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Aberrant expression of IFN-γ in Th2 cells from Th2 LCR-deficient mice

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ABSTRACT

The Th2 locus control region (LCR) has been shown to be a crucial *cis*-acting element for Th2 cytokine expression and Th2 cell differentiation. To study the role of Th2 LCR in *ifng* locus regulation, we examined the expression of IFN-γ in Th2 cells from Th2 LCR-deficient mice. We found IFN-γ to be aberrantly up-regulated. In addition, histone 3(H3)-acetylation and histone 3 lysine 4 (H3-K4)-methylation greatly increased at the *ifng* locus of the Th2 cells. GATA-3 and STAT6 bound to the *ifng* promoter in Th2 cells from the wild type but not from the Th2 LCR-deficient mice, and they directly repressed *ifng* expression in transient reporter assay. Moreover, ectopic expression of GATA-3 and STAT6-VT repressed the aberrant expression of the *ifng* gene and restored repressive chromatin state at the *ifng* locus in Th2 cells from Th2 LCR-deficient mice. These results suggest that expression of the *ifng* gene and chromatin remodeling of the *ifng* locus are under the control of a Th2 LCR-mediated Th2 differentiation program.

1. Introduction

CD4 T cells play important roles in coordination of a variety of immune responses against various pathogens. Several subsets of CD4 T cells, including Th1, Th2, Th9 and Th17 cells, are differentiated from common naive CD4 T cells by T cell receptor (TCR) stimulation and by the influence of appropriate cytokines [1–6]. Th1 cells produce IFN-γ and mediate cellular immunity against intracellular bacteria; Th2 cells produce IL-4, IL-5, and IL-13, and mediate anti-parasite response and also activate B cells, which aid in humoral immune response against extracellular pathogens. Th17 cells produce IL-17A, IL-17F, and IL-22 and mediate immunity against extracellular bacteria and fungi, facilitating inflammation by recruiting neutrophils to inflammation sites. The effector CD4 T cells also cause immunopathologic diseases; Th2 cells cause allergic diseases, and Th1 and Th17 cells play a critical role in some autoimmune and inflammatory diseases.

Different signal transduction pathways and transcription factors play a role in their differentiation to each subset of effector CD4 T cells [1–6]. Th1 cells are induced by IL-12 and IFN-γ, which activate STAT4 and STAT1, respectively, and induce T-bet. Th2 cells are induced by IL-4, which activates STAT6 and induces GATA-3. GATA-3 has been shown to be an essential transcription factor for Th2 differentiation. It is selectively expressed in Th2 cells and induces Th2 cell differentiation [7]. When GATA-3 is ectopically expressed in Th1 cells, it induces *il4* expression and chromatin remodeling at the Th2 cytokine locus, which contains the *il4*, *il5*, and *il13* genes. [8–10]. Deletion of the *gata3* gene in the mouse genome causes complete inhibition of Th2 cell differentiation [11–13]. GATA-3 has been shown to not only induce the Th2 cell program but also block differentiation into other T helper cell types including Th1 and Th17 [14,15]. Although, the function of GATA-3 in Th2 differentiation is well-known, its mechanisms of GATA-3 in chromatin remodeling and in cytokine gene expression are poorly understood.

The expression of subset-specific cytokines is crucial for the differentiation and function of T helper cells. Regulation of the expression of these cytokines has been intensively studied as a model system for gene regulation during cell differentiation [16,17]. In particular, the Th2 cytokine locus has been well studied in the aspects of the gene expression and epigenetic mechanisms underlying cell differentiation [16,17]. Likewise, the *ifng* locus also has been well studied to understand the regulation of gene expression and epigenetic modifications in Th1 cells [6,14,16,18]. Many of the *cis*-elements for regulation of Th2 cytokine genes in the Th2 locus have been identified and studied. These include promoters, enhancers, a silencer, and a Th2 locus control region [16,17]. Among these elements, the Th2 LCR has been shown to coordinately regulate Th2 cytokine genes [16,17]. The Th2 LCR is composed of 4 DNase I hypersensitive sites that are highly conserved between species [19,20]. Th2 LCR-harboring transgenic mice render high-level, differentiation-specific, and copy number-dependent expression to linked transgenes [21]. The Th2 LCR undergoes Th2-specific histone acetylation and DNA methylation during Th2 cell differentiation [16,17]. It has been shown to associates with promoters of the *il4*,

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il5, and *il13* genes by long-range intrachromosomal interactions [22]. More recently, the Th2 LCR has been shown to associate with the *ifng* locus that is present in a different chromosome by interchromosomal interaction [23]. Deletion of the Th2 LCR causes almost complete reduction of Th2 cytokine gene expression, and Th2 LCR-deficient mice are resistant to pathogenesis of asthma [24]. In a previous study, we have found that the expression of GATA-3 and the phosphorylation of STAT6 are specifically defective in Th2 LCR-deficient mice [25]. This study suggests that GATA-3 and STAT6 cooperate with each other to regulate the *gata3* locus, and thus its expression must be regulated by the Th2 LCR-mediated Th2 differentiation program.

