CD28 Costimulation Controls Histone Hyperacetylation of the Interleukin 5 Gene Locus in Developing Th2 Cells*

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* The abbreviations used are: TCR, T cell receptor; Th cells, helper T cells; IFN, interferon; STAT, signal transducers and activators of transcription; IL, interleukin; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; CNS1, conserved noncoding sequence 1; ChiP, chromatin immunoprecipitation; GFP, green fluorescent protein; EGFP, enhanced GFP; RT, reverse transcription; IRES, internal ribosome entry site; CREB, cAMP-response element-binding protein.

Interleukin 5 (IL-5) plays a unique role in allergic inflammatory responses, and the understanding of molecular mechanisms underlying the generation of IL-5-producing cells is crucial for the regulation of allergic disorders. Differentiation of naive CD4 T cells into type-2 helper (Th2) cells is accompanied by chromatin remodeling including hyperacetylation of histones H3 and H4 in the nucleosomes associated with the IL-4, IL-13, and IL-5 genes. Histone hyperacetylation of the IL-5 gene displayed a delayed kinetics compared with that of the IL-4 and IL-13 genes, suggesting a distinct remodeling mechanism for the IL-5 gene locus. Here we studied the role of CD28 costimulation in the generation of IL-5-producing cells and the histone hyperacetylation of the IL-5 gene locus. CD28-costimulation selectively enhanced histone hyperacetylation of the IL-5 gene locus that appeared to be mediated through NF-κB activation and subsequent up-regulation of GATA3. The CD28 costimulation-sensitive histone hyperacetylation spanned almost the entire intergenic region between the IL-5 and RAD50 accompanied with intergenic transcript. Thus, this is the first demonstration that CD28 costimulation controls a chromatin-remodeling process during Th2 cell differentiation.

Upon antigen recognition by T cell receptor (TCR), naive CD4 T cells differentiate into two distinct helper T (Th) cell subsets, Th1 and Th2 cells (1). Th1 cells produce IFNγ and tumor necrosis factor-β and initiate cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and are involved in humoral immunity and allergic responses. The cytokine environment is crucial in controlling the direction of Th cell differentiation (2, 3). For Th1 cell differentiation, IL-12-mediated activation of signal transducer and activator of transcription (STAT) 4 is required, whereas IL-4-mediated STAT6 activation is important for Th2 cell generation (4–6). In addition, TCR stimulation events upon encounter with antigens are also indispensable for both Th1 and Th2 cell differentiation. We reported that efficient TCR-mediated activation of the p366–205, calcineurin, and Ras-extracellular signal-regulated kinase mitogen-activated protein kinase signaling cascade is crucial for Th2 cell differentiation (7–9). Recent studies have identified several transcription factors that control Th1/Th2 cell differentiation (10). Among them, GATA3 appears to be a master transcription factor for Th2 cell differentiation. GATA3 is selectively induced in developing Th2 cells, and the ectopic expression of GATA3 induced Th2 cell differentiation even in the absence of IL-4 or STAT6 (11–14). For Th1 cell differentiation, T-bet was recently identified as a key transcription factor (15).

CD28 costimulation enhances Th2 responses significantly (16, 17). Upon anti-CD28 mAb stimulation, phosphatidylinositol 3-kinase is recruited to CD28 and activated, and then subsequent activation of NF-κB is induced (18–21). It has been reported that GATA3 induction was an outcome of the CD28-induced NF-κB activation in T cells (22, 23). This may be a mechanism by which Th2 responses were enhanced by CD28 costimulation. It is also known that IL-5 production and IL-5-dependent airway inflammation are dependent on NF-κB family members (24–26).

