Temporal and Spatial Changes of Histone 3 K4 Dimethylation at the IFN-γ Gene during Th1 and Th2 Cell Differentiation[1]

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Covalent modification of nucleosomal histones is an important mechanism for cytokine gene regulation in Th1 and Th2 cells. In this study, we analyzed the kinetics of histone H3 K4 dimethylation (H3K4me2) of the IFN-γ gene. Minimal levels of H3K4me2 were found in naive CD4 T cells. After 5 days of differentiation, H3K4me2 levels were elevated in both Th1 and Th2 cells at the −5.3 kb, the promoter, the intronic DNase I hypersensitive sites, and 3′ distal sites including the +9.5 kb and +16 kb sites. Th1 cells maintained high levels of H3K4me2 after longer time of culture. However, in Th2 cells after 14 days, high levels of H3K4me2 were detected only at the −5.3 kb and the promoter, whereas H3K4me2 was lost at the 3′ distal sites and greatly diminished at the DNase I hypersensitive sites. After 28 days, Th2 cells lose H3K4me2 at all sites. Unlike the long-term primary Th2 cells, the Th2 clone D10 showed strong H3K4me2 at the IFN-γ gene with distinctly high levels at the 3′ distal sites. CD4 T cells transgenic for Hlx or infected with T-bet-expressing retrovirus produced IFN-γ and retained high levels of H3K4me2 even after differentiated under Th2 polarizing conditions, suggesting positive roles of these two factors in maintaining high levels of H3K4me2 at the IFN-γ gene. ***The Journal of Immunology,* 2007, 179: 6410–6415.**

Interferon-γ is an immune regulatory cytokine that plays critical roles in both innate and adaptive immune responses (1). Its expression in CD4 T cells is subject to multiple layers of regulation. The transcription factors T-bet and Hlx activate IFN-γ expression in Th1 cells (2–4), whereas GATA-3 suppresses IFN-γ expression in Th2 cells (5–7). *cis*-regulatory elements also contribute to IFN-γ gene regulation. Both positive and negative regulatory elements exist in the promoter proximal region (8, 9). In more distal locations, enhancer elements are found in the conserved noncoding sequences (CNS)[3] (10–14). Some of the CNS have also been shown to have boundary function (11). In 3C analyses, dynamic and distinct interactions between different DNA segments of the IFN-γ locus were found in differentiating Th1 and Th2 cells (15). These findings indicate that distal elements can come in contact with the promoter proximal sequences to form a "transcriptional hub" that acts as the control center of gene expression.

Apart from subset-specific transcription factors, the expression profiles of the IFN-γ gene are "memorized" through chromatin remodeling of the IFN-γ locus. The IFN-γ locus adopts an active chromatin configuration during Th1 cell differentiation and conversely a repressive configuration during Th2 cell differentiation as judged by DNase I hypersensitivities (DHS) (16). To a large degree, the underlying biochemical events in chromatin remodeling are chemical modifications of nucleosomal histones (17). Nucleosomal histones of active genes are hyperacetylated, as well as methylated at specific lysine residues, most notably the lysine 4 of histone H3, whereas inactive genes typically lack such modifications (17).

After a prolonged period of culture, the IFN-γ locus in Th2 cells eventually relocates to the pericentromeric heterochromatin region (18). This suggests that chromatin remodeling at the IFN-γ locus continues after Th2 cells have ceased to express the IFN-γ gene, which ultimately leads to heterochromatin formation. Previous works have documented several types of histone modifications at the IFN-γ gene during Th1 and Th2 cell differentiation (11, 12, 19–26). Importantly, the study by Schoenborn et al. (11) identified elevated levels of the active modification histone H3 lysine 4 (H3K4) methylation at CNS sites distributed over 100 kb of the IFN-γ locus in Th1 cells. In contrast, Th2 cells acquired the repressive modification histone H3 lysine 27 (H3K27) methylation at the IFN-γ locus (11, 25). Despite these comprehensive studies, extended kinetics of histone modifications at the regulatory sites of the IFN-γ gene, particularly in the post differentiation phase, has not been analyzed. Therefore, potentially important differences of histone modifications at the IFN-γ locus between Th1 and Th2 cells may have been overlooked. In this report, we show that the active histone modification, H3K4 dimethylation (H3K4me2) is elevated at critical sites of the IFN-γ gene in both Th1 and Th2 cells at an early time point. However, this modification is gradually lost from 3′ to 5′ in Th2 cells. We further demonstrated that the Th1-specific transcription factors T-bet and Hlx contributed to the maintenance of the high levels of H3K4me2 at the IFN-γ gene. These findings are important for understanding the mechanism of establishing and/or maintaining epigenetic modifications of the IFN-γ gene in Th1 and/or Th2 cells.