In the current study, we investigated the role of the Th2 LCR in regulating the *ifng* locus. We found that the expression of IFN-γ is aberrantly up-regulated in Th2 cells from Th2 LCR-deficient mice. STAT4 and T-bet were not up-regulated in these cells. GATA-3 and STAT6 bound to the *ifng* promoter in Th2 cells from the wild type but not from Th2 LCR-deficient mice, and they directly repressed the *ifng* gene. Thus, our results suggest that the Th2 LCR is essential for regulation of the *ifng* gene through controlling the expression of GATA-3 and STAT6.

2. Materials and methods

2.1. Mice

The generation of Th2 LCR-deficient mice has been described previously [24].

2.2. In vitro differentiation of CD4 T cells

Isolation and *in vitro* differentiation of naïve CD4 T cells into Th1 and Th2 cells has been described previously [26].

2.3. Chromatin immunoprecipitation (ChIP)

Naïve CD4 T cells were stimulated under Th1 or Th2 polarizing conditions as described above. Th1 or Th2 cells ($1–2 \times 10^6$) were cross-linked with 1% formaldehyde and quenched with 0.125 M glycine. Cells were lysed with a lysis buffer (50 mM Tris, pH8.1, 1% SDS, 10 mM EDTA), and sonicated at the high-power setting for 15 min using a Bioruptor sonicator (Diagenode). Under this condition, the average DNA fragment size was approximately 500 bp. Cell extracts were precleared with protein A agarose/salmon sperm DNA (Upstate), and incubated with anti-H3-acetylation (Millipore), a mixture of anti-trimethyl-H3-K4 and anti-monomethyl H3-K4 (Millipore 07-473 and 07-436, respectively), anti-GATA-3 (Santa Cruz, sc-268AC), anti-STAT6 (Chemicon, AB3165), rabbit IgG (Santa Cruz, sc-2027), or normal mouse IgG (Santa Cruz) as a negative control. Antibody-bound chromatin was precipitated by protein A agarose, washed, and eluted with elution buffer (0.1 M sodium bicarbonate, 1% SDS). The chromatin was reverse cross-linked by incubating at 65 °C for 4 h, followed by protease K treatment (100 ng/ml). The amount of precipitated DNA was quantified by real-time PCR using primers as follows: *ifng* promoter F: 5′-CGG GGC TGT CTC ATC GTC-3′, *ifng* promoter R: 5′-CTC GGG ATT ACG TAT TTT CAC AA-3′, *ifng* CNS1 F: 5′-GTT ACC TCC TCC ACC CGT TCA-3′, *ifng* CNS1 R: 5′-GAC AGG ACT ATG ACT CTC TAG-3′, *ifng* CNS2 F: 5′-AAT GCC AGT GGA CTC CTC AGC-3′, *ifng* CNS2 R: 5′-ACA GGG AAT ACA GCC TTG AAG-3′, *ifng* gene F: 5′-GGT CCA AGG TAC AAA GAT GCT-3′, *ifng* gene R: 5′-GAA CTT TGC CTC CCA TTA CTT TA-3′.

2.4. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Naïve CD4 T cells were *in vitro* stimulated into Th1 and Th2 directions as described above. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen). cDNA was synthesized using SuperScript II reverse transcriptase and oligo dT (Invitrogen) according to the manufacturer's protocol. Quantitative PCRs were performed with real-time fluorogenic 5′-nuclease PCR using the 7500 Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions**.** Sequences used for quantitative PCR are as follows.*ifng* sense: 5′-GGATGCATTCATGAGTATTGC-3′, *ifng* anti-sense: 5′-CCTTTTCCGCTTCCTGAGG-3′, *ifng* probe: (FAM)-5′-TTTGAGGTCAACAACCCACAGGTCCA-3′-(Tamra); *hprt* sense: 5′-CTGGTGAAAAGGACCTCTCG-3′, *hprt* anti-sense: 5′-TGAAGTACTCA TTATAGTCAAGGGCA-3′, *hprt* probe: (FAM)5′-TGTTG GATACAGGCC AGACTTTGTTGGAT-3′(Tamra). GATA-3 sense: 5′-AGAACCGGCCCCT TATCAA-3′, GATA-3 anti-sense: 5′-AGTTCGCGCAGGATGTCC-3′, GATA-3 probe: (FAM)-5′-CCAAGCGAAGGCTGTCGGCAG-3′-(Tamra), T-bet sense: 5′-CAACAACCCCTTTGCCAAAG-3′, T-bet anti-sense: 5′-TCCCCCAAGCAGTTGACAGT-3′, T-bet probe: (FAM)-5′-CC GGGAGAACTTTGAGTCCATGTACGC-3′-(Tamra).

