



Published in final edited form as:

*Cytokine*. 2013 April ; 62(1): 91–95. doi:10.1016/j.cyto.2013.02.010.

## IFN- $\gamma$ suppresses permissive chromatin remodeling in the regulatory region of the *IL4* Gene

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### Abstract

In order to develop the most effective T helper type-1 (Th1) immunity, naïve CD4<sup>+</sup> T cells must acquire the capacity to express IFN- $\gamma$  while silencing T helper type-2 (Th2) cytokine-producing potential. An *IL4* gene silencer has been described. However, it is not completely understood how the silencer works. In this study, we examined whether IFN- $\gamma$  can suppress permissive chromatin remodeling of regulatory region of the *IL4* gene. We demonstrate that IFN- $\gamma$  suppressed H3K4 dimethylation at the intronic enhancer region of the *IL4* gene. The IFN- $\gamma$ -mediated suppression of permissive chromatin remodeling was IFN- $\gamma$  receptor-, STAT1-, and T-bet-dependent. Our study reveals a novel mechanism of how Th1 cells silence the *IL4* gene.

### Keywords

IFN- $\gamma$ ; Th1; Silencing *IL4* gene expression; Chromatin remodeling

## 1. Introduction

In order to develop the most effective T helper type-1 (Th1) immunity, naïve CD4<sup>+</sup> T cells must acquire the capacity to express IFN- $\gamma$  while silencing T helper type-2 (Th2) cytokine-producing potential. Ansel *et al.* have identified such a silencer region within the *IL4* gene [1]. Deletion of the *IL4* gene silencer results in a Th2 immune response following

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Author Contributions: J.N. and H.H. are involved in experimental designs, performing experiment, data analysis and manuscript preparation; Y. L., Y.Z. and Z. H. are involved in experimental designs, performing experiments, revising the manuscript.

### Financial Disclosure

Authors have no financial conflict of interest.

*Leishmania major* infection, which would normally trigger a strong Th1 immune response [1].

It has demonstrated that chromatin structure plays a pivotal role in Th2 cytokine gene expression [2]. As naïve CD4<sup>+</sup> T cells differentiate into Th2 cells, the histone molecules that surround the regulatory regions of Th2 cytokine gene loci undergo covalent modifications. These modifications lead to conformational changes in chromatin structure, which allow transcription factors to gain access to cytokine gene loci. More generally, chromatin modifications can include both permissive and repressive modifications. Permissive modifications render the gene locus more accessible to transcription factors, whereas repressive modifications make the gene locus less accessible. Permissive modifications include demethylation of CpG islands in the regulatory regions of a gene, acetylation of the ninth and fourteenth lysine residues of histone 3 (H3K9/14ac), and dimethylation of the fourth lysine residue of H3 (H3K4me2) [3-7]. Repressive modifications include methylation of the ninth lysine residue of H3 (H3K9me) [6], methylation of the twenty-seventh lysine residue of H3 (H3K27me) [8] and methylation of the thirty-sixth lysine residue of H3 (H3K36me) [2].

Transcriptional active regions of the *Il4* gene have also been mapped by DNase sensitivity assay. These DNase hypersensitive sites found in the *Il4* locus includes HSS 0 in the intergenic region between the *Il13* and the *Il4* gene, HSS 1 and HSS 2 in the conserved noncoding sequence 1 (CNS-1) region, HS I in the *Il4* gene promoter region, HS II and HS III in the second intronic enhancer region (IE), and HSV in 3' flanking region of exon 4 [9-11]. Rad50 hypersensitive site 7, the seventh *Il4* hypersensitive site, was found to be a DNA element that supports high-levels of IL-4 expression in Th2 cells [12, 13]. This region is known as the locus control region (LCR). The *Il4* gene silencer has been mapped to DNase hypersensitive site IV within the second consensus non-coding sequence (CNS-2), which is located in the 3' untranslated region of the *Il4* gene. T-bet and Runx3 have been shown to bind to the *Il4* gene silencer, yet chromatin in the silencer region exhibits a permissive configuration rather than a repressive configuration [1]. Thus, it remains incompletely understood how the *Il4* gene silencer keeps the *Il4* gene transcriptionally inactive.

