JBC Papers in Press. Published on May 19, 2014 as Manuscript M113.545210
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M113.545210

Histone Modifications Are Associated with Delta (9)-tetrahydrocannabinol-Mediated Alterations in Antigen-Specific T Cell Responses

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Running Title: THC and histone methylation

Key Words: Epigenetics, Histone methylation, Immunosuppression, Lymphocyte, T cell

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Background: Marijuana has been shown to have an immunomodulatory activity.
Results: ChIP-seq results show genome-wide changes in histone methylation in immune cells treated with THC.
Conclusion: Histone modifications are associated with THC-mediated alterations in antigen-specific T cell response.
Significance: This study provides insights into the potential role of epigenetic changes induced by THC in gene regulation.

ABSTRACT

Marijuana is one of the most abused drugs due to its psychotropic effects. Interestingly, it is also used for medicinal purposes. The main psychotropic component in marijuana, Δ^9-tetrahydrocannabinol (THC), has also been shown to mediate potent anti-inflammatory properties. Whether the immunomodulatory activity of THC is mediated by epigenetic regulation has not been investigated previously. In this study, we employed ChIP-Seq technology to examine the in vivo effect of THC

on global histone methylation in lymph node cells of mice immunized with a superantigen, staphylococcal enterotoxin B (SEB). We compared genome-wide histone H3K4, H3K27, H3K9, H3K36 trimethylation and H3K9 acetylation patterns in such cells exposed to THC or vehicle. Our results showed that THC treatment leads to the association of active histone modification signals to Th2 cytokine genes and suppressive modification signals to Th1 cytokine genes, indicating that such a mechanism may play a critical role in THC-mediated switch from Th1 to Th2. At the global level, a significant portion of histone methylation and acetylation regions were altered by THC. However, the overall distribution of these histone methylation signals among the genomic features were not altered significantly by THC, suggesting that THC activates the expression of a subset of genes while suppressing the expression of another subset of genes through histone modification. Functional classification of these histone marker associated genes showed that these differentially associated genes were involved in various cellular functions, from cell cycle regulation to metabolism, suggesting that THC had a pleiotropic effect on gene expression in immune cells. Together, the current study demonstrates for the first time that THC may modulate immune response through epigenetic regulation involving histone modifications.

Marijuana is the most frequently used illicit substance in the United States (1). In addition, many states in the US have now legalized marijuana use, especially when authorized by a physician, for medical purposes such as alleviation of nausea and vomiting from chemotherapy, wasting in AIDS patients, and chronic pain that is unresponsive to opioids (2, 3). Moreover, two states in the US have legalized marijuana for recreational use. Thus, studies evaluating the risks and benefits of marijuana use are critical.

$^{9}\Delta$-tetrahydrocannabinol (THC), the active psychotropic ingredient of marijuana, mediates its activity through cannabinoid receptors (CB1 and CB2). Cannabinoid receptors are typical transmembrane G protein-coupled receptors. While CB1 is highly expressed in the brain, and to a lower extent in peripheral tissues (4), CB2 is predominant in immune cells (5). Therefore, besides its psychoactive effects, THC can suppress inflammation through activation of cannabinoid receptors on immune cells, using multiple pathways (6-8). THC has been shown to suppress Th1 while promoting Th2 cells (9, 10). In addition, THC induces CD11b+ Gr-1+ myeloid-derived suppressor cells (MDSC) (11-13), as well as Tregs (14), which have been shown to inhibit T cell proliferation. The induction of MDSCs by THC was associated with alterations in microRNA expression (15). Moreover, we also noted that prenatal exposure to THC causes T cell dysfunction in the offspring (16). Together, such data suggested that THC may trigger epigenetic modulations in immune cells.

