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Epigenetic Regulation of Beta2-Adrenergic Receptor Expression in T_H1 and T_H2 Cells

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Abstract

We showed previously that murine naive CD4⁺ T cells and T_H1 cell clones express the beta2-adrenergic receptor (β₂AR), while T_H2 cell clones do not. We report here that naive CD4⁺ T cells that differentiated for 1-5 days under T_H1 driving conditions increased β₂AR gene expression, while cells cultured under T_H2 driving conditions decrease β₂AR gene expression. Chromatin immunoprecipitation revealed that the increase in β₂AR gene expression in T_H1 cells is mediated by an increase in histone 3 (H3) and H4 acetylation, as well as an increase in histone 3 lysine 4 (H3K4) methylation. Conversely, the decrease in β₂AR gene expression in T_H2 cells is mediated by a decrease in H3 and H4 acetylation and a decrease in H3K4 methylation, as well as an increase H3K9 and H3K27 methylation. The histone changes could be detected as early as 3 days of differentiating conditions. Genomic bisulfite sequencing showed that the level of methylated CpG dinucleotides within the promoter of the β₂AR gene was increased in T_H2 cells as compared to naive and T_H1 cells. Collectively, these results suggest that epigenetic mechanisms mediate maintenance and repression, respectively, of the β₂AR gene expression in T_H1- and T_H2-driven

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Authorship Contributions

J.W.M. contributed data and manuscript preparation; L.T.S. contributed data and scientific expertise; R.S.E contributed technical assistance; D.J. contributed statistical analyses; N.M.P. contributed samples for analysis; V.M.S contributed funding, scientific expertise, and manuscript preparation.

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cells, providing a potential mechanism by which the level of β_2 AR expression might be modulated pharmacologically within immune cells and other cell types in which the expression profile may change during a disease process.

Keywords

Beta2-Adrenergic Receptor; CD4; Epigenetics; T_H1 ; T_H2 ; Histone modifications

Introduction

T_H1 cells produce interferon (IFN)- γ to promote effective immune responses that protect the host against the spread of intracellular pathogens, while T_H2 cells produce interleukin (IL)-4 to promote effective immune responses that protect the host against the spread of extracellular pathogens (Abbas et al., 1996). The development of an appropriate effector T cell response is, therefore, critical for the maintenance of homeostasis. The cellular activity of naive and effector $CD4^+$ T cells is regulated primarily by cytokines and costimulatory molecules, as well as by neurotransmitters and hormones (Steinman, 2004). The neurotransmitter norepinephrine is stored in sympathetic nerve terminals that reside within the parenchyma of lymphoid tissue (Kohm et al., 2000) and are in close association with $CD4^+$ T cells (Felten et al., 1998; Felten and Olschowka, 1987). Norepinephrine is released from these nerve terminals after antigen enters the system and binds to the beta2-adrenergic receptor (β_2 AR) that is expressed on immune cells. Engagement of the β_2 AR on an immune cell regulates cellular activity in a cAMP- and PKA-dependent manner (Kohm and Sanders, 2001). Although it was thought that the β_2 AR was expressed on all $CD4^+$ T cells, we now know that $CD4^+$ T cell naive and T_H1 cells express the β_2 AR, while the T_H2 cells do not, as assessed by radioligand binding, immunofluorescence analysis, agonist-induced intracellular cAMP accumulation, and/or reverse transcription-PCR (Ramer-Quinn et al., 1997; Sanders et al., 1997; Swanson et al., 2001). Upon $CD4^+$ T cell activation in the presence of a β_2 AR agonist, the level of IL-2 and IFN- γ production by naive $CD4^+$ T cells and T_H1 cells changed when compared to activated, but unexposed cells, while the level of IL-4 produced by T_H2 cells remained the same (Ramer-Quinn et al., 1997; Sanders et al., 1997; Swanson et al., 2001), supporting the pharmacologic and molecular data that a functional β_2 AR was expressed on naive and T_H1 cells, but not T_H2 cells. Thus, the collective findings suggest that an association may exist between the development of T_H1 and T_H2 cells and their ability to maintain or repress expression of the β_2 AR gene.