Chromatin remodeling of the Th2 cytokine gene loci (IL-4/IL-5/IL-13) occurs during Th2 cell differentiation (27). A highly conserved 400-bp noncoding sequence 1 (CNS1) was identified, and an important role in coordinate expression of Th2 cytokines was revealed (28, 29). More recently, a 3′ distal IL-4 enhancer (V4L) containing an inducible DNase I hypersensitive site was identified (30). Reiner and co-workers (31) report that demethylation of the intron 2 region of the IL-4 gene was associated with cell cycle progression and Th2 cell differentiation (31). We reported that demethylation of this region is regulated by polycomb group genes (32) that are known to
Hyperacetylation of histone H3 and H4 by histone acetyltransferases was suggested to be associated with active chromatin (33). Recently, we and others have demonstrated that histone hyperacetylation of the Th2 cytokine gene loci occurs in developing Th2 cells in a Th2-specific and STAT6-dependent manner (34–36). We demonstrated an essential role for GATA3 in the Th2-specific hyperacetylation (34). We also generated a precise map of the Th2-specific histone hyperacetylation within the IL-13 and IL-4 gene loci and identified a 71-bp conserved GATA3 response element 1.6 kilobase pairs upstream of the IL-13 locus exon 1. This histone hyperacetylation remodeling process could be a major target for the Th2 master transcription factor GATA3 to induce differentiation toward Th2 cells.

Histone hyperacetylation of another Th2 cytokine gene locus, IL-5, occurs in a Th2-specific STAT6- and GATA3-dependent manner with significantly different kinetics compared with that of the IL-4 and IL-13 genes (34). The direction of transcription of the IL-5 gene is opposite to that of IL-13 and IL-4. In addition, the RAD50 gene encoding a DNA repair enzyme is located between the IL-5 and IL-13 gene loci. A differential role for GATA3 in the regulation of promoter activity of the IL-5 gene from IL-4 has been suggested (37–39). These results encourage us to explore possible novel molecular mechanisms that would govern histone hyperacetylation of the IL-5 gene locus.

In the present study we investigated histone hyperacetylation of the IL-5 gene locus in developing Th2 cells cultured with or without CD28 costimulation. A long range CD28-sensitive Th2-specific histone hyperacetylation was detected in the IL-5 and the intergenic region of the IL-5 and RAD50 gene loci. The hyperacetylation was accompanied by CD28-sensitive intergenic transcripts and required high expression of GATA3. A molecular mechanism that governs Th2-specific histone hyperacetylation of the IL-5 gene associated nucleosomes will be discussed.

MATERIALS AND METHODS

Mice—C57BL/6 mice were purchased from SLC (Shizuoka, Japan). STAT6-deficient (KO) mice were kindly provided by Shizuo Akira (Osaka University, Osaka, Japan) (40). All mice used in this study were maintained under specific-pathogen-free conditions and were about 4 weeks old. Animal care was in accordance with the guidelines of Chiba University.

Immunofluorescent Staining and Flow Cytometry Analysis—In general, one million cells were stained with appropriate specific antibodies according to a standard method (41, 42). For intracellular staining, fluorescein isothiocyanate-conjugated anti-IFN-γ antibody (XMG1.2; Pharmering), phosphatidylethanolamine-conjugated anti-IL-4 antibody (1B11; Pharmingen), and allopurinol-conjugated anti-IL-5 antibody (TRFK5; Pharmingen) were used. The detection intracellular IL-13, biotinylated polyclonal anti-IL-13 (R&D Systems), and phosphatidylethanolamine-conjugated avidin were used. Flow cytometry analysis was performed on a FACScalibur (BD Biosciences), and results were analyzed by CELLQUEST software (BD Biosciences).