2.5. Cell transfection and dual luciferase assay

The expression vector for GATA-3 was constructed from the CMV-base expression vector (pCMV-SPORT6). The expression vectors for a constitutively active form of STAT6, STAT6-VT, were a generous gift from Dr. M. Kaplan (Indiana University, USA). Cell transfection into EL4, a mouse thymoma cell line, and measurement of dual luciferase was done as previously described with minor modifications [19].

2.6. Retroviral transduction

$1–2 \times 10^6$ Phoenix-ECO cells were transfected with empty (GFP-RV), RV-GATA3 (generous gift from Dr. K. Murphy), or MIEG3-STAT6-VT vector. Culture supernatants containing high titers of retrovirus were collected after 48 h of transfection. Purified naïve CD4 T cells were activated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) antibodies in the presence of mIL-2 (1 μg/ml) for 24 h. The activated T cells were spin-infected in 1 ml of retrovirus-containing supernatant with polybrene (4 mg/ml) at 2800 rpm for 90 min at 32 °C, and further incubated for 3 days under a Th2 polarizing condition. Transduction efficiency measured based on GFP^+ cells by FACS analysis was 80–90%. Total cells were used in ChIP assay or qRT-PCR.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Differences between groups were determined by a Student's *t*-test.

3. Results

3.1. IFN-γ expression in Th2 LCR deficient cells

To study the role of the Th2 LCR in the regulation of the *ifng* locus, we examined IFN-γ expression using quantitative RT-PCR and ELISA in Th2 LCR-deficient mice. Naïve CD4 T cells were isolated from littermate control mice and conditional Th2 LCR-deficient mice, and differentiated into Th0, Th1 and Th2 cells *in vitro*. The expression of Th1 cytokine IFN-γ has been shown to modestly decrease in Th2 LCR-deficient Th1 cells compared to littermate control cells [21]. Surprisingly, the expression of IFN-γ greatly

increased in Th0 and Th2 cells from Th2 LCR-deficient mice compared to that from control mice (Fig. 1A and B). Although the amount of IFN-γ produced in Th2 cells from Th2 LCR-deficient mice is about 60 times lower than that in Th1 cells from the same mice, the amount is significantly higher (by tenfold) than that in Th2 cells from heterozygote littermate control mice both at the transcript and protein levels. To examine whether this IFN-γ up-regulation was due to changes in the amount of key transcription factors, we analyzed the expression of such in Th1 and Th2 cell differentiation. Expression of Th1-specific factors, T-bet and STAT4 was measured by quantitative RT-PCR. The expression of T-bet and STAT4 was only slightly changed in Th2 LCR-deficient cells compared to heterozygote control cells (Fig. 1C and 1D). We also have shown previously that the protein levels of T-bet, STAT1, phosphor-STAT1, STAT4, and phosphor-STAT4 do not differ in Th2 LCR-deficient Th2 cells compared to wild type Th2 cells, but that expression of GATA-3 is almost completely lost in Th2 LCR-deficient cells [25]. These results strongly suggest that the aberrant IFN-γ expression under the Th2 condition is not due to increased expression of T-bet, STAT1, or STAT4, but may be due to the defective expression of GATA-3 in Th2 LCR deficient cells.

3.2. The ifng locus is in permissive chromatin status in Th2 LCR-deficient Th2 cells

To investigate whether the increase of IFN-γ expression was caused by the change in chromatin remodeling of the *ifng* locus, we analyzed H3-acetylation and H3-K4-methylation statuses, which are markers for transcriptionally active chromatin, of the *ifng* locus. DNA sequence comparison has revealed two conserved noncoding sequences (CNSs) in the *ifng* locus [14,18]. CNS1 is located 5 kb upstream of the *ifng* gene, CNS2 is 17 kb downstream of the *ifng* gene [14,18]. Th1 and Th2 cells from wild type and Th2 LCR-deficient mice were chromatin-immunoprecipitated with antibodies specific for H3-acetylation and H3-K4-methylation, and the precipitated DNA was quantified by quantitative PCR. All the regulatory elements in the *ifng* locus examined in this study were hypo-acetylated at the H3 in wild type Th2 cells compared to wild type Th1 cells. However, H3-acetylation in these elements was greatly increased in Th2 LCR-deficient Th2 cells (Fig. 2A). H3-K4-methylation showed a similar result to H3-acetylation (Fig. 2B). These results suggest that the *ifng* locus is rendered transcriptionally permissive in Th2 LCR-deficient Th2 cells, and that the Th2 LCR plays an essential role in keeping the *ifng* locus inactive in Th2 cells.