Gene expression is regulated primarily by transcription factors that bind to the gene promoter region. Chromatin remodeling, which results from covalent modifications of histones and DNA, influences eukaryotic gene activity by affecting the accessibility of the chromatin to transcription factors and RNA polymerases. Mechanisms involved in chromatin remodeling within the promoter regions of DNA include methylation of CpG residues and histone acetylation, methylation, phosphorylation, ribosylation, and ubiquitination (Eberharter and Becker, 2002; Jaenisch and Bird, 2003; Mutskov and Felsenfeld, 2004). Specifically, panacetylation of histone 3 (H3) and histone 4 (H4) as well as methylation of either lysine 4, 36, or 79 in H3 (H3K4, H3K36, and H3K79) is associated with transcriptionally active genes, while a lack of acetylation or methylation of lysine 9, 20, or 27 (H3K9, H3K20, H3K27) is associated with transcriptionally repressed genes (Margueron et al., 2005). Such epigenetic mechanisms have been reported in post-developmental $CD4^+$ T cells and appear to regulate the level of gene expression for CD4 and CD8 (Bilic et al., 2006; Taniuchi et al., 2002), TCR alleles (Bergman and Cedar, 2004), IFN- γ (Avni et al., 2002; Chang and Aune, 2007; Eivazova and Aune, 2004; Fields et al., 2002), and IL-4 (Agarwal and Rao, 1998; Avni et al., 2002; Chang and Aune, 2007; Fields

et al., 2002; Lee et al., 2002; Makar et al., 2003). Thus, chromatin remodeling plays an intricate role in regulating the expression of genes in each effector CD4⁺ T cell subset as it develops from a naive CD4⁺ T cell.

We proposed that chromatin remodeling may also be involved with the maintenance and repression of β_2 AR gene expression in developing T_H1 and T_H2 cells, respectively. In the present study, we show for the first time that β_2 AR gene transcription was differentially regulated in effector CD4⁺ T cells that were derived from a naive CD4⁺ T cells activated with either anti-CD3/anti-CD28 or a specific antigen in the presence of IL-12 or IL-4 to promote Th1 and Th2 development, respectively. Moreover, in comparison to naive CD4⁺ T cells, a lack of histone acetylation, an increase in the level of H3K9 and H3K27 dimethylation, and the methylation of CpG dinucleotides within the proximal promoter region of the β_2 AR gene occurred specifically in T_H2-driven cells, while pan-H3/H4 acetylation and H3K4 dimethylation increased in T_H1-driven cells. Thus, our findings show that epigenetic changes such as histone acetylation and methylation along with DNA methylation maintain expression of the β_2 AR on T_H1 cells, but repress expression on T_H2 cells.

Methods

Animals

Female pathogen-free Balb/c mice were obtained from Taconic Farms (Germantown, NY), housed in the American Association Accreditation of Laboratory Animal Care (AAALAC)-accredited Animal Research facility at The Ohio State University (Columbus, OH). Mice were housed in microisolator cages and provided autoclaved food and water ad libitum and used between 6-12 weeks of age. All experiments complied with the Animal Welfare Act and the National Institutes of Health (Bethesda, MD) guidelines for the care and use of animals in biomedical research.

T Cell Clones

The specificity and source of all T cell clones have been described previously (Sanders et al., 1997). T_H1 cell clones included AR100.9, D1.1, and HDK-1. Th2 cell clones included CDC35, LNT-1, and LNT-4. Viable cells were obtained before use by centrifugation over Lympholyte-M (Cedarlane Laboratories, Ltd. Ontario, Canada) 8-14 days after antigen activation. Clones were maintained in IL-2-containing medium and were used at least 3 days after initial IL-2 exposure. All clones tested negative for the presence of Mycoplasma contamination (Invitrogen, Carlsbad, California). For activated samples, 1×10^7 cells of each T cell clone were stimulated for 8 hrs on plates coated with 5 μ g/ml hamster anti-mouse CD3 (clone 145-2C11, kindly provided by Dr. William Lee, Wadsworth Institute, Albany, NY) prior to RNA and/or DNA isolation.

Isolation of CD4⁺ T cells

Naive CD4⁺ T cells were isolated from the spleens of Balb/c mice as described previously (Kohm et al., 2000) with minor changes. Red blood cells were removed with ammonium chloride treatment for 4 min at 37°C and then washed with Hanks Buffered Salt Solution. Naive CD4⁺ T cells were isolated using the CD4⁺ CD62L⁺ T Cell Isolation Kit sorted on an AutoMacs machine following the manufacturer's directions (Miltenyi Biotec). Briefly, following red blood cell lysis, cells were incubated with an antibody cocktail designed to magnetic-bead label non-CD4⁺ cells. The cells were sorted using the AutoMacs machine and the negative fraction was retained for further incubation with anti-CD62L-coated magnetic beads. The cells were sorted again on the AutoMacs machine and the positive

fraction was retained and the purity of CD4⁺CD62L⁺ T cells was verified by Flow Cytometry (>95%). Approximately 10×10⁶-10×10⁷ cells were obtained for each experiment.