Cell Cultures and in Vitro T Cell Differentiation—IL-5-producing cells, stimulation with anti-TCR and anti-CD28 mAbs transferred to new dishes and cultured for another 5 days in the presence of immobilized anti-TCR mAb, soluble anti-CD28 mAb, and the cytokines present in the initial culture. To enhance the generation of IL-5-producing cells, stimulation with anti-TCR and anti-CD28 mAbs was performed during the second culture for 5 days. This procedure is slightly different from that used in our previous report (16). Where indicated, wortmannin (Calbiochem) was added to the culture at the doses of 30 or 300 nm for the first 5 days. In vitro differentiation was then assessed by intracellular cytokine staining with anti-IL-4, anti-IL-13, and anti-IFN-γ or by ELISA as described (42).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed using human H3 CHIP assay kits (17-245: Upstate Biotechnology) as described (34). Anti-GATA3 Ab (H-8, Santa Cruz Biotechnology) was used for precipitation. Where indicated, GFP-negative retrovirus-infected cells were sorted by flow cytometry and subjected to ChIP assay. Several primer pairs for ChIP assay were described previously (34, 42). The primer pairs newly generated are as follows: IL-5 1-F, 5′-CACAGTTGTTGGAATATTCTCATT-3′; IL-5 1-R, 5′-GGGTAAACCTGAGTCATCTCG-3′; IL-5 2-F, 5′-GGTTAGGAGACCCCATCTACCA-3′; IL-5 2-R, 5′-GGAAGGCCTGACATTCA-3′; IL-5 3-F, 5′-GGTTTGCTGTCATGACGCCCGCTC-3′; IL-5 3-R, 5′-GCAGGGAGCTTGAGACCTAG-3′; IL-5 4-F, 5′-GCCCTTGGGAGTTCGATCC-3′; IL-5 4-R, 5′-GCCTCAGAGGACCCCATCAAGTATA-3′.

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CACTGAGCTGCCTGGCGCCGT. A detailed protocol for detection of intergenic transcripts was described previously (34). The primers used are the same as those used in ChIP assay.

RESULTS

Costimulation with Anti-CD28 mAb Enhances the Generation of IL-5- and IL-13-producing Cells—The aim of this study was to clarify the molecular mechanisms that control chromatin remodeling of the IL-5 gene locus during Th2 cell differentiation. We first assessed the role for CD28 costimulation in the generation of IL-5-producing Th2 cells. Freshly prepared CD4 T cells from young adult (4 weeks) B6 mice were cultured in vitro with immobilized anti-TCR in the presence of agonistic anti-CD28 mAbs (37.51) to effect stimulation. The IL-5/IL-4 profiles of CD4 T cells cultured under Th1- or Th2-skewed conditions are depicted in Fig. 1A. As can be seen, the generation of IL-5-producing cells (both IL-5/H11001 and IL-5/H11002 fractions) cultured under conditions described above, and restimulation was done with anti-TCR mAb for 8 h. The amounts of IL-5, IL-13, IL-4, and IFNγ in the culture supernatant were measured by ELISA. WT, wild type.

Concurrently, the amount of cytokines produced by developing Th2 cells cultured with CD28 costimulation was assessed by ELISA (Fig. 1, D and E). As expected, CD28 costimulation significantly enhanced the production of IL-5 and IL-13, whereas the effects on the production of IL-4 and IFNγ were marginal. The production of Th2 cytokines (IL-5, IL-13, and IL-4) was all STAT6-dependent regardless of the presence or absence of CD28 costimulation (Fig. 1E). Taken together these results suggest that the generation of IL-5- and IL-13-producing cells was Th2-specific, STAT6-dependent, and more sensitive to CD28 costimulation as compared with that of IL-4-producing cells.

Dynamics of Histone H3 Hyperacetylation of the IL-5 Gene Locus in Developing Th2 Cells Cultured with CD28 Costimulation—To clarify whether CD28 costimulation enhances histone hyperacetylation of the IL-5 gene locus during Th2 cell differentiation, we first examined the kinetics of acetylation of the IL-5 promoter, IL-4 promoter, and RAD50 promoter regions using a ChIP assay with anti-acetylhistone H3 Ab. Developing Th1 and Th2 cells cultured with CD28 costimulation were harvested 2, 3.5, 5, and 7 days after stimulation. The results of this analysis are presented as the relative band intensity of each group normalized with band intensity of the corresponding input DNA as shown in the right panel of Fig. 2. Two days after stimulation, low but significant levels of increase in acetylation occurred at all of the regions tested, and basically no difference between the three culture conditions was noted. However, as shown in Fig. 2A, the levels of histone acetylation associated with the IL-5 promoter increased significantly after cultivation for more than 3 days under Th2-skewed conditions, particularly in those cultures with CD28 costimulation. In contrast, the levels of histone hyperacetylation in the IL-4 promoter were increased day by day, and no significant difference was detected in the presence or absence of CD28 costimulation. The levels of histone hyperacetylation in the RAD50 gene promoter region were increased equivalently under these three culture conditions. These results suggest that histone hyperacetylation of the IL-5 gene locus is more sensitive to CD28...
costimulation than that of the IL-4 locus.