We previously showed that IFN- $\gamma$  is essential in maintaining the Th1 phenotype by actively suppressing the *Il4* gene transcription potential [14]. We further demonstrated that both STAT4 and T-bet are required for silencing the IL-4-producing potential in committed Th1 cells [15, 16]. However, it has not been determined if IFN- $\gamma$  can suppress permissive remodeling of regulatory regions in order to silence the *Il4* gene. Here, we demonstrate that IFN- $\gamma$  can directly suppress permissive chromatin remodeling in the regulatory region of the *Il4* gene.

## 2. Materials and methods

### 2.1. Animals and cell cultures

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). T-bet-deficient (*Tbx21*<sup>-/-</sup>) mice [17], *IFNGR*<sup>-/-</sup> mice [18], and *STAT1*<sup>-/-</sup> mice [19] were also purchased from The Jackson Laboratory and maintained in the animal facility of National Jewish Health (Denver, CO). Naïve CD4<sup>+</sup> T cells were purified from the spleen and lymph nodes of these various strains of mice using the CD4<sup>+</sup>CD62L<sup>+</sup>T Cell Isolation Kit (Miltenyi Biotec Inc, CA). Purified naïve CD4<sup>+</sup>T cells ( $0.2 \times 10^6$ ) were stimulated with irradiated T cell-depleted APC ( $0.8 \times 10^6$ ) in 2 ml of complete RPMI medium supplemented with IL-2 (30 U/ml), anti-CD3 (2C11, 3  $\mu$ g/ml), and anti-CD28 antibody (3  $\mu$ g/ml) for 2 to 7 days as described in more detail previously [16]. Briefly, for Th1-inducing conditions, IFN- $\gamma$  (20

ng/ml, BD-PharMingen, San Diego, CA), and anti-IL-4 antibody (11B11, 10  $\mu$ g/ml) were used; and for Th2-inducing conditions, IL-4 (5 ng/ml, BD-PharMingen), anti-IL-12 antibody (10  $\mu$ g/ml), and anti-IFN- $\gamma$  antibody (XMG, 10  $\mu$ g/ml) were added; and for neutralized conditions anti-IL-4 antibody (10  $\mu$ g/ml), anti-IL-12 antibody (10  $\mu$ g/ml), and anti-IFN- $\gamma$  antibody (10  $\mu$ g/ml) were added (used as a control group). Anti-IL-4 antibody, anti-IL-12 antibodies, and anti-IFN- $\gamma$  antibody were prepared by our lab from hybridoma culture supernatants using HiTrap Affinity Protein G column according to manufacturer's instructions (GE Healthcare Life Science, Piscataway, NJ). The qualities of these purified antibodies were tested using ELISA as well as Th1 and Th2 priming [14-16, 20].

All animal protocols were approved by the Institutional Animal Care and Users Committee of National Jewish Health (IACUC#AS2703-03-14).

## 2.2. Intracellular staining of cytokine expression

Intracellular staining of IL-4 and IFN- $\gamma$  was carried as described previously [15]. Briefly, the primed cells were washed and stimulated with PMA (50 ng/ml) and ionomycin (1  $\mu$ M) in the presence of 2  $\mu$ M of monensin (Calbiochem, La Jolla, CA). After 6 hours of stimulation, cells were fixed with 4% paraformaldehyde and stained with APC-labeled anti-CD4, FITC-labeled anti-IFN- $\gamma$  and/or PE-labeled anti-IL-4 (11B11) monoclonal antibody (BD-PharMingen). Samples were collected using FACScan or FACScalibur (Becton-Dickinson) and analyzed using FlowJo software (Tree Star Inc, Ashland, OR). All FACS data were analyzed using a CD4<sup>+</sup> gate and dead cells were excluded from FACS data using a low Forward Scatter.