Enrichment of IL-4⁺ or IFN-γ⁺ Cells

Single-cell suspensions of in vitro-differentiated T cells (approximately 50×10⁶) were harvested following the completion of a week of activation. The cells were stimulated for 4hrs with phorbol 12-myristate 13-acetate (PMA, 50mg/ml) and Ionomycin (500ng/ml) (both from Sigma, St. Louis, MO) in the presence of Brefeldin A (1μg/ml) (BD Biosciences, San Jose, CA). Cells were then washed in PBS containing 0.1% BSA and 0.02% sodium azide then fixed and permeabilized using the Cytofix/Cytoperm solution (BD Biosciences) according to manufacturers directions. The cells were then resuspended in 1X Perm/Wash buffer (BD Biosciences) and incubated with either rat anti-mouse IL-4 (clone BVD6-24G2) or rat anti-mouse IFN-γ (clone XMG1.2) (both from eBiosciences, San Diego, CA) for 30 min at 4°C. Following the incubation, the cells were washed twice with 1X Perm/Wash buffer and resuspended in PBS containing 0.1% BSA. The top 10% (MFI) IL-4⁺ or IFN-γ⁺ cell populations were sorted using a BD FACSria flow cytometer for analysis.

T_H1/T_H2 Culture Conditions

Naive CD4⁺ T cells were cultured on plates coated with 5μg/ml hamster anti-mouse CD3 (clone 145-2C11) and 1μg/ml hamster anti-mouse CD28 (clone 37.51.3F3) in complete RPMI. To promote T_H1 differentiation, cells were activated in the presence of 20-150 U/ml of human recombinant IL-2 (BRB Preclinical Repository, National Cancer Institute), 5ng/ml mouse recombinant IL-12 (eBioscience, San Diego, CA), and 10μg/ml rat anti-mouse IL-4 (clone 11B.11, BRB Preclinical Repository, National Cancer Institute). To promote T_H2 differentiation, cells were cultured in the presence of 20-150 U/ml human recombinant IL-2, 10μg/ml mouse recombinant IL-4 (eBioscience), and 5μg/ml rat anti-mouse IFN-γ (clone XGM1.2, eBioscience). For generation experiments, cells were stimulated every 5 days under the same conditions.

Chromatin Immunoprecipitation (ChIP)

ChIP analysis was carried out as described previously (Podojil et al., 2004). Briefly, naive, activated, or sorted cells were collected and fixed with formaldehyde solution. Cells were then lysed in a series of lysis buffers and nuclei were collected and the suspension was sonicated using a Branson sonifier 250 (Emerson, Danbury, CT) to lyse the nuclear membranes and shear the DNA. The collected chromatin was precleared with Protein A/G agarose beads (Oncogene), the beads were removed, and the chromatin was incubated at 4°C overnight with the following antibodies/conditions: rabbit anti-mouse dimethylated H3K9, rabbit anti-mouse dimethylated H3K4, rabbit anti-mouse monomethylated H3K27, rabbit anti-mouse-acetyl-H3, rabbit anti-mouse-acetyl-H4 (all from Millipore), rabbit isotype control (Cell Signaling, 2729S), or no antibody. Immune complexes were precipitated with Protein A/G agarose beads. An input control sample was obtained from the supernatant of either a no antibody or isotype control sample. The samples were adjusted to 0.5% SDS, 100μg/ml RNase A, and 200μg/ml Proteinase K and then incubated at 55°C for 3 hrs followed by an overnight incubation at 65°C to reverse the formaldehyde crosslinks. The DNA was precipitated using phenol-chloroform and then amplified using the Platinum Taq PCRx Enhancer system (Invitrogen) and the following primer set: β₂AR-3P (sense) 5'-GCAGCCCCAGATTTCTCTCT-3' and β₂AR-4P (antisense) 5'-GACTCCTGGAAGCTTCATTCA-3' to amplify the promoter region -248 to 105.

Bisulfite Genomic Sequencing

The DNA from 2.5×10^6 resting T_H1 or T_H2 clone cells and primary cells were collected using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturers' instructions. One μ g of DNA was subjected to bisulfite modification using the CpGenome DNA Modification Kit (Intergen, Purchase, NY) according to the manufacturers' instructions as published previously (Smith et al., 2006). PCR was performed on 100ng of bisulfite-treated DNA with one of the following primer pairs: Distal: β_2 ARp-b7 (sense) 5'-GGAGATAGTTGTTRTTTTAGAAATGAG-3' and β_2 ARp-b8 (antisense) 5'-ACCCTAACTAAAACACCAAAAAAAA-3' (299bp product); Proximal: β_2 ARp-b9 (sense) 5'-TGTTTTAGTTAGGGTAGTTGGAAGG-3' and β_2 ARp-b10 (antisense) 5'-AACTATACTAAAATACRTCCTACACA-3' (315bp product). The PCR products were then cloned and transformed using the TOPO-TA Cloning Kit for Sequencing (Invitrogen) according to the manufacturers' instructions. DNA samples were then sequenced using an ABI sequencer with Big Dye Terminators (Applied Biosystems). For Wild-type untreated DNA sequencing, the primer sets used were as follows: β_2 ARp-1P (sense) 5'-CTTATGTGTGTTTCTTTCTCCTG-3' and β_2 ARp-2P (antisense) 5'-GCACCAGAGAGAGAAATCTGG-3' (positions -581 to -222); β_2 ARp-3P (sense) 5'-GCAGCCCCAGATTTCTCTCT-3' and β_2 ARp-4P (antisense) 5'-GACTCCTGGAAGCTTCATTCA-3' (positions -248 to 105).

Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously (Podojil and Sanders, 2003). Briefly, a common master mix [LightCycler-FastStartDNA SYBR Green I (Roche, Mannheim, Germany), 2mM MgCl₂, 0.5 μ M gene-specific primer] was used and the concentration of gene-specific cDNA was quantified using a standard curve diluted 1:10 for a concentration range of 1ng/ml to 1fg/ml. A melting curve was generated after each real-time reaction and samples were run on a 1.0% agarose gel to ensure that only one gene-specific PCR product was generated. Real-time PCR was performed using the Roto-gene 2000 Real-time Cycler (Phenix Research Products; Hayward, CA). The following primers were used: β -actin 5'-TACAGCTTCACCACCACAGC-3' and 5'-AAGGAAGGCTGGAAAAGAGC-3' (annealing temperature, 60°C, 128-bp product); β_2 AR 5'-TGTTACACCGAGGAGACTTGCTGT-3' and 5'-TGAGGTTTTGGGCGTGGAATC-3' (annealing temperature, 58°C); IL-4 5'-CGAAGAACACCACAGAGAGTGAGCT-3' and 5'-GACTCATTCATGGTGCAGCTTATCG-3' (annealing temperature, 58°C, 180-bp product); and IFN- γ 5'-TTAGCCAAGACTGTGATTGCGG-3' and 5'-GAGCGAGTTATTGTTCATTCGGG-3' (annealing temperature, 56°C, 323-bp product).

Statistics

Data were analyzed by ANOVA to determine whether an overall statistically significant change existed. Certain *p* values were calculated using either a Bonferroni post hoc test for comparison of more than two treatment groups or a Students *t* test for comparison between two treatment groups. Statistically significant results were determined by a *p* value of ≤ 0.05 .

To determine whether the two different cell conditions showed different DNA methylation rates, we determined the number of sequence locations that methylation occurred for each cell sample. By using this count as the primary outcome, we assumed a Poisson distribution for the counts. We did one analysis for all the data across the passages of cells (1-6 weeks). The poisson regression model estimated with effect for the T_H1 *versus* T_H2 conditions and the effects over weeks of reactivation as well. We also tested an over dispersion parameter to determine whether Poisson assumption was providing too small an error variance. For simplification of interpretation, we also modeled the dichotomy of whether or not methylation occurred at any site using logistic regression. In these models, the treatment

condition effects, the passage effects, and the interaction between the two were estimated. This interaction effect estimated the trend in the difference between the two cell conditions as a linear function of the passages. We expected an increasing difference between the two conditions from 1-6 passages. Models were estimated separately for proximal and distal regions. All significance tests were double-sided with $\alpha=0.05$. Analyses such as t-tests or repeated measures models were used for other comparisons. Statistically significant results were determined by a p value of ≤ 0.05 .

Results

Differential β_2 AR mRNA expression

Our laboratory reported that the β_2 AR is differentially expressed by murine T_H1 and T_H2 clones (Ramer-Quinn et al., 1997; Sanders et al., 1997), and that the β_2 AR transcript is detectable in naive $CD4^+ CD62L^+$ splenic T (T_HN) cells (Swanson et al., 2001). To determine if β_2 AR gene expression was also transcriptionally regulated when murine T_H1 and T_H2 cells were newly derived from freshly isolated T_HN cells cultured in vitro for 1-5 days under either T_H1 or T_H2 promoting conditions using immobilized anti-CD3 and soluble anti-CD28. To confirm that the culture conditions were indeed driving the cells to differentiate to either T_H1 or T_H2 type cells, we measured IFN- γ and IL-4 mRNA, respectively. The data show that primary T_HN cells cultured under T_H1 -promoting conditions increased and maintained the level of β_2 AR mRNA over 5 days of culture when compared to naive cells (Fig. 1A), which was associated with increased IFN- γ mRNA production (Fig. 1B). In contrast, cells cultured under T_H2 -promoting conditions initially expressed an increased level of β_2 AR mRNA that decreased during the subsequent days of culture as IL-4 mRNA production increased (Fig. 1A, C).