Enhanced Histone Hyperacetylation Induced with CD28 Costimulation—Next, we examined the acetylation status of the DNA regions corresponding to IL-5 promoter, IL-5 intron, IL-13 intron, IL-4 promoter, CNS1, IL-4 V_A enhancer, RAD50 promoter, and IFNγ promoter. CNS1 and IL-4 V_A enhancer regions were previously described to contain regulatory elements or DNase I hypersensitive sites (28, 30). Histone hyperacetylation induced in these regions, except for two controls (RAD50 and IFNγ), occurred in a Th2-specific manner as reported previously (34). As can be seen in Fig. 3A, the levels of acetylation in the region of IL-5 promoter and IL-5 intron were significantly enhanced by the presence of CD28 costimulation. In contrast, those of other regions were all unaffected. The effect of CD28 costimulation on the histone hyperacetylation within the IL-5 gene loci was analyzed more precisely with a series of primer pairs within the IL-5 genes as shown in Fig. 3B. CD28 costimulation significantly enhanced the levels of histone hyperacetylation at all regions within the IL-5 genes tested (Fig. 3C).

Next, we assessed the STAT6 dependence of the CD28 costimulation-induced enhancement of the acetylation (Fig. 3D). Similar to other regions (IL-13 intron, IL-4 promoter,
CNS1, and IL-4 V_A enhancer), histone hyperacetylation of the IL-5 promoter locus was not induced in STAT6-KO T cell cultures even in the presence of CD28 costimulation. Thus, STAT6 is critical for the Th2-specific histone hyperacetylation of the IL-5 gene locus induced by anti-TCR stimulation and CD28 costimulation.

Enhanced Production of IL-5 and Histone Hyperacetylation of the IL-5 Gene Locus Induced by CD28 Costimulation Are Dependent on NF-κB Activation—CD28 costimulation is known to induce phosphatidylinositol 3-kinase activation and the subsequent activation of NF-κB. We tested the effect of wortmannin, a phosphatidylinositol 3-kinase inhibitor, on the CD28-induced enhancement of the generation of IL-5-producing cells (43). The generation of IL-5-producing cells in the culture with CD28 costimulation was decreased in the presence of wortmannin in a dose-dependent manner (Fig. 4A). In contrast, the generation of IL-4-producing cells was not affected at any doses of wortmannin tested. Concurrently, the effect of wortmannin on the production of cytokines was assessed by ELISA (Fig. 4B), and as expected, the enhanced production of IL-5 with CD28 costimulation was sensitive to wortmannin. IL-13 production was also slightly decreased; however, the levels of IL-4 were not changed by the presence of 30 to 300 nM wortmannin.

Next, we assessed the role for NF-κB activation in the CD28-induced enhancement of the generation of IL-5-producing cells and histone hyperacetylation of the IL-5 gene locus. A mutant form of IκBα (IκBαM), which inhibits the NF-κB activation (44), was inserted in an IRES-GFP retroviral construct, and the

![Fig. 3. Hyperacetylation of histone H3 of the IL-5, IL-13, and IL-4 gene loci in developing Th2 cells cultured with CD28 costimulation.](image-url)