Epigenetic modification has been implicated in the establishment and maintenance of differential gene expression in T cells (17). DNA methylation and histone modifications are common epigenetic pathways leading to alterations in gene expression. Epigenetic modifications have been shown to regulate T cell differentiation by modifying the chromatin at the related genes such as Ifn-γ, Foxp3 and IL-4 (18). Genome wide histone modification studies using ChIP-Seq method in human T cells have linked histone methylation patterns to the specific gene activity in different T cell subtypes (17, 19-21). Histone methylation mainly occurs on the lysine and arginine residues, and lysines can be mono-, di- or tri- methylated. Histone H3 methylation on lysine 4, lysine 9, lysine 27 and lysine 36 are among the most extensively studied histone methylations (22). In general, histone H3 lysine 4 trimethylation (H3K4me3) in the promoter region is associated with transcription activation, while histone H3 lysine 27 trimethylation (H3K27me3) within the promoter region is associated with transcription repression. However, H3K4me3 and H3K27me3 that seem to be associated with opposite functions can co-exist in the same regions. This so called "bivalent domains" has been shown in embryonic stem cells and T cells, and is proposed to lead to activation or suppression (23-25). Histone lysine 36 trimethylation (H3K36me3) has been linked to the transcription elongation and is enriched in the body of active transcripts (26, 27). Histone lysine 9 methylation (H3K9me3) has been linked to the

silencing of gene. This mark is enriched in the telomeric region and terminal repeats (19, 27-29). However, it has been shown that H3K9me3 is also enriched in many promoters (30). Histone acetylation in general is associated with gene activation. One of the most well study histone acetylation markers is Histone H3 acetylation at lysine 9 (H3K9ac), which is enriched near the transcription start site (TSS) of highly expressed genes (31).

Staphylococcal enterotoxin B (SEB) is a bacterial superantigen that triggers a massive Th1-cytokine storm leading to lethal toxic shock syndrome (32). In this study, we investigated the effect of THC on SEB-induced T cell activation *in vivo* and determined whether THC modifies global histone methylation in activated immune cells. Using ChIP-Seq approach, we compared genome-wide H3K4me3, H3K27me3, H3K36me3, H3K9me3 and H3K9ac patterns in SEB activated popliteal lymph node (LN) cells in mice with or without THC pre-treatment. Our data showed that a significant portion of histone methylation and acetylation regions are altered by THC treatment at the genomic level. However, the associated methylation markers, not the H3K9ac marker, in key Th1/Th2 cytokine genes are altered by THC treatment, which is consistent with the ability of THC to induce a shift in Th1-Th2 balance. Moreover, we identified many other genes whose expression may be regulated by THC through histone modification.

EXPERIMENTAL PROCEDURES

Mice and cell isolation Female C57BL/6J mice were purchased from NIH (Frederick, MD). 6-7 weeks old mice received intraperitoneal injection of THC (Sigma, 20mg/kg of body weight) or same amount of vehicle as described previously (33). Twenty four hours later, the mice received the same treatment again. Two hours after the second treatment, 10µg of staphylococcal enterotoxin B (SEB) in 50µl of PBS was injected in each foot pad (2 foot pads per mouse). Mice were euthanized 1d, 3d or 5d after SEB challenge. Popliteal lymph nodes (LN) were collected and single cell suspension was prepared in RPMI1640 cell culture medium. We used pretreatment regimen with THC because SEB triggers an acute cytokine storm, and moreover, such studies would indicate how marijuana abuse would alter the immune response when exposed to an infectious agent.

Staining and FACS analysis for intracellular markers LN cells were cultured in complete RPMI in the presence of 1 nM PMA (Sigma Aldrich, St. Louis, MO), 1 µM calcium ionophore (Sigma) and 2 µM protein transport inhibitor Monensin (Biolegend, San Diego, CA) for 4 h. Cells were washed and resuspended in FACS buffer (PBS containing 2% FBS and 0.1% sodium azide). Fc receptors were blocked by adding anti-mouse CD16/CD32 (10 µg/ml) followed by surface staining for CD4. Intracellular staining for cytokines IL-4 and IFN-γ was performed using leukocyte activation cocktail with BD Golgiplug (BD biosciences, San Jose, CA) according to manufacturer's instructions. Intranuclear staining for TBX21, GATA3 and Ki67 was done using fix/perm reagent kit from Biolegend. Anti-mouse CD16/CD32 mAbs (Fc-block), PE-Cy7-conjugated anti-mouse CD4, and APC-conjugated anti-mouse IL-4 antibodies were purchased from BD biosciences. Cells were analyzed in BC FC 500 flow cytometer.