Materials and Methods

Animals and cell culture

Inbred mice were purchased from National Cancer Institute. DO11. $10C\alpha^{-/-}$ mice were bred at the University of Rochester animal facility.

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[3] Abbreviations used in this paper: CNS, conserved noncoding sequences; DHS, DNase I hypersensitivities; H3K4, histone H3 lysine 4; H3K27, histone H3 lysine 27; ChIP, chromatin immunoprecipitation.

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Table I. *IFN-γ PCR primers used in this study*

Name of the Amplicon	5′-Primer Sequence-3′
IFN-γ −5.3 kb	Forward GAACGGGTGGAGGAGGTAACA
	Reverse GCCAATAGTGAGCCTGCAGAA
IFN-γ Pro	Forward GCAAAGTAACTTAGCTCCCCCCA
	Reverse CCAGAAACAGCCATGAGGAAGAG
IFN-γ DHS I	Forward CTGGTCCCCAGTCATTTTGAGA
	Reverse AACATTAAGAACTTTGCCTCCCA
IFN-γ DHS II	Forward AGAAGTAAGTGGAAGGGCCCAGA
	Reverse GATTTTCATGTCACCATCCTTGG
IFN-γ DHS III	Forward GTGTTAGTGGAAAGAGCAGTGGG
	Reverse TGGACTGCATAGCACTAGAGGCTA
IFN-γ +9500	Forward CAAATGAAAATGTTGTCTATGTGGAA
	Reverse TCCCTTTTTATACTGTAATTCTCTTTTCAC
IFN-γ +12500	Forward GATATCTGACATGCAACTCCTGAGA
	Reverse AGAATCTTTCAGGGTAAGGCACAA
IFN-γ +14000	Forward TAGTGCGACCAGAATCTGAATTTG
	Reverse ATGTTCTGGAGATAAGACACAGAGTGA
IFN-γ +15000	Forward CTCACCAGAGGCAAAGCTTCA
	Reverse ATGAGATACTTTTCCAACTCCAATTTAAAC
IFN-γ +16000	Forward TGAACTTGGAGCTTGCCATTT
	Reverse TGTATGGAGTGCCCTCAATGAA

Hlx transgenic mice were described before (4). All animal work was conducted in compliance with regulations stipulated by the University of Rochester Committee for Animal Research. Procedures for naive $CD4^+$ T cell, APC preparation, Th1 and Th2 cell differentiation and retroviral infection of T cells were described previously (4). The T-bet retroviral vector T-bet-RV was a gift from Dr. L. Glimcher and Dr. S. Szabo. T cells from C57BL/6 were stimulated with 2.5 μg/ml ConA (Boehringer Mannheim), T cells from DO11.10$C\alpha^{-/-}$ mice were stimulated with $OVA_{323–39}$ peptide (0.5 μg/ml). For a 14-day culture, cells were restimulated at day 7. For long-term cultures of Th1 or Th2 cells of the C57BL/6 origin, naive CD4 cells (5×10^4/ml) were stimulated with irradiated (10,000 rad) alloantigenic TA3 cells (5×10^4/ml) in a 96-well tissue culture plate in RPMI 1640 medium containing 10% FBS, 50 μM 2-ME and IL-2 (1 ng/ml). For Th1 differentiation, the medium was supplemented with IL-12 (1 ng/ml), and for Th2 differentiation with IL-4 (2 ng/ml) as previously described (27). Culture of D10 (D10.G4.1) and AE7 cells was described before (28). IL-4 and IFN-γ expression in CD4 T cells was detected by intracellular cytokine staining or ELISPOT as described (4, 27, 29).

Chromatin immunoprecipitation (ChIP)

ChIP was performed essentially as described in the Upstate Biotechnology protocol with minor modifications. After sonification, chromatin solution was cleared by centrifuge. To recover more soluble chromatin, the pellet was resuspended in 30 ul of 3M L-proline (Fisher Scientific). The soluble fractions were combined, diluted with nine volumes of the immunoprecipitation buffer. Typically chromatins of ~$1–2 \times 10^6$ cells were used for each experiment. Before immunoprecipitation, the sample was precleared with 30 μl of salmon sperm DNA blocked Protein A agarose beads (Upstate Biotechnology) for 3 h. The chromatins were immunoprecipitated with α-H3K4me2 Abs (07–030) or normal rabbit IgG (Upstate Biotechnology, Lake Placid, NY). An aliquot of the chromatin was saved as input control. The amounts of input chromatins were quantified by PCR using the input chromatin DNA serially diluted at 1, 1/10, 1/100 as templates. The immunocomplexes were pulled downed with Protein G agarose beads. After reversing crosslinking and proteinase K digestion, the chromatin DNA was recovered by Phenol:chloroform extraction and ethanol precipitation.