A, developing Th1 and Th2 cells cultured with CD28 costimulation were prepared and subjected to ChIP assays with the indicated primer pairs. B, schematic representation of the IL-5 gene locus and the IL-13 and IL-4 gene loci is shown with the location of the specific primers used in panel A and C. C, a ChIP assay with the indicated primer pairs within the IL-5 gene locus was done as in panel A. Three independent experiments were performed with similar results. D, a ChIP assay with STAT6-KO-developing Th2 cells was done as in panel A. Two independent experiments were performed with similar results.
Enhanced production of IL-5 and histone hyperacetylation of the IL-5 gene locus induced by CD28 costimulation were dependent on NF-κB activation. A, effect of wortmannin on the generation of IL-5-producing cells under Th2-skewed culture conditions with CD28 costimulation. CD4 T cells were cultured under the conditions described in Fig. 1 in the presence of the indicated doses of wortmannin. The intracellular IL-5/IL-4 profiles are shown. The numbers represent the percentages of the cells present in each quadrant. B, cytokine production profiles of the cells prepared under the same condition as described in panel A are shown. C, freshly prepared splenic CD4 T cells were stimulated with anti-TCR and anti-CD28 and infected with a retrovirus pMX-IRES-GFP (GFP) or pMX-IκBα-M-IRES-GFP encoding a mutant form of IκBα (IκBαM). Seven days after infection GFP+ cells were sorted and subjected to immunoblotting with an anti-IκBα Ab that reacts with both wild type IκBα and IκBαM. The position of IκBαM is indicated by an arrowhead. Also non-infected Th2 cells (Th2) were included. Tubulin was used as a loading control. D, freshly prepared splenic CD4 T cells were stimulated under the indicated conditions and infected with a retrovirus encoding a mutant form of IκBα (IκBαM) with EGFP. Seven days after infection, the cultured cells were restimulated, and intracellular IL-5/IL-4 profiles of electronically gated GFP positive populations were determined. The percentages of cells present in the each quadrant are shown. E, effect of ectopic expression of IκBαM on histone H3 hyperacetylation in IL-5, IL-4, and RAD5 gene loci. Retrovirus-infected CD4 T cells were prepared as described in panel C. One million GFP+ infected cells were then collected by cell sorting and the acetylation status of histone H3 was determined by ChIP assay. Relative band intensities measured by densitometry are shown in the right. At least three independent experiments were done in each (A–D) with similar results.
Fig. 5. The generation of IL-5-producing cells and histone hyperacetylation of the IL-5 gene locus were highly dependent on the expression levels of GATA3. A, freshly prepared CD4 T cells were cultured for 16 and 32 h under the conditions indicated, and the protein expression levels of GATA3 and tubulin were determined by immunoblotting with specific mAbs. The lysates from 3 × 10^6 (upper for GATA3) and 0.3 × 10^6 (lower for tubulin) cells were loaded per lane. The results are representative of three independent experiments. Arterial densitometric units are depicted under each band. B, the effect of wortmannin on the induction of GATA3 was assessed. The experiments as in panel A were done in the presence of wortmannin (300 ng/ml). C, freshly prepared CD4 T cells were cultured for 12 h under the conditions indicated, and total RNA was prepared. The transcription levels of GATA3, exon 1b of GATA3, and β-actin were determined by semiquantitative RT-PCR analysis with 3-fold serial dilution of template cDNA. Shown are the PCR product bands. Arbitrary densitometric units are indicated. Three independent experiments were done with similar results. D, freshly prepared CD4 T cells were stimulated under Th1-skewed conditions and were infected on day 2 with retrovirus encoding GATA3 bicistronically with EGFP (pMx-GATA3-IRES-GFP). The expression levels of GATA3 in the indicated populations sorted using GFP fluorescence were assessed by immunoblotting with anti-GATA3 Ab. Non-infected developing Th1 and Th2 cells were also included for comparison. Arterial densitometric units are indicated. E, three days after the infection as in D, the cells were restimulated, and intracellular IL-5/IL-4 and IFN-γ/IL-4 profiles of electronically gated GFP− (gate G1), GFP^{low} (gate G2), and GFP^{high} (gate G3) populations were determined. The percentages of cells present in the each quadrant are shown. F, the cells present in the G1, G2, and G3 gate prepared as in panel D were sorted on day 5 by a cell sorter, and the acetylation status of histone H3 was determined by ChIP assay. G, relative band intensities (Ac-H3/input DNA) of each group in panel E are shown. The results are representative of three independent experiments.