ChIP and ChIP-Seq ChIP was performed using Simple ChIP-enzymatic Chromatin IP Kit (Cell signaling, #9003). Briefly, cells were diluted to $5x10^6$ cells/ml in the cell culture medium, and 37% formaldehyde was added to a final concentration of 1% to cross link histone and DNA. After 10 min incubation at room temperature, formaldehyde was quenched by adding glycine to a final concentration of 125mM. Cells were then pelleted and washed with cold PBS for 2 times. $5x10^6$ cells were resuspended in 500µl of Micrococcal Nuclease buffer and digested with 2000 units of the enzyme for 20 min at 37°C. Nuclei were pelleted by centrifugation at 13,000rpm for 1 min and resuspended in 1ml ChIP buffer. Nuclear membrane was disrupted by brief sonication (2 sets of 10-second pulses) and lysates were clarified by centrifugation at 10,000rpm for 10 min. The supernatant was used for chromatin immunoprecipitation. The ChIP antibodies were purchased from Abcam (Cambridge, MA). They were: H3K4me3 (ab1012), H3K27me3 (ab6002), H3K9me3 (ab8898), H3K36me3 (ab9050) and H3K9ac (ab12179). Ten µg of antibody was used for each IP. Antibodies were incubated with the sample at 4°C for overnight with rotation. After the addition of bead, samples were incubated for

another 2 h at 4°C with rotation. After washing, the immunoprecipitated chromatin was eluted from the bead and the cross link was reversed by proteinase K digestion. DNA was then purified using spin columns and quantified. For ChIP-Seq, the library was constructed using Illumina's Chip Sequencing sample preparation kit (#1003473) according to the manufacturer's instruction. Briefly, 10 ng of ChIP enriched DNA was used for each library construction. First the DNA fragments were repaired to phosphorylated blunt ends using T4 DNA polymerase, Klenow polymerase and T4 polynucleotide kinase. After DNA fragments were purified using Qiagen PCR purification kit (Qiagen #28104), an "A" base was added to the 3' end of the blunt DNA fragment by Klenow fragment (3' to 5' exo-) at 37°C for 30 min. The product was purified by MinElute purification kit (Qiagen #28004). Sequencing adapters were ligated to the ends of DNA fragments using DNA ligase at room temperature for 15 m followed by purification with MinElute PCR Purification kit. The product was then separated in 2% agarose gel to remove excess adaptors and to select a size range of library. The fragments with size range from 150bp to 250bp were excised and purified using QIAquick Gel Extraction Kit (Qiagen #28704). The library was then amplified by limited PCR (16 cycles) using primers provided by the kit. The concentration and distribution of the library were determined by Agilent Bioanalyzer 2100. The library was sequenced by illumina HiSeq2000 at Tufts University Genomic core facility.

Data Analysis HiSeq2000 platform generated single-end reads with a read length of 50bp. Raw sequencing reads in FASTQ format were mapped to mouse genome build mm9 using Bowtie software by allowing two mismatches in the read (34). The mapped reads (SAM file) were then filtered and only uniquely mapped reads were used for the downstream analysis. SICER was used for the peak calling (35, 36). The peak calling parameters were 200bp window size and 600bp gap size except for H3K4me3 and H3K9ac in which 200bp window size and 200bp gap size were used. The statistic threshold value (E-value) was set as 0.01. The peaks (in WIG file format) were visualized in the UCSC genome browser (http://genome.ucsc.edu/). SICER generated scoreisland files were used to draw circular overall methylation picture using R program. The correlation heat map of these signals was generated using DiffBind software (37). Distribution of signal in various genomic features was calculated using CEAS software (38). Promoter region was defined as 3kb upstream of transcription start site (TSS). Annotation for peak associated genes was performed using peak2gene program in Cistrome (39). Genes with the center of H3K4me3, H3K9me3 and H3K9ac located within 3kb up- or down- stream of their TSS were identified as H3K4me3 and H3K9me3 associated genes. Since H3K27me3 had broad peaks, its associated genes were identified as the peak center located within 5kb up-and down-stream of TSS. H3K36me3 associated genes were identified as the peak center located within 3kb downstream of TSS. Biological functions of those genes were classified using the PANTHER Classification System (www.pantherdb.org).

Quantitative Real-time PCR Popliteal lymph nodes were collected and homogenized, and total RNA was isolated using Trizol (Invitrogen). RNA was reverse transcribed into cDNA using random primer and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. The relative abundance of gene expression was determined by Real-time PCR using Gapdh as the internal reference.