3.3. GATA-3 and STAT6 directly bind to the ifng promoter and repress the ifng gene

The results above showed that IFN-γ expression increased and the chromatin status of the *ifng* locus changed to permissive state in Th2 LCR-deficient Th2 cells. Since deletion of the Th2 LCR does not likely influence STAT4 and T-bet directly, and since it has been shown that STAT6 and GATA-3 bind to the *ifng* promoter in the pre-

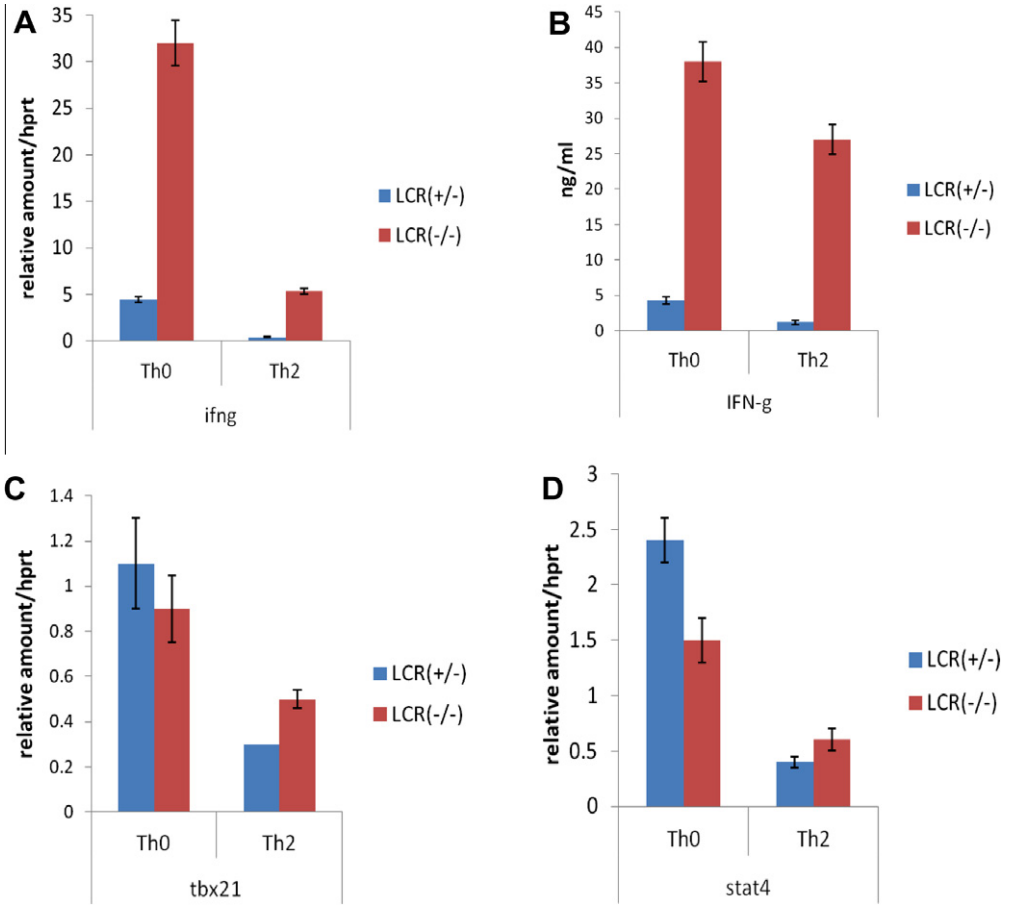

Fig. 1. Expression of IFN-γ and subset-specific transcription factors in Th1/Th2 cells from littermate control and Th2 LCR KO mice. Naïve CD4 T cells were isolated from littermate control and Th2 LCR-deficient (LCR KO) mice, stimulated and differentiated *in vitro*. IFN-γ expression was measured by quantitative RT-PCR (A) and by ELISA (B). Under the Th0 or Th2 condition, Th1-specific transcription factors *tbx21* (C), and *stat4* mRNA (D) was measured by quantitative RT-PCR. Error bar shows standard deviation ($n = 3$).