To determine if more differentiated T_H2 cells, as determined by the level of intracellular IL-4 expression, expressed lower levels of β_2 AR mRNA, cells cultured under T_H2 -promoting conditions were collected and sorted for high IL-4 expression and low IL-4 expression. Total mRNA was collected from the sorted populations and analyzed for β_2 AR mRNA expression. The data show that high IL-4-expressing T_H2 -driven cells transcribed less β_2 AR mRNA compared to low IL-4-expressing cells (Fig. 2A).

To determine if differential β_2 AR expression occurred under physiological T_H1 - or T_H2 -promoting conditions, we measured the level of β_2 AR mRNA expression in T_H1 and T_H2 cell lines that were generated to afford protection against a specific tumor antigen in vivo. In vivo clearance of a reticulum cell sarcoma (RCSX) tumor is dependent on the ability of $CD4^+$ T cells to interact and recruit leukocytes that participate in tumor clearance (Simmons et al., 2005). The IFN- γ -producing T_H1 cell lines, C2 and A312, and the IL-4-producing T_H2 cell line WB4 were selected for their ability to recognize γ -irradiated lymphoma cells in vitro, as well as their ability to recognize RCSX tumor cells in vivo (Simmons et al., 2005). Following re-activation of the cell lines in vitro, total mRNA was isolated and analyzed for the level of cytokine and β_2 AR mRNA expression using quantitative real time PCR (Fig. 2B). A higher level of β_2 AR mRNA expression was detected in IFN- γ -producing C2 and A312 cells as compared to the very low level detected in IL-4-producing WB4 cells.

Histone modifications in the β_2 AR promoter

Histone modification is a well-studied mechanism of gene regulation. Acetylation of histones 3 and 4 occurs on many residues and is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Strahl and Allis, 2000). Most data suggest that acetylation of H3 and H4 plays a fundamental role in regulating gene transcription while unacetylated DNA is silenced (Kornberg and Lorch, 1999; Struhl, 1998). Acetylation of

specific lysine residues varies from gene to gene and throughout cell development, thus we examined the pan-acetylation status of H3 and H4. The level of total H3 or H4 acetylation in T_H1 and T_H2 cultures was examined in cells after one week in culture was variable (data not shown). However, cells from either T_H1- or T_H2-promoting conditions sorted for IFN- γ or IL-4 protein expression, respectively, revealed a distinct level of pan-H3 and H4 acetylation when compared to T_HN cells (Fig. 3). T_H2 cells, which do not express the β_2 AR, had low levels of H3 acetylation (Fig. 3A) and no detectable levels of H4 acetylation (Fig. 3B) when compared to T_H1 cells, which do express the β_2 AR. Interestingly, T_HN cells expressed low levels of H3 acetylation and relatively high levels of H4 acetylation. These findings suggest that histones in the β_2 AR promoter of the T_HN and T_H1 cells are acetylated, which opens the β_2 AR gene to transcription, while the T_H2 cells have very little H3 or H4 acetylation, which has been associated with a lack of transcription.

More specifically, histone methylation is a very early mechanism by which cytokine expression is regulated in differentiating T_H1 and T_H2 cells (Chang and Aune, 2007). Surprisingly, some of these changes can be detected within hours to days after initial CD4⁺ T cell exposure to T_H1- or T_H2-promoting conditions, which was previously described in the promoters for *Ifng* and *Il4* (Chang and Aune, 2007). To determine if histone methylation changes were occurring within the β_2 AR promoter region, we used ChIP to examine the methylation level of H3K4, which is associated with open chromatin, and H3K9 and H3K27, which are associated with closed chromatin. Because the peak changes in the cytokine genes occurred at 3 days of culture in T_H1- or T_H2-promoting conditions, we examined histone changes in the β_2 AR promoter on day 3. Uncultured T_HN cells contained a low level of both H3K4 and H3K9 methylation within the β_2 AR promoter (Fig. 4A, B). CD4⁺ T cells cultured under T_H1-promoting conditions showed enriched H3K4 methylation (Fig. 4A) while cells cultured under T_H2-promoting conditions showed enriched H3K9 methylation in the β_2 AR promoter region after 3 days of culture (Fig. 4B). Methylation of H3K27 was only detected in cells cultured under T_H2-promoting conditions (Fig. 4B). These findings suggest that methylation of the histones may be an early mechanism by which expression or repression of the β_2 AR gene is mediated.