vectors were introduced into developing Th2 cells cultured with CD28 costimulation. The expression of the introduced IκBαM was confirmed by immunoblotting with an anti-IκBα Ab that reacts with both wild type IκBα and IκBαM (Fig. 4C). Substantial amounts of endogenous IκBα were detected in the GFP^{+} population of mock pMX-IRES-GFP-infected cells (GFP) and non-infected Th2 cells (Th2). Also, substantial amounts of IκBαM were detected in the IκBαM-infected GFP^{+} population (IκBαM). As previously reported, the upper band, indicated by an arrowhead, is IκBαM (45). The amount of endogenous IκBα in the IκBαM-infected cells was found to be reduced, probably as a result of the failure of NF-κB activation (46). The percentages of IL-5- and IL-4-producing cells in the GFP-positive infected cell population were determined (Fig. 4D). As can be seen, the numbers of IL-5-producing cells were decreased (12.4 ± 23.4 to 6.8 ± 6.1%) by the expression of IκBαM. Interestingly, the percentages of IL-4-producing cells were not significantly affected by IκBαM expression (10.6 ± 12.4 versus 20.5 ± 6.8%). The acetylation status of the IL-5 promoter, IL-4 promoter, and RAD50 promoter regions was assessed in the developing Th2 cells infected with IκBαM vector, and significant down-regulation of hyperacetylation in the IL-5-related nucleosomes was detected (Fig. 4E). Again, the introduction of IκBαM did not inhibit the acetylation levels of the IL-4- and RAD50-related nucleosomes, suggesting that NF-κB activation is preferentially involved in the process of hyperacetylation of the IL-5 gene locus.

The Generation of IL-5-producing Cells and Histone Hyperacetylation of the IL-5 Gene Locus Are Highly Dependent on the Expression Levels of GATA3—It is reported that the inhibition of NF-κB activity results in reduced GATA3 expression and Th2 cytokine production in developing but not committed Th2 cells (23). To examine the possible involvement of GATA3 in the CD28-induced enhancement of histone hyperacetylation of the IL-5 gene locus, we assessed the protein expression levels of GATA3 in developing Th2 cells cultured with CD28 costimulation. The GATA3 levels were clearly increased by the presence of CD28 costimulation at the 16- and 32-h time points (Fig. 5A). The increase was abrogated by the presence of wortmannin (Fig. 5B). Furthermore, the transcriptional levels of GATA3 as assessed by semiquantitative RT-PCR were significantly higher in the Th2 cell culture with CD28 costimulation (Fig. 5C). We also examined the transcriptional expression of GATA3 exon 1a and 1b (47). Although the expression of exon 1a transcript was undetectable in these developing Th2 cells, that of exon 1b was moderately enhanced in the presence of CD28 costimulation.

To examine the correlation between GATA3 expression and histone hyperacetylation of the IL-5 gene locus, we introduced
GATA3 into CD4 T cells stimulated under Th1-skewed conditions using a retroviral vector (pMX-IRES-EGFP) encoding GATA3 bicistronically with EGFP (pMX-GATA3-IRES-EGFP). The expression of GFP and GATA3 protein in the GATA3-infected T cells is depicted in Fig. 5. The expression levels of GATA3 in GFPhigh (expressing high levels of GATA3, G3) population were ~2-fold as compared with those of GFPlow (expressing low levels of GATA3, G2) population and equivalent to those in Th2 cells. Next, the levels of IL-5- and IL-4-producing cells were compared between GFP- (no GATA3 expression, G1) and GFP+ (expressing high levels of GATA3, G3) populations. The results are representative of three independent experiments.