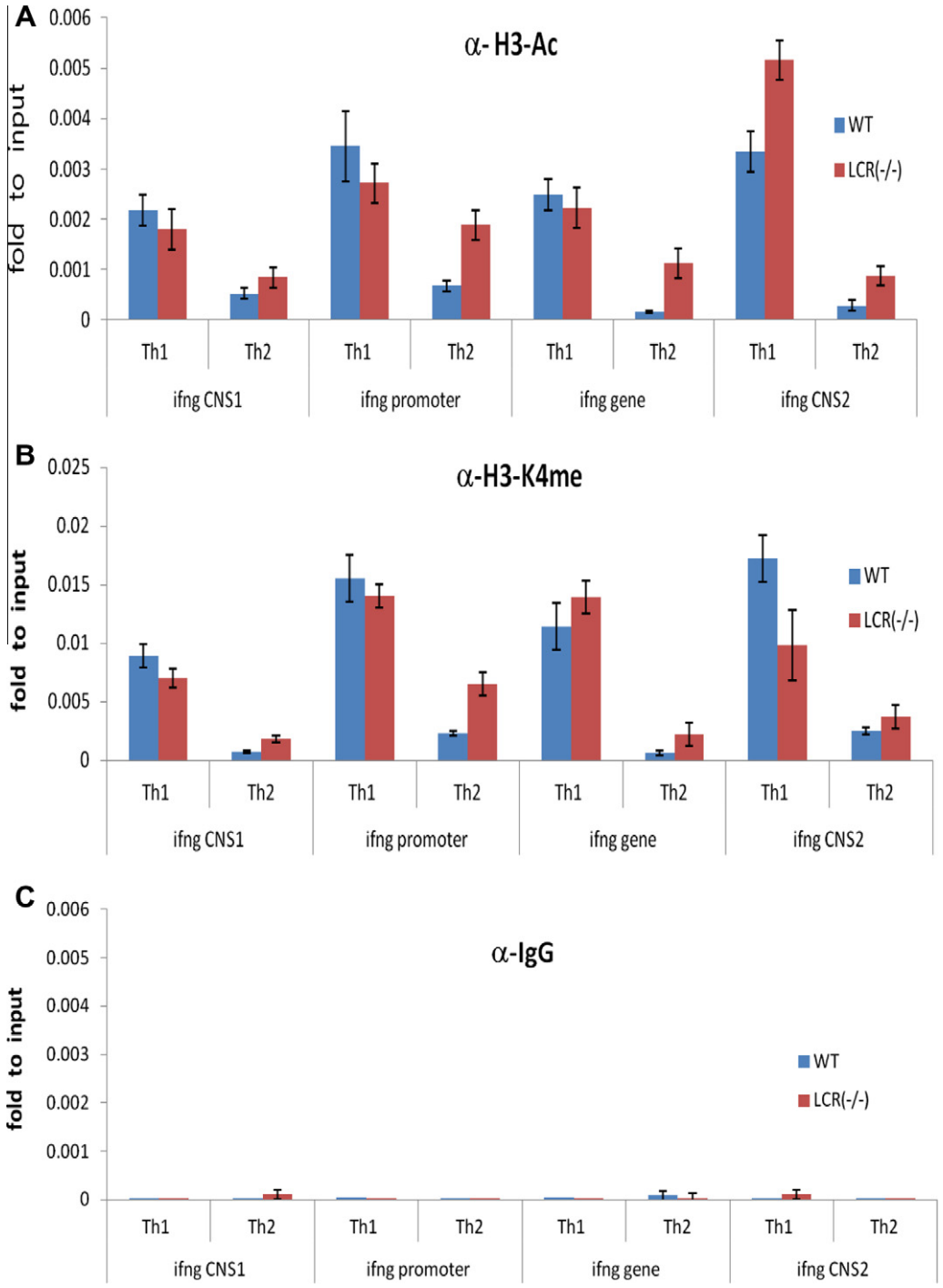

Fig. 2. H3-acetylation and H3-K4-methylation patterns in the *ifng* locus in Th1/Th2 cells from wild type and Th2 LCR KO mice. Naïve CD4 T cells were isolated from wild type (WT) or Th2 LCR-deficient (LCR KO) mice, stimulated, and differentiated *in vitro*. (A) H3-acetylation pattern was measure by ChIP using H3-acetylation-specific antibody. (B) H3-K4-methylation pattern was analyzed by ChIP using H3-K4-methylation specific antibodies. (C) A mock experiment was measured in parallel with (A and B) using control IgG antibody. Precipitated DNA was quantified by quantitative PCR. Relative amount of precipitated DNA to input DNA is shown on the *y*-axis. Error bar shows standard deviation ($n = 3$).

vious study by Chang et al. [14], we hypothesized that the up-regulation of IFN-γ in Th2 LCR-deficient Th2 cells might be caused by defective binding of STAT6 and GATA-3 to the *ifng* locus. To test this hypothesis, first, we searched for candidate GATA-3 and STAT6 binding sites in the *ifng* promoter. GATA-3 and STAT6 binding sites were present in the *ifng* promoter regions. These binding sites are highly conserved in various mammalian species (Fig. 3A). Next, we examined the GATA-3 and STAT6 binding to the *ifng* promoter by chromatin immunoprecipitation with specific antibodies for GATA-3 and STAT6 (Fig. 3B and C). GATA-3 bound to the *ifng* promoter in a Th2-specific manner, and this binding increased with time (Fig. 3B). Interestingly, Th2-specific GATA-3 and STAT6 binding to the *ifng* promoter was greatly reduced in Th2 LCR-deficient mice (Fig. 3C), suggesting that this binding is influenced by the absence of the Th2 LCR.