PCR detection of ChIP DNA

PCR primers from different locations of the mouse IFN-γ gene were designed using Primer Express software (Applied Biosystems). The sequences of the primers are shown in Table I. The primers for the IL-4 promoter were described before (19). The primers were purchased from Integrated DNA Technologies. The chromatin DNA was amplified by PCR using the following conditions: 94°C, 3 min followed by 35 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. More than three independent experiments were performed for each sample. For quantification, the DNA samples of the chromatins precipitated with anti-H3K4me2 Abs or normal rabbit IgG (mock) were amplified by PCR. The gel pictures were taken using the VersaDoc Imaging System (BioRad), and the Quantity One software was used to detect specific DNA bands on agarose gel and measure the band intensities. The specific amount of amplification of an anti-H3K4me2 Ab precipitated chromatin DNA sample was determined as (Band Intensity of Specific Ab-Band Intensity of Mock)/Band Intensity of Input. We arbitrarily define one unit of the H3K4me2 as the specific amount of amplification of the IFN-γ promoter region using the DNA template of the chromatins precipitated with the anti-H3K4me2 Abs from normal naive CD4 T cells.

Results

H3K4me2 modification at the promoter and intronic DHS sites of the IFN-γ gene

We analyzed a 20 kb genomic region of the IFN-γ locus (Fig. 1*A*) for H3K4me2, which is an epigenetic mark of active genes that is believed to be associated with regulatory DNA sequences (30, 31). Because many regulatory signals converge at the gene promoter, we first chose to analyze the H3K4me2 status of the IFN-γ gene promoter in Th1 and Th2 cells. In our in vitro Th1 and Th2 differentiation cultures, CD4 T cells acquired their characteristic cytokine expression profiles after 4–5 days of culture (Fig. 1*B*) (28, 32). Therefore, the chromatin fragments of Th1 and Th2 cells of the B6 origin after 5 days of differentiation were immunoprecipitated with anti-H3K4me2 Abs. The IFN-γ promoter in the immunoprecipitated chromatin DNA was detected by PCR using primers derived from the previously characterized IFN-γ promoter (33). Unexpectedly, H3K4me2 was detected at the IFN-γ promoter in both Th1 and Th2 cells (Fig. 1*C*, left). We then analyzed H3K4me2 modification at the intronic DHS sites (DHS I, II, and III). The DHS II exists in naive CD4 T cells, whereas DHSI and DHSIII are induced during Th1 differentiation (16). The H3K4me2 was detected at all three DHS sites again in both Th1 and Th2 cells (Fig. 1*C*, right).

H3K4me2 in early Th1 and Th2 cells was elevated compared with naive CD4 T cells

One possible explanation for the similar levels of H3K4me2 in the day 5 Th1 and Th2 cells is that this modification pre-exists in the naive CD4 T cells. We therefore analyzed the H3K4me2 levels at the IFN-γ gene in the naive CD4 T cells and compared them with those in the day 5 Th1 and Th2 cells. In addition to the promoter and the intronic DHS sites, we included the −5.3 kb region that was recently described as an enhancer (12) and additional sites distal to the 3′ end of the IFN-γ gene. The additional 3′ distal sites