Fig. 6. Long range histone hyperacetylation and intergenic transcripts in the intergenic region of the IL-5 and RAD50 loci in developing Th2 cells with CD28 costimulation. A and B, splenic CD4 T cells were stimulated under the indicated conditions for 7 days, and a ChIP assay was performed. Shown are the PCR product bands for each primer pair (A) and the relative band intensities (B). The results are representative of three independent experiments. The location of GRE-IL-5 is indicated in panel B, kb, kilobase. WT, wild type. C and D, freshly prepared CD4 T cells from B6 and STAT6-KO mice were stimulated under the indicated conditions for 2 days and total RNA was prepared. RNA samples were treated with RNase free DNase I to eliminate any possible genomic DNA contamination, reverse-transcribed (RT+), and then subjected to PCR with the indicated primer pairs. RT- represents PCR without reverse transcription. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The numbers of the primer pairs are the same as those used in panel A. The intensity of bands of the highest concentration was measured, and relative intensities to the β-actin bands are shown in panel D. The results are representative of three independent experiments. E and F, the GATA3 introduced cells as in Fig. 5D were sorted (GFP+, gate G1; GFPlow, gate G2; and GFPhigh, gate G3), and subjected to ChIP assay with indicated primer pairs. The relative intensity (Ac-H3/Input DNA) of each band is shown in panel F. The results are representative of two independent experiments.
The generation of IL-5-producing cells was greatly increased in the fraction of high GATA3-expressing cells (G3) compared with that of low GATA3-expressing cells (G2) (24.5 ± 18.2 versus 8.2 ± 3.8%). The difference in the percentage of IL-5-producing cells was about 4-fold. In contrast, slight (−25%) increases in the generation of IL-4-producing cells were detected (G3, 28.7 ± 24.5%, versus G2, 34.6 ± 8.2%). As for the IFNγ-producing cells, a GATA3 dosage-dependent decrease was observed (Fig. 5E, right). No significant IFNγ/IL-5 double-producing cells were detected (data not shown).

To assess the acetylation status of histones in the GATA3-introduced developing T cells, GFP, GFPlow, and GFPhigh cells prepared as above were purified by cell sorting and subjected to ChIP assay. Histone hyperacetylation of the Th2 cytokine loci (IL-5 promoter, IL-5 intron, IL-13 intron, IL-4 promoter, CNS1, IL-4 Vα enhancer) was significantly higher in GATA3-expressing cells (G2 and G3) compared with GATA3 non-expressing cells (G1) (Fig. 5F). The levels of histone hyperacetylation were increased concomitantly with the increase in the expression of GATA3 (compare G2 and G3) in the IL-5 gene locus (IL-5 promoter and IL-5 intron). No such increase was detected in the IL-4- and IL-13-related nucleosomes. These results suggest that the generation of IL-5-producing cells and histone hyperacetylation of the IL-5 gene locus are highly dependent on the expression levels of GATA3.

Long Range Th2-specific Hyperacetylation Detected in the Intergenic Region of the IL-5 and RAD50 Gene Loci Is Enhanced by the Presence of CD28 Costimulation—A series of primer pairs between the IL-5 and RAD50 loci were generated, and the acetylation status of the nucleosomes associated with IL-5 and RAD50 loci was analyzed. The actual band patterns of each ChIP assay (Fig. 6A) and the relative band intensities (Ac-H3/Input DNA) of the 14 selected primer pairs (Fig. 6B) are depicted. A long range Th2-specific hyperacetylation was observed from 400 bp upstream of the RAD50 exon 1 (corresponding to primer 5) to the end of IL-5 exon 4 (primer 14). The acetylation levels of all regions tested were significantly increased in the presence of CD28 costimulation. These results indicate that almost all histones from 400 bp upstream of the RAD50 exon 1 to the end of IL-5 exon 4 (primer 14) are selectively hyperacetylated under Th2-skewed culture conditions and are sensitive to CD28 costimulation.

Intergenic Transcription Is Detected throughout the Intergenic Region between the IL-5 and RAD50 Gene Loci—We demonstrated that the intergenic transcription throughout the IL-4 and IL-13 gene loci was accompanied by histone hyperacetylation (34). Thus, we examined the transcription of the intergenic region between the IL-5 and RAD50 gene loci. Interestingly, considerable amounts of transcripts were detected throughout the intergenic region, and the levels were significantly enhanced in the presence of CD28 costimulation (Fig. 6, C and D). In addition, we examined whether the intergenic transcripts were STAT6-dependent or not. STAT6-deficient CD4 T cells were used in paral-
CD28-mediated Enhancement of Hyperacetylation of the IL-5 Gene

lel. Only base-line levels of intergenic transcripts were detected. These results suggest that intergenic transcripts are induced throughout the intergenic region between the IL-5 and RAD50 gene loci in a Th2-specific and STAT6-dependent manner and are sensitive to CD28 costimulation.