To examine whether the increase of IFN-γ in Th2 LCR-deficient Th2 cells is due to direct repression of the *ifng* gene by GATA-3 and STAT6, we transfected a reporter construct containing the *ifng* pro-

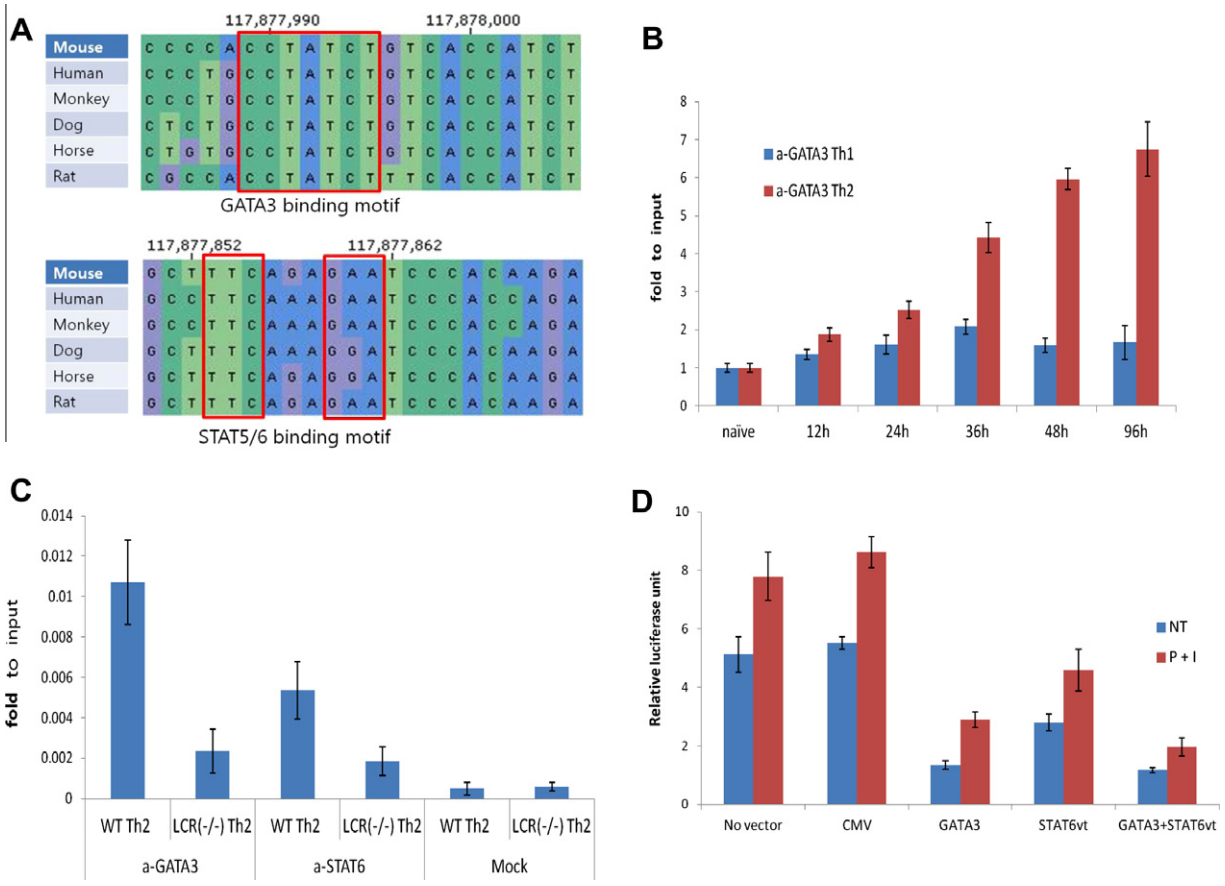

Fig. 3. Binding of GATA-3 and STAT6 on the *ifng* promoter and repression of the *ifng* promoter by these proteins. (A) VISTA (visualization tool for alignment, http://www.genome.lbl.gov/vista/index.shtml) was used to analyze highly conserved *ifng* promoter sequences between human, mouse, rat, cow, dog, and monkey. Red-squared regions represent the GATA-3 binding motif (WGATAR) and STAT5/6 binding motif (TTCNNNGAA). (B) Naïve CD4 T cells were isolated from C57BL/6 mice, stimulated, and differentiated *in vitro*. Binding of GATA-3 on the *ifng* promoter was analyzed by ChIP in a time course using specific antibodies. (C) Naïve CD4 T cells were isolated from WT and LCR KO mice, stimulated and differentiated *in vitro*. Binding of GATA-3 and STAT6 on the *ifng* promoter was analyzed by ChIP using specific antibodies. Precipitated DNA was quantified by quantitative PCR. Relative amount of precipitated DNA to input DNA is shown on the y-axis. Error bar shows standard deviation ($n = 3$). (D) Expression vectors of GATA-3 and/or STAT6-VT were transfected by electroporation into EL4 cells with reporter constructs containing the *ifng* promoter and the luciferase gene, and rested overnight. Cells were then stimulated with PMA (0.5 ng/ml) and ionomycin (1 μM) for 4 h, and luciferase activity was measured. Transfection efficiency was normalized by dual luciferase activity. Error bar shows standard deviation ($n = 3$).