Methylation of CpG Dinucleotides in the β_2 AR promoter

Along with histone modifications, DNA methylation of CpG dinucleotides is a more permanent mechanism by which the activity of a specific promoter is regulated (Hmadcha et al., 1999; Holliday and Pugh, 1975; Kang et al., 1999). To determine if differential methylation of CpG dinucleotides within the β_2 AR promoter occurred in T_H1 and T_H2 cells, 3 experimental approaches were used, namely methylation-sensitive restriction endonuclease (MSRE) digestion, methyl-sensitive PCR (MSP) of the β_2 AR promoter, and bisulfite genomic sequencing. Preliminary results using MSRE and MSP indicated that methylation of CpG dinucleotides within both the proximal (location -181 to 56) and distal (-481 to -196) regions of the promoter the β_2 AR promoter occurred in T_H2, but not T_H1 cells (data not shown). To confirm the MSRE and MSP data that indicated DNA methylation had occurred and to identify the locations of methylated CpG dinucleotide pairs within the β_2 AR promoter region, we used bisulfite genomic sequencing to analyze T cell clones and primary T cells cultured in T_H1- or T_H2-driving conditions for several weeks. The data revealed a distinct pattern of CpG dinucleotide methylation in both the proximal (location -181 to 56) region of the promoter, as well as within the transcription start site (map shown in Fig. 5A). Analysis of T cell clones showed that the cytosine residues were methylated in T_H2, but not T_H1 clones, while the level of CpG dinucleotide methylation was undetectable in T_HN (Fig. 5B). In contrast to the striking difference found in the pattern of CpG dinucleotide methylation in T cell clones, the pattern was not as distinct in primary effector cells. However, the level of methylation in primary T_H2 cells was significantly

greater than that measured in primary T_H1 cells ($p=0.036$) (Fig. 5B). The mean frequency of methylation was 3 times larger in the T_H2 cells as compared to T_H1 cells within the proximal region (Fig. 5B). The proximal (Fig. 5B) region of the β_2 AR promoter expressed a low level of methylation throughout several weeks of re-activation, and no passage by culture interaction was found. Taken together, these data identify CpG dinucleotide methylation in the β_2 AR promoter sequence of primary T_H2 cells and clones, suggesting that this may be the mechanism by which permanent repression of the β_2 AR gene is induced.

Discussion

Gene silencing mediated by epigenetic mechanisms is important for regulating appropriate gene expression as a cell differentiates during early development (Li, 2002). As a naive CD4⁺ T cell differentiates post-developmentally, epigenetic mechanisms are activated to promote appropriate cytokine gene expression in resulting T_H1 and T_H2 cells (Baguet and Bix, 2004; Chang and Aune, 2005, 2007; Zhou et al., 2004). We hypothesized and show for the first time in the present study that similar changes occur in the β_2 AR promoter during CD4⁺ T cell differentiation to either a T_H1 or T_H2 and include modifiable (histone modifications) and more permanent (DNA methylation) epigenetic changes.

To test these hypotheses, we examined histone modifications as well as DNA methylation during CD4⁺ T cell differentiation. These data show that within the β_2 AR proximal promoter H3K9 and H3K27 dimethylation and the frequency of methylated CpG dinucleotides (both modifications are classically indicative of closed chromatin) increased in T_H2 cells as compared to T_HN and T_H1 cells. In contrast, T_H1 cells showed an increase in pan-H3/H4 acetylation and H3K4 dimethylation, which are indicative of open chromatin, and very little DNA methylation when compared to T_HN and T_H2 cells. Taken together, these findings suggest that chromatin remodeling in the β_2 AR gene promoter is one of the mechanisms by which β_2 AR gene expression is regulated in CD4⁺ T cells as they differentiate under T_H1- or T_H2-driving conditions.

Our observations revealed a pattern of histone methylation that is not entirely black or white. While pan-H3/H4 acetylation and H3K4 dimethylation are associated with open chromatin and gene expression, these modifications are not entirely absent within the β_2 AR promoter in T_H2 cells, which lack β_2 AR expression. Conversely, H3K9 dimethylation is more often associated with closed chromatin and gene repression, yet this modification is still detectable at low levels in the T_H1 cells, which express the β_2 AR. These methylation findings may be due to the analysis in the present study of unsorted populations of cells in which undifferentiated cells were still present. While the culture conditions are intended to drive the cells towards a T_H1 or T_H2 phenotype, we determined by flow cytometry that this is not the case for all cells (data not shown). In addition, several other types of histone modifications, such as acetylation, phosphorylation, ribosylation, and ubiquitination (Eberharter and Becker, 2002; Jaenisch and Bird, 2003; Mutskov and Felsenfeld, 2004) may also be occurring as a mechanism to control expression of the β_2 AR. Our data on pan-H3/H4 acetylation, which is often associated with gene transcription, was more prevalent in T_H1-driven and sorted cells while it was nearly absent in T_H2-driven and sorted cell populations. Ultimately, a pattern of distinct histone modifications would create a 'histone code' to determine chromatin remodeling and gene expression (Strahl and Allis, 2000).