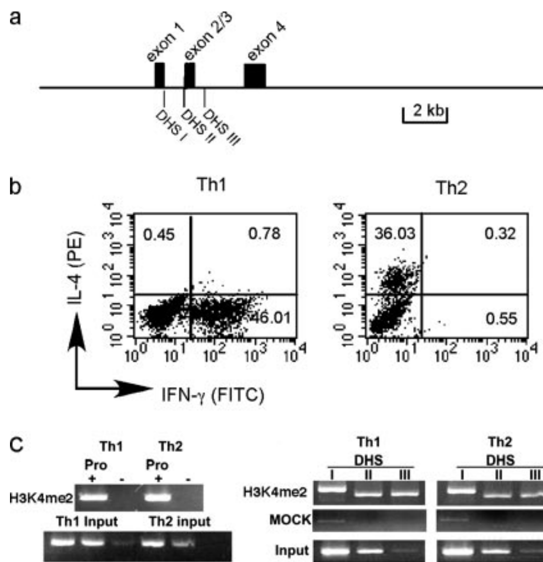

FIGURE 1. H3K4me2 modification at the IFN-γ gene in Th1 and Th2 cells after 5 days of differentiation. *a*, Genomic region analyzed in this study, showing the structure of the IFN-γ gene. DHS, DNase I hypersensitive site. *b*, Cytokine staining of in vitro differentiated Th1 and Th2 cells. Th1 and Th2 cells from C57BL/6 mice differentiated for 5 days under their polarizing conditions were analyzed by intracellular staining of IL-4 and IFN-γ. Dot plots of the gated CD4 T cells are shown. Numbers show the percentages of each cell population. *c*, H3K4me2 modification at the promoter (Pro) and the intronic DHS sites of the IFN-γ gene. Th1 and Th2 cells differentiated for 5 days were used for ChIP analysis. DNA samples from chromatin fragments immunoprecipitated with α-H3K4me2 Ab (+) or normal rabbit IgG (-) (mock) were amplified by PCR with primers derived from the mouse IFN-γ promoter and the intronic DHS sites I-III. Input chromatin DNA was serially diluted at 1, 1/10, 1/100 for semiquantitative PCR using primers derived from the promoter.

were included because a recent 3C conformational study suggested that the 3′ distal region of the IFN-γ gene may contain regulatory sequences (15). The selection of the 3′ distal sites was based on the loosely defined consensus nucleosome positioning sequence with high CpG contents that are potential targets of DNA methylation and gene silencing (34–36).

To quantify the levels of the H3K4me2 modification at each sites, we measured the intensities of the specific DNA bands. After being normalized to the input controls, the band intensities relative to that of the promoter in the naive CD4 T cells are plotted to show the amounts of H3K4me2 modification. As shown in Fig. 2*A*, except for the +9.5 kb site, low levels of H3K4me2 were detected at all sites in the naive CD4 T cells. Compared with the naive CD4 T cells, the H3K4me2 levels were elevated at most sites of the IFN-γ gene in the day 5 Th1 cells (Fig. 2*B*). Similar elevation was observed at the −5.3 kb, promoter, and the intronic DHS sites in the day 5 Th2 cells, whereas the H3K4me2 at the 3′ distal sites was slightly weaker in the Th2 cells than in the Th1 cells (Fig. 2*B*).

Partial Loss of H3K4me2 modification in Th2 cells after 14 days of culture

Although cytokine expression patterns are established after one round of TCR stimulation in the differentiation cultures, the chromatin structure of cytokine genes could undergo further remodeling following subsequent T cell stimulations (18). We therefore examined the H3K4me2 levels at the IFN-γ gene after two rounds of stimulation. As expected, after two rounds or 14 days of culture, the Th1 and Th2 cells exclusively expressed IFN-γ or IL-4, respectively (Fig. 3*A*). In the Th1 cells, high levels of H3K4me2 were maintained at different sites of the IFN-γ gene. In the Th2 cells, strong H3K4me2 modification was detected at the –5.3kb site and the promoter. The H3K4me2 was still detectable at the DHS sites but at significantly reduced levels compared with those in day 5 Th2 cells. In contrast, the H3K4me2 was completely lost at the distal 3′ sites in the day 14 Th2 cells (Fig. 3*B*).

Complete loss of H3K4me2 in Th2 cells after 28 days of culture

The data on the day 14 Th2 cells indicated that the H3K4me2 modification at the IFN-γ gene could be lost in a progressive manner. To test this possibility, we analyzed the H3K4me2 modification after 28 days of culture. For such long-term culture, we used allogenic stimulation to activate the T cells. Thus, B6 naive CD4 T cells were differentiated by allogeneic stimulation under polarizing conditions for 14 days, then restimulated and cultured for additional 2 wk. The Th1 or Th2 phenotype of the differentiated cells was confirmed by ELISPOT analysis of the IL-4 and IFN-γ expression (Fig. 4*A*). Like the day 5 and day 14 Th1 cells, the H3K4me2 modification was detected at the different sites of the IFN-γ gene in the day 28 Th1 cells. In sharp contrast, the H3K4me2 was lost at all sites in the day 28 Th2 cells (Fig. 4*B*). The loss of the H3K4me2 modification in the day 28 Th2 cells was gene specific because the H3K4me2 modification could still be detected at the IL-4 promoter of the same Th2 samples (Fig. 4*C*).