CD28 Costimulation-sensitive Hyperacetylation in the Intergenic Region of the IL-5 and RAD50 Gene Loci Is Dependent on the Levels of GATA3 Expression—Next, we examined the correlation between the levels of GATA3 expression and histone hyperacetylation of the intergenic region. The retrovirus-induced GATA3-expressing cells shown in Fig. 5D were used to compare the acetylation status between GFP-/H11002 and GFP+/H9260 expressing cells (G1), GFPlow (expressing low levels of GATA3, G2), and GFPhigh (expressing high levels of GATA3, G3) populations (Fig. 6E). The relative intensity (Ac-H3/input DNA) of each acetylation band of GATA3-introduced cells is shown in Fig. 6F. As expected, the levels of acetylation in the high GATA3-expressing cells (G3) were significantly higher than those of low and no GATA3-expressing cells (G2 and G1, respectively), suggesting that histone hyperacetylation of the intergenic region requires a high level expression of GATA3.

**DISCUSSION**

In this report we demonstrated that CD28 costimulation controls Th2-specific histone hyperacetylation of the IL-5 gene locus. CD28-mediated activation of NF-κB and the resulting enhancement of GATA3 induction appeared to be a mechanism by which histone hyperacetylation of the IL-5 gene locus was efficiently induced. This regulation was IL-5 gene-specific because the effect of CD28 costimulation was not observed in the acetylation of the IL-13 or IL-4 gene loci. A long range CD28-sensitive histone hyperacetylation with transcripts was detected in the IL-5 and intergenic region between the IL-5 and RAD50 gene.

The generation of IL-5- and IL-13-producing cells and the production of these cytokines were enhanced by CD28 costimulation of the differentiation culture (Fig. 1). A similar conclusion was drawn from the experiments with wortmannin (Fig. 4, A and B). As for histone hyperacetylation, however, CD28 costimulation affected only the IL-5 gene locus (Fig. 2 and 3). The transcription of IL-5 and IL-13 is known to be highly dependent on GATA3 as compared with that of IL-4 (11, 48, 49). An efficient transcription of IL-5 or IL-13 may require the dependence on the levels of GATA3. Although the reason for the difference is not clear at this time, the nature of putative GATA response elements responsible for the IL-5 gene acetylation could be distinct from that of conserved GATA3 response element (34). There is 60% homology in the DNA sequence around the upstream region of human RAD50 gene compared with mouse, but we did not identify any conserved GATA binding motifs. However, there are several GATA binding motifs present in both mouse and human, suggesting a possible targeting of GATA3 to this region.

Hyperacetylation of the histone H3 (K9/14) and H4 (K5/8/12/16) is associated with transcriptionally active chromatin (33). However, acetylation of the histone H3-K9/14 does not always correlate with histone H4 acetylation (56). Furthermore, methylation of histone H3-K4 appears to be correlated with active chromatin (57). In the study we focused on the acetylation status of histone H3-K9/14. Thus, further analysis of histone H4 and histone H3-K4 methylation will be required to provide a more detailed view of the chromatin remodeling of the IL-5 gene locus.

In conclusion, we have demonstrated a possible molecular mechanism that controls histone hyperacetylation of the IL-5 gene loci. Characteristic features of chromatin remodeling of the IL-5 gene locus as compared with those of IL-13 and IL-4 were revealed to be the differential involvement of CD28 costimulation and sensitivity to the levels of GATA3 protein. This study is the first to provide evidence that CD28 costimulation controls chromatin remodeling during Th2 cell differentiation.

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CD28 Costimulation Controls Histone Hyperacetylation of the Interleukin 5 Gene Locus in Developing Th2 Cells
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