Bird and colleagues demonstrated that histone modifications of cytokine genes are cell-cycle dependent and that an increased number of cell divisions is required for T_H2 differentiation as compared to T_H1 differentiation (Bird et al., 1998). Our results are consistent with this report because, like the histone modification data, the level of β_2 AR mRNA was not reduced to undetectable levels, even in sorted populations or tumor-driven (comparable to *in vitro* re-

stimulated) cells, indicating that the cells may not be fully differentiated. Furthermore, following multiple weeks of in vitro culture under T_H2-driving conditions (data not shown), the level of β_2 AR mRNA remained at low, but detectable levels, as well. In addition, we found that DNA methylation in the β_2 AR promoter was variable and inconsistent in T_H2-driven cells, although significant after several weeks of re-stimulation. Incomplete differentiation would be even more discernable when examining human CD4⁺ T cells, as several studies have suggested that human T cells do not differentiate to the degree of murine T cells (Abbas et al., 1996; Romagnani, 1994, 2000). Our data from cells differentiated in vitro by T_H1- or T_H2-driving tumors (Fig. 2B) suggests that the greatest levels of differentiation may be reached during disease states and that during these disease states, proper differentiation of CD4⁺ T cells and proper expression of the β_2 AR may be of utmost importance.

The epigenetic changes measured within the β_2 AR proximal promoter in the present study occurred in T_HN cells that were activated under conditions that differed only in the cytokines introduced to direct differentiation, i.e., IL-12 and IL-4 for T_H1 and T_H2 differentiation, respectively. This difference suggests, but does not prove, that IL-12 and IL-4, as well as possibly the IFN- γ released by the developing T_H1 cells, may activate specific transcription factors that participate in promoting the chromatin remodeling that is associated with differential expression of β_2 AR mRNA and protein by T_H1 and T_H2 cells. However, the mechanism responsible for linking these events is unknown. The chromatin remodeling that is associated with both cytokine gene expression and repression in T_H1 and T_H2 cells is promoted by the activation of the transcription factor GATA3 by IL-4 (Lee et al., 2001; Lee et al., 2003), STAT-4 by IL-12, and/or STAT1 and T-bet by IFN- γ (Lee et al., 2006; Murphy and Reiner, 2002). Our findings suggest that the absence or presence of IL-12, IFN- γ , and IL-4 are associated with the onset of chromatin modifications within the β_2 AR promoter of developing T_H1 and T_H2 cells. Therefore, it is possible that the transcription factors activated by these cytokines might also play a role in promoting the onset of chromatin modifications that occur within the β_2 AR promoter of each effector subset.

The physiological relevance for differential β_2 AR expression by T_H1 and T_H2 cells is unknown. However, stimulation of the β_2 AR on either the effector T_H1 cell or the naive CD4⁺ T cells cultured under T_H1-driving conditions causes a change to occur in the level of IFN- γ produced (Ramer-Quinn et al., 2000; Swanson et al., 2001). If such changes occurred under physiological conditions, e.g. if the neurotransmitter NE, which is the endogenous ligand for the β_2 AR, is released during times of stress or upon antigen exposure (Kohm et al., 2000) and causes an increase in the level of IFN- γ within the microenvironment of an activated T_HN cell, then this might influence the direction of naive CD4⁺ T cell differentiation and/or the subsequent development of T_H1-biased pathologies, including inflammation, tissue damage, and autoimmune diseases (Firestein, 2003). Thus, understanding a mechanism that exists for regulating the level of expression for the β_2 AR might prove useful. Furthermore, since the elevation of cAMP within a T_H2 cell is reported to increase the level of IL-4 produced (Lacour et al., 1994), a β_2 AR-induced increase in cAMP in T_H2 cells might exacerbate an IL-4-dependent response (Fedyk et al., 1996), such as might occur during allergic asthma when an individual either is stressed and norepinephrine is released at a higher level than level or is using a β_2 AR agonist for bronchodilation therapy. Thus, understanding the epigenetic mechanisms that regulate β_2 AR expression may lead to the development of novel targeted therapies for limiting or enhancing T_H1 and T_H2 cell responsiveness to norepinephrine or a β_2 AR agonist. In addition, such an understanding may provide one more tool with which to therapeutically regulate the development and progression of various T_H1- and T_H2-mediated pathologies,

and perhaps provide insight into novel mechanisms by which β_2 AR expression might be regulated in cells outside of the immune system itself.