moter linked to the luciferase gene together with the expression vectors of GATA-3 or STAT6-VT (a constitutively active form of STAT6), into EL4 cells, and stimulated with PMA and ionomycin. GATA-3 greatly repressed transcriptional activity of the *ifng* promoter in both resting and stimulated conditions, and STAT6-VT also modestly repressed the *ifng* promoter activity (Fig. 3D). It seems that GATA-3 played a greater repressive role than did STAT6 at the *ifng* promoter. This result strongly suggests that IFN-γ expression is repressed by GATA-3 and STAT6 through direct transrepression of the *ifng* promoter.

3.4. GATA-3 and STAT6 repressed the aberrant expression of the ifng gene and restored the repressive chromatin state at the ifng locus

To examine the role of GATA-3 and STAT6 in the aberrant expression of the *ifng* gene and in the de-repressed chromatin state at the *ifng* locus in Th2-LCR deficient Th2 cells, we enforced expression of GATA-3 or STAT6-VT by retroviral transduction in these cells. Ectopic expression of GATA-3 or STAT-6 VT reduced the aberrant expression of the *ifng* gene (Fig. 4A) and restored the repressive chromatin state at the *ifng* locus in Th2-LCR-deficient Th2 cells (Fig. 4B and C). This result suggests that GATA-3 and STAT6 are responsible for repression of the *ifng* locus. Taken together, the results in this study support our hypothesis that IFN-γ expression is negatively regulated by GATA-3 and STAT6, which are regulated by a feed-forward mechanism of the Th2 LCR.

4. Discussion

In this study, we showed that repressed IFN-γ expression is reversed in Th2 LCR-deficient Th2 cells. We also showed that the *ifng* locus is in a permissive chromatin state in Th2 LCR-deficient Th2 cells, and that GATA-3 and STAT6 bindings are defective in these cells. These results suggest that IFN-γ expression in Th2 cells is repressed by GATA-3 and phosphorylated STAT6, and that the levels of these proteins are determined by the Th2 LCR.

GATA-3, the master transcription factor for Th2 cell differentiation, not only induces the Th2 cell program, but also blocks the differentiation into other T helper cell types including Th1 and Th17 [14,15]. The binding of GATA-3 and STAT6 to the *ifng* promoter was observed in the previous study by Chang et al. [14]. Fields et al. have shown that GATA-3 induces repressive histone deacetylation and methylation at the *ifng* locus in Th2 cells [27]. Here, we provided a more detailed molecular mechanism of *ifng* locus regulation. We showed that GATA-3 and STAT6 directly bind to the *ifng* promoter and transrepress *ifng* expression. Chromatin remodeling ability of GATA-3 at the Th2 cytokine locus has been shown previously [8–10]. STAT6 has also been shown to be re-