RESULTS

THC attenuated SEB-induced cell proliferation and immune response in vivo SEB is a superantigen that triggers robust T cell activation. While THC is known to induce a switch in Th cell differentiation from Th1 to Th2 (9, 10), we attempted to corroborate these studies using SEB so that we could use the same cells for epigenetic analysis. To this end, C57BL/6J mice were pretreated with THC or vehicle as described previously (33), on day 0 and 1, and two hours later, 10μg of staphylococcal enterotoxin B (SEB) was injected in each foot pad. Draining popliteal lymph nodes were harvested and cells analyzed 1d, 3d and 5d after SEB challenge. SEB exerted the most robust effect on $CD4^+$ T cell proliferation 3d after the treatment as determined by total cell number (Fig.1a) and Ki67 staining (Fig.1b), and THC attenuated cell proliferation at all those time points. Therefore, we chose 3d time point for the following study. In SEB+vehicle treated mice,

there was significant enlargement of draining popliteal lymph nodes (LN) with high cell yield (~13 million cells) (Fig.1a), when compared to LN from naïve mice (~1million; data not shown), indicative of strong T cell proliferation caused by SEB. In contrast, in SEB+THC treated mice, there was a significant decrease in total cell numbers as well as Ki67 positive cell number (Fig.1b). We next determined the numbers of Th1 and Th2 cells by double-staining the cells for CD4 and IFN-γ/Tbx21 to detect Th1 cells; or CD4 and IL-4/GATA3 to detect Th2 cells using flow cytometry. Based on the percentages of these cells as detected by flow cytometry, we calculated the absolute numbers of Th1 or Th2 cells respectively. In THC pretreated mice, numbers of $CD4^+$ T cells TBX21 positive (Fig.1c) and IFN-γ positive (Fig.1d) were significantly lower than those in vehicle treated mice. On the other hand, IL-4 positive (Fig.1e) and GATA positive (Fig.1f) $CD4^+$ T cells were increased in THC treated mice. The proliferation of Th1 and Th2 cells was further analyzed by FACS analysis for the expression of Ki67 on IFN-γ (Th1) or IL-4 (Th2) positive $CD4^+$ T cells. Compared with vehicle treated mice, cells from THC treated mice had a decreased $Ki67^+$ Th1 population (Fig 1, g, h) and an increased $Ki67^+$ Th2 population (Fig1.i, j). These results were consistent with what were shown in our previous studies and those of others that exposure to THC suppresses Th1 while enhancing Th2 response (10, 40, 41).

Genome-wide histone H3 methylation profile in SEB activated lymph node cells To determine whether THC exerts its immunosuppressive function through epigenetic modifications, and to see if it has a global effect on histone modifications, we employed ChIP-Seq method to examine genome-wide histone H3 trimethylation pattern at Lys4, Lys9, Lys27 and Lys36 sites as well as acetylation at Lys9 site in draining popliteal lymph node cells from mice that received SEB+vehicle or SEB+THC. In our experiment, each ChIP library generated 150-210 million reads. Approximately, 60-70% of those reads were uniquely mapped to the mouse genome (mm9). A graphical display of H3K4me3, H3K27me3, H3K36me3, H3K9me3 and H3K9ac profiles across the whole genome is presented in Fig 2a-e. Although the overall signal level of each histone marker did not differ significantly between vehicle and THC treated cells, the distribution of the signal was altered as demonstrated by correlation analysis (Fig 2f). These results suggested that THC did not alter the overall activity of these histone modification enzymes while genes associated with these histone markers were altered by THC treatment. We further examined the expression of major histone methyltranferase, demethylase, acetyltransferase and deacetylase that are known to control these histone modifications (42, 43). The expression of these enzymes did not differ significantly as determined by real time PCR (Fig 2 g). The unique and common genomic regions (intervals) containing these histone markers between vehicle and THC treated cells were further analyzed. Among these, while the occurrence of H3K36me3 markers was most abundant, H3K9me3 had the fewest number of signal regions (Fig 3a). For H3K4me3, H3K27me3 and H3K36me3, there were more common regions than unique regions, while for H3K9me3 and H3K9ac, there were more unique regions. A representative histone methylation profile on a region of Chromosome 1 is shown in Fig 3b. In general, the H3K9ac, H3K4me3, H3K9me3 had narrow signal peaks while H3K27me3 and H3K36me3 had much broader peaks. These typical patterns were consistent with previous reports (19, 27). These results demonstrated that exposure to THC during an immune response to antigens such as SEB *in vivo* could alter histone modification, particularly H3K36me3, H3K9me3 and H3K9ac, thereby influencing global gene expression.