Distinct histone modifications of the IFN-γ locus in D10 cells

In light of the complete loss of the H3K4me2 modification of the IFN-γ gene in the day 28 Th2 cells, we wished to determine whether this was also true for the in vitro maintained Th2 clone

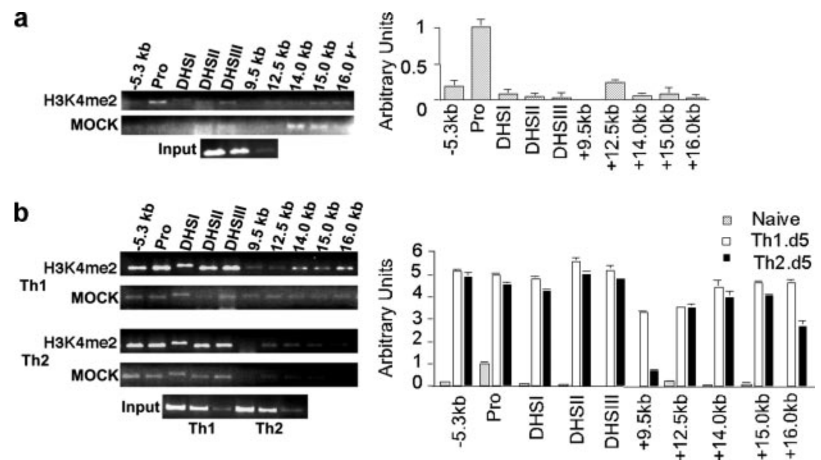

FIGURE 2. Comparison of H3K4me2 modification at the IFN-γ gene between the naive CD4 T cells and the day 5 Th1, Th2 cells. *a*, Fresh isolated naive CD4 T cells ($CD62L^{high}CD44^{low}$) and (*b*) Th1, Th2 cells as in Fig. 1 were used for ChIP analysis. PCR primers were derived from different sites at the IFN-γ gene as indicated. The left panels show a representative of agarose gel pictures of the PCR products. The right panels are quantitative presentations of the PCR amplification at the different sites of the IFN-γ gene as described in the *Materials and Methods*. The amount of amplification at each site is presented as band intensity relative to that of the IFN-γ promoter in the naive CD4 T cells.

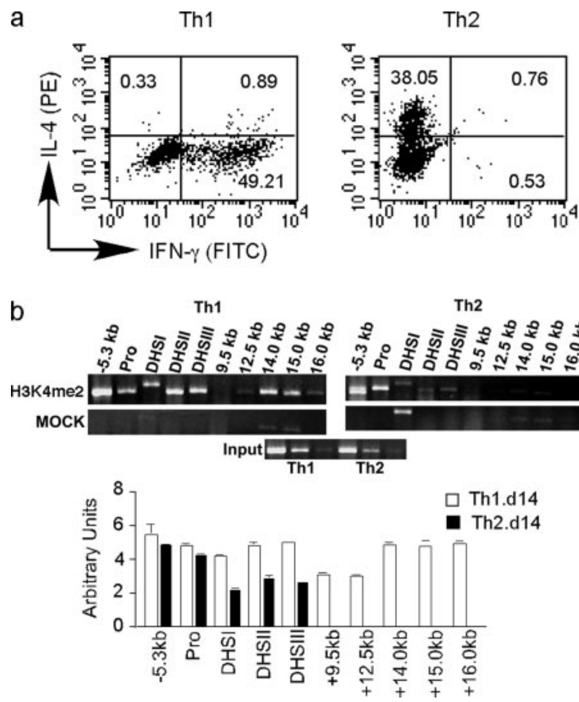

FIGURE 3. H3K4me2 modification at the IFN-γ gene in day 14 primary Th1 and Th2 cells. *a*, Th1 and Th2 cells were cultured for 14 days, and analyzed for IL-4 and IFN-γ expression by intracellular cytokine staining. Dot plots of the gated CD4 T cells are shown. Numbers show the percentages of each cell populations. *b*, On day 14, resting Th1 and Th2 cells were analyzed by ChIP for H3K4me2 modification at the indicated positions of the IFN-γ gene. Upper panels show the representatives of the agarose gel pictures, the lower panel shows the band intensities relative to that of the IFN-γ promoter in the naive CD4 T cells.

D10 (D10.G4.1) that did not express IFN-γ (data not shown). For comparison, we also analyzed the Th1 clone AE7. Although AE7 showed H3K4me2 modification similar to the primary Th1 cells,