## 2.3. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay was performed using Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology Lake Placid, NY) according to manufacturer's protocol. Briefly, cells ( $1 \times 10^6$ ) were treated with 1% formaldehyde for 10 minutes at 37°C and lysed in 200  $\mu$ l of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1), freshly supplemented with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml of pepstatin A, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin. Cell lysates were sonicated and diluted 10 fold in 2 ml of dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). Twenty microliter (10% input) of lysates was set aside for input measurements. Lysates were pre-cleaned by incubating with 50  $\mu$ l of protein A agarose beads (50% slurry). To immunoprecipitate dimethyl-histone H3 lysine 4, anti-dimethyl-histone H3 lysine 4 antibody (Upstate Biotechnology) was added to 100  $\mu$ l of lysate and incubated at 4°C for 1 hour. As a control, normal rabbit IgG was added to the other 100  $\mu$ l of lysate. The immunocomplex was captured by incubating 50  $\mu$ l of protein A agarose beads at 4°C overnight. The beads were subsequently washed for 3-5 minutes by low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl) once, followed by high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) once. The washed beads were further washed with LiCl immune complex wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1) once and TE Buffer, (10 mM Tris-HCl, 1 mM EDTA pH 8.0) twice. After wash, immunocomplex was eluted in 500  $\mu$ l of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>) at room temperature. Then crosslinking was reversed by adding 20  $\mu$ l of 5 M NaCl and incubating at 65°C for 4 hour. Proteins were digested by Proteinase K (10  $\mu$ l of 0.5M EDTA, 20  $\mu$ l of 1 M Tris-HCl pH6.5, 2  $\mu$ l of 10 mg/ml Proteinase K). The eluted DNA was purified by phenol/chloroform extraction and precipitated by ethanol with 20  $\mu$ g of glycogen at -70°C for 30 minutes. The precipitated DNA was dissolved in 50  $\mu$ l of TE buffer. The amount of DNA precipitated by the antibody was determined by Real-Time PCR. Ten

percent of input was also used for Real-Time measurement of IE or silencer. Ct values of diluted input were adjusted to 100% of input by subtracting 3.322 cycles (i.e.,  $\log_2$  of 10) from the Ct value of diluted input. Real-time PCR primer sequences were as follows: IE, forward: 5'-GGGTGTGAATAAGCCATATTG-3', reverse: 5'-CCCAGCGTTTACATGAGC-3' [5]; Silencer, forward: 5'-TGCCACATGAAATACCAGC-3'; reverse: 5'-GCATACCTTCCCTGATTGGC-3' [1]. The DNA amount precipitated by anti-H3K4me2 antibody was calculated as % input using the following formula: % of input =  $2^{\Delta Ct} \times 100$ , where  $\Delta Ct = Ct_{input} - Ct_{IP}$  [21].

## 2.4. Statistical analysis

All of the error bars in this report represent the standard deviation. The difference between two samples was analyzed using Student's *t* test.

## 3. Results

### 3.1. IFN- $\gamma$ suppresses permissive chromatin remodeling in the intronic enhancer region

Our previous work has demonstrated that IFN- $\gamma$  played critical role in stabilizing the Th1 phenotype. In the absence of IFN- $\gamma$  signaling, Th1 cells retained the capacity to produce IL-4, indicating that the *Ii4* gene is not silenced in Th1 cells in the absence of IFN- $\gamma$  signaling [14, 20]. Although an *Ii4* silencer has been found, it remains to be determined whether IFN- $\gamma$  can actively suppress permissive chromatin remodeling in addition to target the *Ii4* silencer through T-bet and Runx3.

Purified naïve CD4<sup>+</sup> T cells did not express IFN- $\gamma$  or IL-4 protein as measured by intracellular staining (Fig. 1). Th1- or Th2-inducing conditions used throughout this study led to highly polarized Th1 and Th2 cells, while neutralized conditions (with addition of antibodies to Th1- and Th2-inducing cytokines) did not significantly enhance IL-4-producing or IFN- $\gamma$ -producing capacity (Fig. 1), indicating that the Th1 and Th2 polarization protocol we used in this study, which is identical to that used in many of our previous studies [14, 16, 20, 22], is a highly reproducible process.