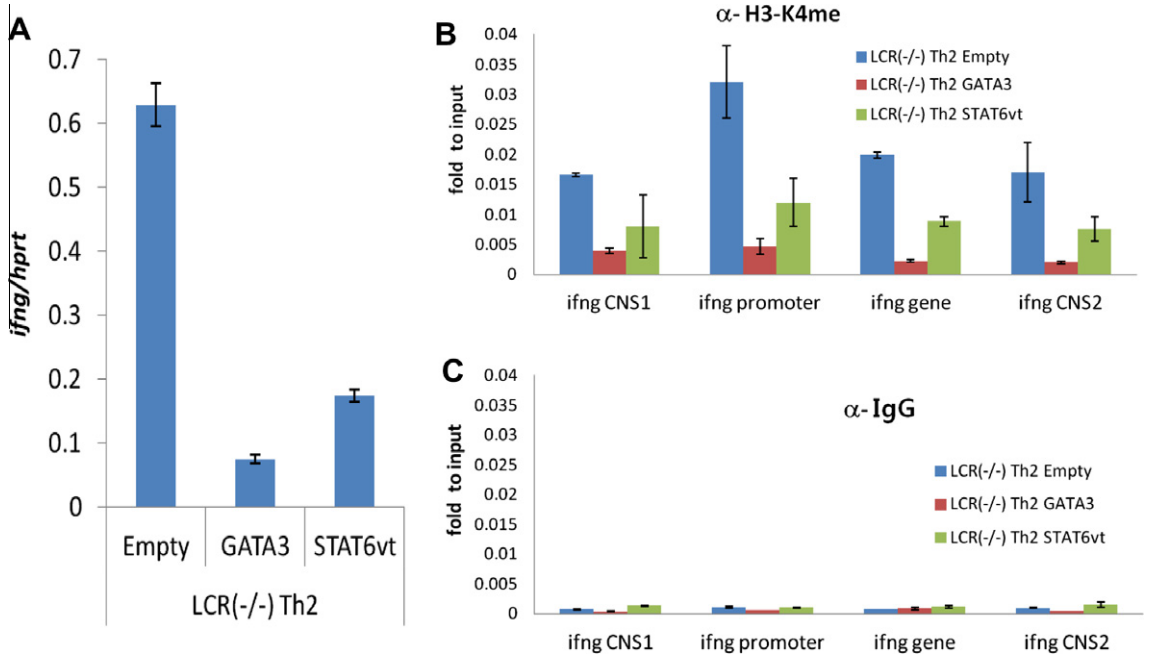

Fig. 4. Reduced expression of the aberrant *ifng* expression and restoration of the repressed chromatin state by GATA-3 and STAT6-VT at the *ifng* locus in Th2 LCR-deficient Th2 cells. Naïve CD4 T cells from Th2 LCR-deficient mice were stimulated and transduced with control (empty), GATA-3, or STAT6-VT retroviral vector, and differentiated under a Th2-polarizing condition. Expression of the *ifng* gene was measured by quantitative RT-PCR (A), and H3-K4-methylation state at the *ifng* locus was measured by ChIP using a mixture of anti-H3-K4-me1 and anti-H3-me3 antibodies (B), or by a control IgG antibody (C).

quired for chromatin remodeling of the *ifng* locus by Chang et al. [14]. They have shown that H4-acetylation and H3-K9-me2 (marker for active transcription) increased at the *ifng* locus in STAT6 KO Th2 cells compared to wild type Th2 cells. Our H3-acetylation and H3-K4-methylation results showed that the chromatin at the *ifng* locus in Th2 LCR-deficient cells was in a permissive state. Since both GATA-3 and STAT6 bind to the *ifng* promoter, both may be crucial for chromatin remodeling of the *ifng* locus. Moreover, since the simultaneous binding sites of GATA-3 and STAT6 are concentrated on the *ifng* promoter region and its vicinity in Th2 cells [14], it is plausible that the cooperative binding of GATA-3 and STAT6 to these small regions may be essential for transrepression of *ifng* gene expression and for maintaining repressive chromatin status at the *ifng* locus in Th2 cells.

Previously, we have shown that Th2 LCR-deficient cells have defective Th2 cell differentiation manifested by inhibition of Th2 cytokine expression and repressed chromatin status at the Th2 cytokine locus [24], and that deletion of the Th2 LCR causes defects in the expression of the *gata3* gene and chromatin remodeling at the *gata3* locus [25]. In this study, we showed that the *cis*-element Th2 LCR is not only required for coordinate expression of Th2 cytokine genes including *il4*, *il5* and *il13*, but also suppresses *ifng* gene expression via GATA-3 and STAT6, which are regulated by a feed-forward mechanism of the Th2 LCR. Thus, our results suggest that the Th2 LCR regulates both Th1 and Th2 loci either directly or indirectly to implement the Th2 differentiation program.

The results of this study support our hypothesis that the Th2 LCR-mediated differentiation program inhibits the *ifng* locus. However, other explanations for these results are also possible. For example, interchromosomal interaction of the Th2 LCR with the *ifng* locus may influence IFN-γ expression. The Th2 locus has been shown to associate with the *ifng* locus by interchromosomal interaction [23]. This association is poised in naïve CD4 cells and displaces upon Th1/Th2 differentiation. The absence of the Th2 LCR may cause the failure of marking the *ifng* locus for suppression of IFN-γ expression at the naïve CD4 cell stage, and as a result the *ifng* locus may fail to be suppressed in subsequent Th2-driven programs in Th2 LCR-deficient Th2 cells. Further work will be necessary to test this possibility.

In conclusion, our study suggests that the expression of the key Th2-specific transcription factors GATA-3 and STAT6 negatively regulate *ifng* expression by a feed-forward mechanism under the direction of the Th2 LCR during Th2 differentiation. This study may provide important information on the fundamental process of Th2 cell differentiation, which in turn may assist in developing a therapeutic strategy for curing Th2-mediated diseases such as allergy and asthma. Detailed mechanisms await elucidation in further study.

Acknowledgments

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