Distribution of histone H3 methylation signal in genomic features The distribution of histone markers was analyzed according to mouse genomic features. H3K4me3 was the most enriched in the promoter regions compared to others whereas, H3K27me3 was mostly located in the gene body and intergenic regions. H3K36me3 was mainly found within the gene body and H3K9me3 in the intergenic region (Fig 4a). Furthermore, H3K4me3 was significantly increased near the transcription start site (TSS) of genes, and its signal density decreased near the transcription termination site (TTS). This pattern was consistent with the notion that H3K4me3 is found in transcriptionally active promoters and is associated with gene activation. There was a dip of H3K4me3 signal density right before TSS (Fig

4b,c). Similar observation has been made by others and this dip is thought to be due to the nucleosome loss in active genes (19). H3K27me3 level was lower near the TSS and the signal was increased after the TTS (Figure 4 b,c). This might be due to the reduced H3K27me3 modification in the active genes because H3K27me3 has been suggested to repress gene expression (44, 45). H3K36me3 signal was low before the TSS and after TTS, but was enriched in the gene body, which was consistent with the indication that H3K36me3 associates with the transcription elongation (26). H3K9me3 has been implicated in gene repression. Its signal density decreased slightly near the TSS. Although the signal pattern of these histone methylation markers were similar in SEB+vehicle and SEB+THC treated cells as sorted according to genomic feature, many regions were differentially associated with these markers, suggesting a different set of gene was expressed in these two samples. H3K9ac is associated with the promoter region of active genes. However, in SEB activated lymph cells, its signal near the TSS was decreased, suggesting that SEB might affect histone acetylation or deacetylation enzymes. This pattern was reversed by THC treatment (Fig 4. b, c) and H3K9ac was enriched near the TSS site as expected.

Genes associated with histone methylation markers Because H3K4me3, H3K27me3, H3K9me3 and H3K9ac near the TSS are associated with gene activity, we identified genes that had these methylation signals near their TSS (Fig 5a). Genes that had H3K36me3 signal in their transcript body were also identified. Overall, more genes were associated with H3K4me3 and fewer genes were associated with H3K36me3 and H3K9me3 signals in the THC exposed cells relative to vehicle treated cells. The number of genes that associated with H3K27me3 was similar between the SEB+vehicle treated and SEB+THC treated samples. Most genes with H3K4me3, H3K27me3 or H3K36me3 modification were common between the two samples. However, most of H3K9me3 associated genes were unique to the vehicle treatment. Similarly, there were more H3K9ac that were unique to the vehicle or THC treatment. Biological function classification showed that those genes were involved in a variety of pathways, from cell cycle to metabolism (Fig 5b), suggesting that THC might have a much broader biological impact. The bivalent domains of H3K4me3 and H3K27me3 have been suggested to play a regulatory role in the differentiation in embryonic stem cells and T cells. There are a significant number of active genes that have both H3K4me3 and H3K27me3 in their promoters (25, 46). In this study, we also found that a significant number of genes had both H3K4me3 and H3K27me3 signal present near their TSS (Fig 5a), suggesting that those genes might not be permanently activated or suppressed, rather more finely regulated. While a large number of genes with this bivalent modification in their promoters were common to SEB+vehicle or SEB+THC treatment, a good number of such genes were unique to THC treatment. The supplemental data shows the list of genes that were differentially associated with these histone markers.

Histone methylation pattern and gene expression Since THC has been shown to shift the balance of Th1 and Th2, we examined these histone markers in the genomic regions of some of the Th1 and Th2 cytokines to determine whether their associated histone makers. IFN-γ is one of the most potent pro-inflammatory cytokines induced by SEB and THC is known to suppress IFN-γ expression (9, 14, 47), as was also seen in the current study (Fig 1b,c). In cells from SEB+vehicle treated mice, the promoter of Ifn-γ was found to be associated with both active H3K4me3 and suppressive H3K27me3 signals. Its gene body also had active H3K36me3 signal. In cells from SEB+THC treated mice, H3K4me3 and H3K36me3 diminished, indicating that the expression of Ifn-γ was suppressed (Fig 6a). TBX21 is the transcription factor that controls the expression of Ifn-γ which was decreased in SEB+THC exposed cells (Fig 1c). Correlating with this observation, we noted that the active signal H3K4me3 was present in the promoter region of Tbx21 in the SEB+vehicle treated cells but absent in the SEB+THC treated cells (Fig 6a). On the other hand, IL-4 and IL-5, markers of Th2 cells, had H3K27me3 signal in their promoters in the SEB+vehicle treated cells but lacking in SEB+THC cells (Fig 6b). Interestingly, H3K9ac marker was not found in the promoter regions of these genes in either vehicle or THC treated sample (data not shown). This result suggested that histone methylation, not histone H3K9