We used a chromatin immunoprecipitation (ChIP) assay to examine the status of dimethylation modification of the fourth lysine residue of histone 3 (H3K4me2) in a regulatory region of the *Ii4* gene in CD4<sup>+</sup> T cells that were primed under neutralized conditions, Th1-inducing or Th2-inducing conditions for either two or four days. Representative real-time PCR amplification plots of the immunoprecipitated IE region prepared from CD4<sup>+</sup> T cells primed under neutralized conditions, Th1-inducing or Th2-inducing conditions were shown in Fig. 2A. We found that naïve CD4<sup>+</sup> T cells showed very little H3K4me2 modification in the intronic enhancer region (IE) of the *Ii4* gene. Two days after culturing under Th2-inducing conditions, the cultured CD4<sup>+</sup> T cells displayed elevated levels of H3K4me2 modification in the IE region. Four days after Th2-priming, H3K4me2 modification was dramatically increased. CD4<sup>+</sup> T cells that were primed under neutralized conditions also showed increased levels of H3K4me2 modification albeit to less degree than CD4<sup>+</sup> T cells that were primed under Th2-inducing conditions. We interpret that the neutralized condition-induced H3K4me2 modification was mediated by TCR activation. We observed that in the presence of IFN- $\gamma$ , H3K4me2 modification in the IE region was suppressed (Fig. 2B). We also observed a similar pattern for the acetylation of the H3K9/14 residues modification in the IE region of the *Ii4* gene (data not shown). These data suggest that suppression of H3K4me2 modification in the IE region was actively mediated by IFN- $\gamma$ .

### 3.2. IFN- $\gamma$ depends on STAT1 and T-bet to suppress permissive chromatin remodeling in the intronic enhancer region

To analyze the pathway involved in IFN- $\gamma$ -induced suppression of permissive chromatin remodeling in the IE region of the *Ii4* gene, we first tested the H3K4me2 modification in IFN- $\gamma$  receptor deficient CD4<sup>+</sup> T cells and found that the H3K4me2 modification in the IE region of the *Ii4* gene of IFNR<sup>-/-</sup> CD4<sup>+</sup> that were primed under Th1 conditions for 7 days was greatly increased, demonstrating that IFN- $\gamma$  directly suppresses H3K4me2 modification (Fig. 3). Representative real-time PCR amplification plots of the IE region were shown in Fig. S1. IFN- $\gamma$  has been shown to exert its biological functions in either a STAT1-dependent or STAT1-independent manner. We found that STAT1<sup>-/-</sup> CD4<sup>+</sup> T cells regardless they were under Th1 or Th2-inducing conditions showed markedly increased levels of H3K4me2 modification in the IE region (Fig. 4A). However, elevated levels of H3K4me2 modification were not found in the silencer region of STAT1<sup>-/-</sup> CD4<sup>+</sup> T cells primed under Th1 or Th2-inducing conditions. On the contrary, the H3K4me2 modification was reduced significantly in the silencer region of STAT1<sup>-/-</sup> CD4<sup>+</sup> T cells that were primed under Th1-inducing conditions compared to that of WT Th1 cells (Fig. 4B). Together, these data demonstrate that IFN- $\gamma$  depended on STAT1 to suppress the H3K4me2 modification.

Our previous work showed that T-bet was required for maintaining the silencing state of the *Ii4* gene in committed Th1 cells; in the absence of T-bet, CD4<sup>+</sup> T cells transcribed the *Ii4* gene even they were primed under Th1-inducing conditions [15]. However, it is not clear how T-bet maintains the silencing state of the *Ii4* gene. To determine whether T-bet mediates the IFN- $\gamma$ -induced suppression of H3K4me2 modification, we examined H3K4me2 modification in the IE region of the *Ii4* gene in both WT and T-bet<sup>-/-</sup>CD4<sup>+</sup> T cells. We showed that IFN- $\gamma$ -induced suppression of H3K4me2 modification was abolished in the absence of T-bet (Fig. 5). The amounts of IE fragment immunoprecipitated by isotype control antibody ranged from 0.005% to 0.03% of input (data not shown because values were too low to be reflected in the figures). Thus, we concluded that IFN- $\gamma$  depended on T-bet to suppress the H3K4me2 modification in the IE region of the *Ii4* gene.

