding site in CGRE for activity.

MLL and Menin bound to the CGRE locus in stimulated primary human CD4+ effector/memory T cells (Fig. 6). MLL is known to have intrinsic histone methyl transferase activity for histone H3-K4 (35, 36), and the methylation of histone H3-K4 is important for the maintenance of acetylation of histone H3-K9. Because we have already shown that c-Myb can bind MLL through Menin (28), it is possible that histone modification of the Th2 cytokine locus occurs through c-Myb and MLL. A notable decrease in the methylation of histone H3-K4 at the CGRE gene locus was observed after c-Myb silencing in human primary CD4+ effector/memory T cells.
The function role of GATA-3 in Th1 and Th2 differentiation. 


Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Table 1

The primers for ChIP assay at Figure 5AB

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<td>5’-CATTGGCTGCTCTCTTTATGAGTGA-3’</td>
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