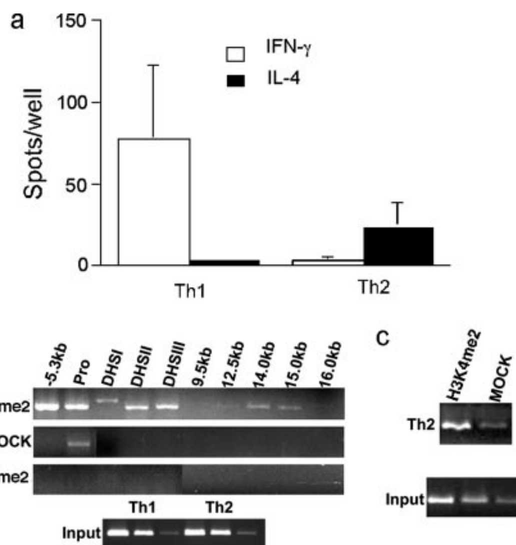

FIGURE 4. H3K4me2 modification at the IFN-γ gene in day 28 primary Th1 and Th2 cells. *a*, C57BL/6 Th1 and Th2 generated by allogeneic stimulation for 28 days were analyzed for IL-4 and IFN-γ expression by ELISPOT. *b*, The same cells were analyzed by ChIP for H3K4me2 modification at the IFN-γ gene. *c*, The immnoprecipitated chromatin DNA of the Th2 cells was also amplified with primers derived from the IL-4 promoter. Representatives of agarose gel pictures of PCR products are shown.

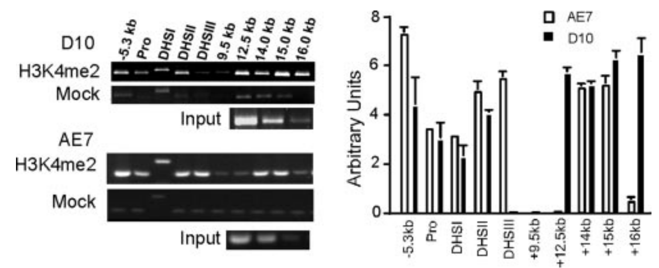

FIGURE 5. H3K4me2 modification at the IFN-γ gene in D10 and AE7 cells. Chromatin fragments of D10 and AE7 cells were analyzed by ChIP. Immunoprecipitated chromatin DNA was amplified by PCR with primers derived from the different sites of the IFN-γ gene. The left panels show representatives of the agarose gel pictures, the right panel shows the band intensities relative to that of the IFN-γ promoter in the naive CD4 T cells.

D10 cells unexpectedly showed strong H3K4me2 modification. In D10 cells, we detected the H3K4me2 modification at −5.3 kb, promoter, the intronic DHS and the 3′ distal sites. The H3K4me2 modification at the 3′ distal sites was particularly strong (Fig. 5). This pattern was different from that of the day 5 primary Th2 cells, where the H3K4me2 levels at the 3′ distal sites were much weaker than those at the 5′ regions (Fig. 3B).

Hlx transgene maintained high levels of H3K4me2 at the IFN-γ gene in CD4 T cells differentiated under Th2 polarizing conditions

During Th1 cell differentiation, the transcription factors Hlx and T-bet are induced, which are the key regulators of IFN-γ expression (2–4, 29). Therefore, it is reasonable to assume that these two transcription factors contribute to the maintenance of the high levels of the H3K4me2 modification at the IFN-γ gene. To investigate the role of Hlx in the maintenance of the H3K4me2 levels, naive CD4 T cells from the CD4-Hlx transgenic mice or wild type littermates were differentiated under Th2-polarizing conditions for 14 days. After the differentiation, CD4 T cells from the CD4-Hlx transgenic mice expressed high levels of IFN-γ whereas the wild type CD4 T cells expressed only IL-4. IFN-γ was expressed alone or simultaneously together with IL-4 in the Hlx transgenic CD4 T cells (Fig. 6*A*). As expected, the CD4 T cells from the wild type littermates had completely lost the H3K4me2 at the 3′ distal sites, and showed diminished H3K4me2 levels at the intronic DHS sites. In contrast, the transgenic CD4 T cells maintained the H3K4me2 at the 3′ distal sites, as well as high levels of the H3K4me2 at the DHS sites (Fig. 6*B*). Thus, the transgenic CD4 T cells showed a pattern of the H3K4me2 similar to that of the Th1 cells.

Retroviral transfer of T-bet induced IFN-γ expression and H3K4me2 in CD4 T cells differentiated under Th2-polarizing conditions

We further investigated the role of T-bet in maintaining the high levels of H3K4me2 at the IFN-γ gene. CD4 T cells from DO11.10C$\alpha^{-/-}$ mice were stimulated for Th2 differentiation and infected with retrovirus bicistronically expressing T-bet and GFP (T-bet-RV) or GFP alone (GFP-RV). The infected cells were sorted after 1 wk and restimulated for another week. As analyzed by intracellular cytokine staining (Fig. 7*A*), Th2 cells infected with the control virus did not express IFN-γ. In contrast, a large number of cells infected by T-bet expressing virus became IFN-γ producers. The cells were then analyzed for H3K4me2 at the IFN-γ gene on day 14. As expected, significant H3K4me2 was only detected at the –5.3kb and the promoter in cells infected with the control virus. However, in cells infected with the T-bet expressing retrovirus