## 4. Discussion

In this study, we tested if IFN- $\gamma$  can suppress permissive chromatin remodeling of regulatory regions other than the silencer region in order to silence the *Ii4* gene. Based on the experimental results obtained from primary cultures, we conclude that IFN- $\gamma$  can actively suppress permissive chromatin remodeling in the IE region of the *Ii4* gene. Th1 and Th2 cell lines or clones, as the result of repeated stimulation under Th1 or Th2-inducing conditions, exhibit more stable phenotype [23, 24][25]. Measuring H3K4me2 modification in the IE region of Th1 cell lines or clones could further strengthen our conclusion. Furthermore, it would be interesting to test whether IFN- $\gamma$  suppresses H3K4me2 modification in the regulatory regions of the *Ii13* genes in Th1 cells.

Th1-inducing conditions has been shown to induce elevated levels of H3K4me2 modification to histone molecules in the regulatory regions of the *Iifng* gene [26]. Authors of the paper showed that during the early phase of Th1/2 priming, levels of H3K4me2 modification in the regulatory regions of the *Iifng* gene were elevated in both Th1 and Th2 cells. After 28 days, H3K4me2 modification in the regulatory regions of the *Iifng* gene in Th2 cells disappeared, suggesting that Th2-inducing conditions act slowly to suppress H3K4me2 modification to histone molecules in the regulatory regions of the *Iifng* gene [26]. On the other hand, we observed a much faster action of IFN- $\gamma$  on suppressing H3K4me2 modification in the IE region of the *Ii4* gene in Th1 cells. Consistently, we note that the fast IFN- $\gamma$  action on H3K4me2 modification was IFNGR1-, STAT1- and T-bet-dependent. Our investigation reveals a novel mechanism by which IFN- $\gamma$  can suppress *Ii4* gene

transcription. These new findings might fill the gap in our understanding of how the silencers can interact with other regulatory regions of the *Il4* gene to achieve silencing of the *Il4* gene. For example, it is likely that a silencer is needed for recruiting enzymes that can induce repressive modifications into other distal regulatory regions of the *Il4* gene, such as IE. Binding of T-bet and Runx3 to the silencer region could provide a contact point for long-distance interactions between the silencer region and other distal regulatory regions of the *Il4* gene. This long-distance interaction is a prerequisite for recruiting repressive remodeling enzymes, such as HDAC1, to the IE region. Long-distance interaction has been documented for the *Il4* gene. Spilianakis *et al.* showed that binding of GATA3 and STAT6 to the LCR resulted in a long-distance interaction of LCR with the *Il4* promoter, which is located 30 kb away from the LCR [12]. Further studies using the *Il4* silencer-deficient mice are needed to verify whether long-distance interaction between the silencer and the IE region can indeed induce reduction in permissive remodeling.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by the National Institutes of Health (RO1 AI048568 and RO1 AI059170).

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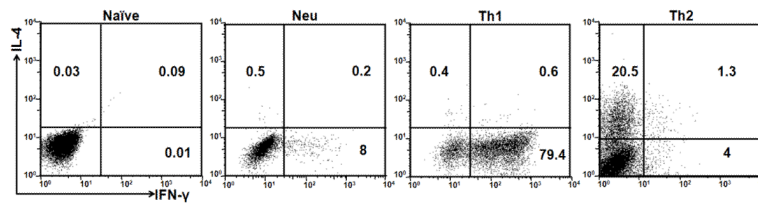
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### Highlights

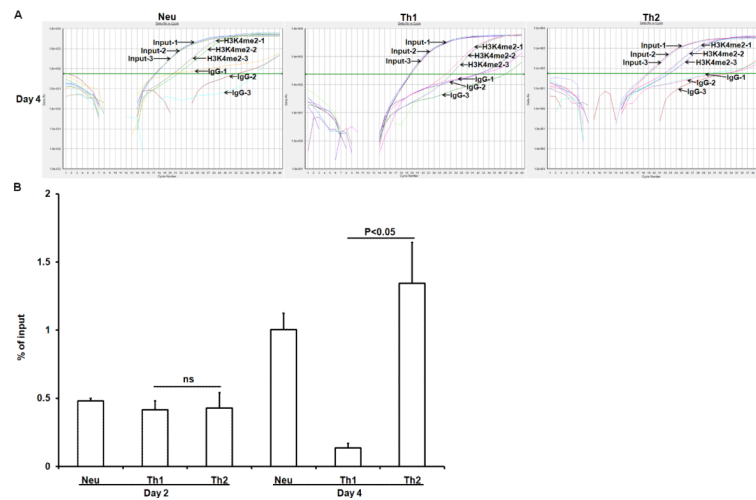
1. We demonstrate that IFN- $\gamma$  suppressed H3K4 dimethylation at the intronic enhancer region of the *IL4* gene.
2. The IFN- $\gamma$ -mediated suppression of permissive chromatin remodeling was IFN- $\gamma$ -R-, STAT1-, and T-bet-dependent.
3. Our study reveals a novel mechanism of how Th1 cells silence the *IL4* gene.