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Figure 1. β_2 AR mRNA is expressed in TH1 cells, but not in TH2 cells derived from primary THN cells *in vitro*

Naive CD4⁺ T cells were activated under TH1- and TH2-driving conditions for 0-5 days. Cells were collected each day and total RNA was isolated from TH1 (circles) and TH2 (squares) as well as THN cells (day 0). (A.) β_2 AR, (B.) IFN- γ and (C.) IL-4 mRNA was measured by quantitative real time PCR. The data were normalized to β -actin and represent the mean mRNA level (fg/ml) $\pm$ SEM from three independent experiments.

Figure 2. $\beta_2\text{AR}$ mRNA expression in sorted and highly differentiated cells *in vitro* and *in vivo* (A.) Primary effector T cells cultured for 1 week under $\text{T}_{\text{H}2}$ -driving conditions were sorted for the level of intracellular IL-4 expression. Total RNA was then isolated from low (open bar) and high (closed bar) IL-4-producing cells. (B.) Total RNA was isolated from primary tumor antigen-specific Th1 (C2 and A312, open bars), and Th2 (WB4, closed bar) cells. The level of $\beta_2\text{AR}$ and β -actin mRNA was analyzed by quantitative real-time PCR. The data were normalized to β -actin and represent the mean $\beta_2\text{AR}$ mRNA (fg/ml) $\pm$ SEM from three independent experiments.

Figure 3. Histone acetylation changes in the β_2AR promoter in T_H1 - and T_H2 -driven $CD4^+$ T cells

(A.) Primary effector T cells cultured for 1 week under T_H1 - or T_H2 -driving conditions were sorted for the level of intracellular IFN- γ or IL-4 protein expression, respectively. Cells were collected, fixed, and analyzed by ChIP for pan (A.) H3 or (B.) H4 acetylation. Isolated DNA was analyzed by PCR and agarose gel separation for the β_2AR promoter. Data from one representative experiment of two independent experiments is presented as the fold difference from naive levels of acetylation. BLD, Below Level of Detection.

Figure 4. Histone methylation changes in the β_2 AR promoter in T_H1- and T_H2-driven CD4⁺ T cells

Primary naive CD4⁺ T cells were cultured under T_H1- or T_H2-promoting conditions as described previously. Before culture (T_HN, gray bars) or after three days of culture (T_H1, white bars; T_H2, black bars), cells were collected, fixed, and analyzed by ChIP for (A.) H3K4 (open chromatin) and (B.) H3K9/H3K27 (closed chromatin) dimethylation. Isolated DNA was analyzed by PCR and agarose gel separation for the β_2 AR promoter. Data from three independent experiments are presented as the level of histone methylation normalized to the input DNA control sample $\pm$ SEM. BLD, Below Level of Detection.

Figure 5. CpG dinucleotides in the proximal β_2 AR promoter show an increased rate of methylation in T_H2 cells as compared to T_H1 cells

(A.) Schematic of the β_2 AR gene and proximal promoter region (-181 to +56) that contains 19 CpG dinucleotides and was amplified by the primer set β_2 ARp-b9/b10 (B.) DNA was isolated from the resting T_H1 cell clones D1.1, HDK, AR100.9 and the resting T_H2 cell clones CDC35, LNT-1, LNT-4, bisulfite treated, and the proximal β_2 AR promoter was sequenced. Murine naive $CD4^+$ T cells were activated as described previously. Cells were reactivated weekly for 4-5 weeks under polarizing conditions. DNA was isolated from naive T cells and each week from T_H1 and T_H2 cells, bisulfite treated, and analyzed as with the T cell clones. Each circle is representative of a CpG dinucleotide within the proximal β_2 AR promoter. Open and filled circles represent unmethylated or methylated status of the CpG dinucleotide, respectively. Data are representative of 6 to 10 cloned sequences.

Figure 6. Epigenetic changes in the proximal β_2 AR promoter

Naive CD4⁺ T cells activated in the presence of IL-12 or IL-4 will differentiate into T_H1 or T_H2, respectively. As these cells differentiate, the histones and DNA of the proximal β_2 AR promoter will undergo many changes. In a T_H1 cell (left side of figure), the tail of histones 3 and 4 will become acetylated at several amino acids and the tail of histone 3 will become methylated at residue Lysine 4. In addition, the chromatin will remain open and unmethylated, allowing access to transcription factors that can bind to the DNA to increase transcription and ultimately expression of the β_2 AR on the T_H1 cell. In a T_H2 cell (right side of figure), the tail of histones 3 and 4 will be stripped of any acetylation and residues Lysine 9 and 27 will be methylated. The chromatin will be closed and become methylated, which decreases DNA access to transcription factors and results in decreased β_2 AR transcription and expression on the T_H2 cell.