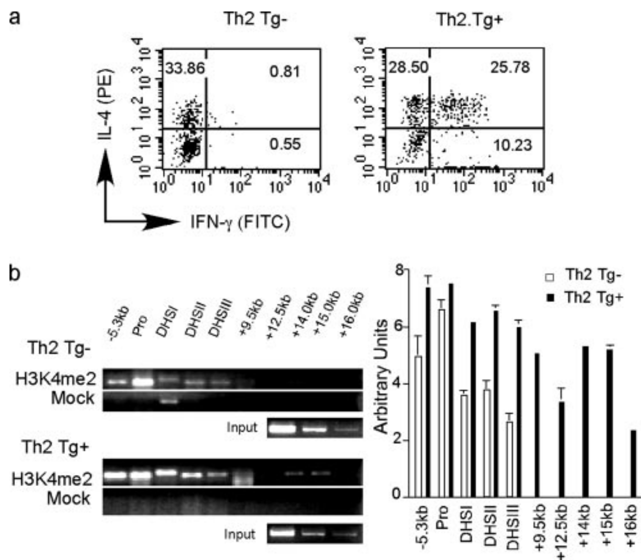

FIGURE 6. Hlx transgene maintained IFN-γ expression and the H3K4me2 modification in CD4 T cells differentiated under Th2 polarizing conditions. *a*, Naive CD4 T cells were isolated from the CD4-Hlx transgenic or the wild type littermates. The cells were stimulated for Th2 differentiation for 14 days, and analyzed for IL-4 and IFN-γ expression by intracellular cytokine staining. Dot plots of the gated CD4 T cells are shown. Numbers in the plots indicate the percentages of each cell populations. *b*, CD4 T cells differentiated under Th2 polarizing conditions were analyzed by ChIP for H3K4me2 modification at the IFN-γ gene. The left panels show representatives of the agarose gel pictures and the right panel shows the band intensities relative to that of the IFN-γ promoter in the wild-type naive CD4 T cells.

high levels of H3K4me2 were detected at most of the sites (Fig. 7*B*), in a pattern similar to those of the Th1 cells and the Hlx transgenic CD4 T cells differentiated under Th2-polarizing conditions.

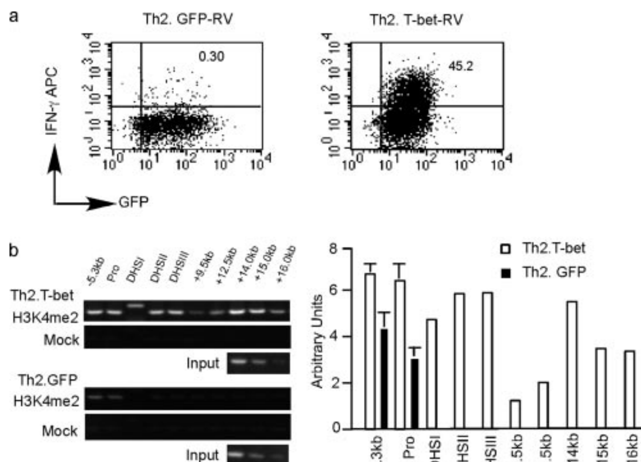

FIGURE 7. Retroviral expression of T-bet in CD4 T cells differentiated under Th2 polarizing conditions induced IFN-γ expression and maintained H3K4me2 modification. (*a*) CD4 T cells from DO11.10C$\alpha^{-/-}$ μιχε ωερε στιμυλατεδ φορ Τη2 διφφερεντιατιον. The cells were infected with either control retrovirus (GFP-RV) or retrovirus bicistronically expressing T-bet and GFP (T-bet-RV). After 1 wk of differentiation, infected cells were sorted and restimulated for an additional 1 wk. The cells were then analyzed for IFN-γ expression by intracellular cytokine staining. Dot plots of gated GFP$^+$ CD4 T cells are shown. Numbers in the plot indicate percentages of IFN-γ positive cell populations. *b*, On day 14, the infected cells were also analyzed by ChIP for H3K4me2 at the IFN-γ gene. Representatives of agarose gel pictures of the PCR products and the band intensities relative to that of the IFN-γ promoter in the naive CD4 T cells are shown.