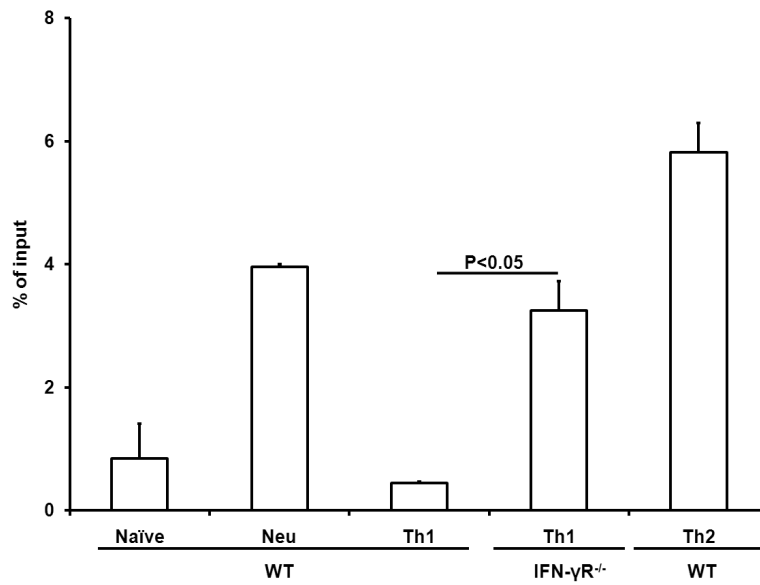


**Fig. 1.**

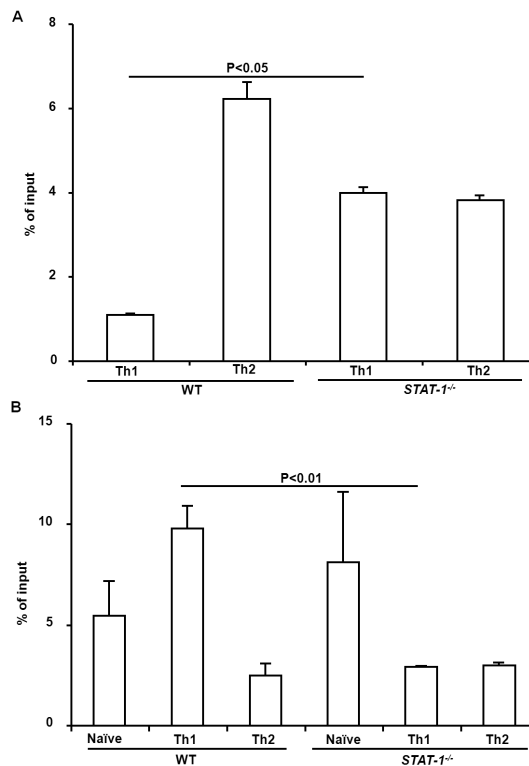
Th1- and Th2-inducing conditions lead to highly polarized IFN- $\gamma$  expression and IL-4 expression, respectively. Naïve CD4<sup>+</sup> T cells were not primed or primed under neutralized conditions [indicated as Neu, these conditions contained anti-IFN- $\gamma$  antibody (10  $\mu$ g/ml) and anti-IL-12 antibody (10  $\mu$ g/ml)], Th1-inducing conditions [indicated as Th1), these conditions contained IFN- $\gamma$  (20 ng/ml) plus anti-IL-4 antibody (10  $\mu$ g/ml)] or Th2-inducing conditions [indicated as Th2, these conditions contained IL-4 (5 ng/ml) plus anti-IFN- $\gamma$  antibody (10  $\mu$ g/ml) and anti-IL-12 antibody (10  $\mu$ g/ml)] for 7 days. IL-4 and IFN- $\gamma$  protein expression was analyzed by intracellular staining. The numbers inside the FACS plots indicate the positive percentage within the gated CD4<sup>+</sup> T cells.



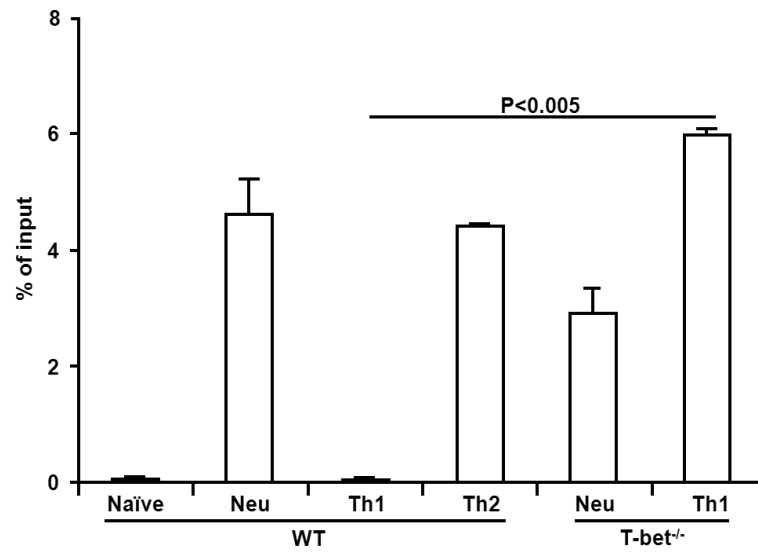
**Fig. 2.** IFN- $\gamma$  suppresses H3K4me2 modification in the IE region of the *Il4* gene. Naïve CD4<sup>+</sup> T cells from C57BL/6 mice were primed under neutralized conditions, Th1-inducing conditions or Th2-inducing conditions for either 2 or 4 days. The resultant cells were harvested, cross-linked, and sonicated. H3K4me2 modification was measured by immunoprecipitation with anti-dimethylated lysine 4 residue antibody. The amount of immunoprecipitated IE fragment was measured by Real-Time PCR and shown as the percentage of input DNA. Values represent mean  $\pm$  SD of triplicates. Ns= not significant. Data from one experiment represent two independent experiments with similar results.



**Fig. 3.** IFN- $\gamma$  directly suppresses H3K4me2 modification. Naïve CD4<sup>+</sup> T cells from C57BL/6 and *IFN $\gamma$ R<sup>-/-</sup>* mice were cultured for 7 days under the conditions indicated. The resultant cells were harvested, cross-linked, and sonicated. H3K4me2 modification was measured by immunoprecipitation with anti-dimethylated lysine 4 residue antibody. The amount of immunoprecipitated IE fragment was measured by Real-Time PCR and shown as the percentage of input DNA. Values represent mean  $\pm$  SD of duplicates. Data from one experiment represent two independent experiments with similar finding.

**Fig. 4.**

IFN- $\gamma$  depends on STAT1 to suppress H3K4me2 modification in the IE region but not the silencer region. (A) Naïve CD4<sup>+</sup> T cells from C57BL/6 and *STAT1*<sup>-/-</sup> mice were cultured for 7 days under the conditions indicated. The resultant cells were harvested, cross-linked, and sonicated. H3K4me2 modification was measured by immunoprecipitation with anti-dimethylated lysine 4 residue antibody. The amount of immunoprecipitated IE fragment was measured by Real-Time PCR and shown as the percentage of input DNA. (B) The amount of immunoprecipitated silencer fragment was measured Real-Time PCR. Values represent mean  $\pm$  SD of triplicates. Data from one experiment represent two independent experiments with similar results.



**Fig. 5.** IFN- $\gamma$  depends on T-bet to suppress H3K4me2 modification. Naïve CD4<sup>+</sup> T cells from C57BL/6 and *Tbx21*<sup>-/-</sup> mice were cultured for 7 days under the conditions indicated. The amount of immunoprecipitated IE fragment was measured. Values represent mean  $\pm$  SD of duplicates. Data from one experiment represent two independent experiments with similar results.