Discussion

Methylation is a form of thermodynamically stable modification of nucleosomal histones, which could be a definitive mark for stably active or repressed genes. To understand epigenetic regulation of the IFN-γ gene in Th1 and Th2 cells, we have studied H3K4me2, a histone modification of active genes. We found minimal levels of H3K4me2 at the −5.3 kb, the promoter, intronic DHS sites, and 3′ distal sites of the IFN-γ gene in naive CD4 T cells. The levels of this modification were elevated in both Th1 and Th2 cells after a relatively short time of differentiation. It was then maintained at high levels in Th1 cells, but gradually lost in Th2 cells. Several reported studies have examined histone modifications at the IFN-γ gene and the Th2 cytokine gene cluster (12, 20, 24, 37–40). These studies examined histone modifications in Th1 and Th2 cells differentiated over a short period time. Our study is the first to examine the kinetics of the remodeling of histone modifications over a prolonged period of time. Our data demonstrated that histone modification at the IFN-γ gene was a progressive event that continued after the cytokine expression pattern had already been established. Earlier studies have identified H3K4me2 at the IFN-γ gene in day 3 Th1 and Th2 cells (11, 12). Our data with the day 5 Th1 and Th2 cells are consistent with their findings. In addition, we have examined several 3′ distal sites (+9.5, +12.5, +14 and + 15kb) that were not included in the earlier reports, and found significant H3K4me2 at these locations. However, future studies would be required to understand the functional significance of the H3K4me2 for IFN-γ gene expression.

During Th1 cell differentiation, the transcription factor Hlx and T-bet are induced. These two transcription factors control Th1 cell differentiation and IFN-γ gene expression, therefore are most likely to play a role in maintaining the high levels of the H3K4me2 modification at the IFN-γ gene. Our studies provided evidence to support this possibility. When expressed as a transgene in CD4 T cells, Hlx prevented the silencing of the IFN-γ gene in CD4 T cells after they differentiated under Th2-polarizing conditions, which resulted in large numbers of cells producing both IL-4 and IFN-γ as well as cells producing IFN-γ only. Similarly, retroviral expression of T-bet induced IFN-γ expression in CD4 T cells differentiated under Th2-polarizing conditions. Instead of showing loss of H3K4me2, both the Hlx- and T-bet-expressing cells continued to have high levels of H3K4me2 at the IFN-γ gene after 14 days of differentiation under Th2 condition. These results demonstrated that high levels of H3K4me2 correlated with the active state of the IFN-γ gene and argued for a positive role of Hlx and T-bet in maintaining H3K4me2 levels at the IFN-γ gene in Th1 cells.

In Th2 cells, substantial H3K4me2 at the IFN-γ gene was found after 5 days of differentiation. This finding was somewhat unexpected but consistent with the study by Shnyreva et al. (12). Furthermore, in many other recent studies that identified active histone modifications, for example, in the cluster of the globin genes (41), the mating type locus in yeast (42), the *Hoxd4* locus (43), active histone modifications do not always correlate with gene expression. Therefore, we believe that for the IFN-γ gene, the lack of H3K4me2 modification is not an essential condition for silencing its expression in the day 5 Th2 cells. Two recent studies have examined H3K27 methylation at the IFN-γ locus (11, 25). H3K27 methylation at the IFN-γ locus could occur as early as 2 days of Th2 cell differentiation. Therefore, in the Th2 cells differentiated for 2–5 days, H3K27 methylation and the recruitment of Polycomb proteins may play a more important role in silencing the IFN-γ gene. After a longer time of culture, Th2 cells lose H3K4me2 at the IFN-γ gene progressively in a direction from 3′ to 5′. By day 28, the IFN-γ gene in Th2 cells became devoid of H3K4me2 at all sites examined. The functional implication of the loss of H3K4me2

remains to be addressed. One may speculate that the gradual loss of H3K4me2 could contribute to perpetuating the repressed state of the IFN-γ gene in the long-term Th2 cells.

Unlike the long-term primary Th2 cells, the in vitro maintained, semi-immortalized Th2 clone D10 showed strong H3K4me2 at the IFN-γ gene. Compared with the Th1 cells as well as the day 5 Th2 cells, D10 cells showed a distinct pattern of H3K4me2 modification. In the Th1 and the day 5 Th2 cells, strong modifications were positioned at the 5′ and the transcribed region, which may help correctly steer the transcription machinery to drive IFN-γ gene expression. In contrast, in D10 cells strong active modifications were found at the 3′ distal region. This feature was also observed in a macrophage cell line that does not express IFN-γ (data not shown). Given the clear difference between D10 cells and the long-term primary Th2 cells, D10 cells, though widely used to study cytokine gene regulation in Th2 cells, may not be substituted for primary Th2 cells in studies of histone modifications at the IFN-γ gene.

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Disclosures

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