acetylation correlated with THC mediated Th1-Th2 shift in SEB activated lymph cells. The mRNA expression of Ifn-γ, Tbx21, IL-4 and IL-5 was further validated by real time PCR (Fig 6d). We also noted that IL-2, involved in T cell proliferation, had suppressive H3K27me3 in its promoter region in THC treated cells (Fig 6b), which correlated with decreased mRNA expression (Fig 6d).

Besides these genes that are known to be regulated by THC, we also found other genes that were distinctively associated with active and suppressive methylation marks in vehicle or THC treated cells. For example, the promoter of Brca2, a tumor suppressor gene, had H3K4me3 and H3K27me3 signal in the SEB+vehicle and SEB+THC treated cells, respectively, suggesting that the expression of this gene might be suppressed by THC. On the contrary, Cbx-1, a member of the heterochromatin protein family, had K3K27me3 signal in its promoter in the SEB+vehicle treated cells, but had K3K4me3 signal in the SEB+THC treated cells (Fig 6c). Real time PCR results showed that Brca2 expression was indeed reduced while Cbx-1 was increased with THC treatment (Fig 6d). These validations indicated that histone methylation determinations in this study correlated well with expected gene expression changes. THC also induces apoptosis in immune cells. In macrophages and T cells, THC has been shown to act by inducing Caspase-1(48). Consistent with this, in SEB+THC treated cells, Caspase-1 had H3K4me3 and H3K36me3 in its promoter and gene body respectively (Fig 6e).

A recent study showed that THC reduces Th17 (49). However, in this study, these histone methylation markers were not associated with Rorc which regulates Th17 (data not shown). To determine whether Th17 is regulated by THC, we examined Rorc by real time PCR. The expression of Rorc was decreased in THC treated cells (Fig 6), suggesting THC modulates immune response by other histone modifications or by other mechanisms.

Besides protein coding genes, THC treatment also altered histone methylations in many noncoding RNAs. Long noncoding RNAs (lncRNAs) and miRNAs are important regulators of gene expression (50). For example, in the SEB+vehicle treated mice, there was a strong H3K36me3 signal in the transcript of Bic/miR-155, while no signal was detected in SEB+THC treated cells (Fig 6e), suggesting that THC down regulates Bic/miR-155 in the superantigen activated LN cells. Another example is miR-212 and miR-132 cluster. These two miRNAs are encoded from the intron of a non-coding transcript. Eighteen transcription start sites have been identified from 3kb to 30bp upstream of these miRNAs based on miRBase (www.mirbase.org). The suppressive marker, H3K27me3 was present in all these transcription start sites in the SEB+vehicle treated cells, but not in the SEB+THC treated cells, suggesting that the suppressed expression of these miRNAs in SEB activated lymph cells was reversed by THC treatment.

DISCUSSION

The immune response and the establishment of functionally specialized immune cell lineages are controlled by multiple transcription factors as well as epigenetic modifications, and these epigenetic modifications can be altered by various environmental factors or bioactive drug components. In this study, we examined the effect of THC on 4 histone methylation markers and 1 histone acetylation marker across the whole genome in SEB superantigen activated lymph node cells *in vivo*. A significant amount of histone modification clusters were found to be unique to THC treatment. These results suggested that THC could specifically activate or suppress the expression of genes.

THC has been shown to have anti-inflammatory and immunosuppression property and induce apoptosis of immune cells (40). Indeed, the size of the popliteal lymph node was smaller and the cell number was lower in SEB+THC treated mice than that in the SEB+vehicle treated mice. The histone methylation pattern in several pro-inflammatory and anti-inflammatory cytokines was consistent with data which indicated that THC suppressed pro-inflammatory cells such as Th1. H3K27me3, the suppression marker, was the only signal present in the promoter of Ifn-γ in the SEB+THC treated sample in this study, and the expression of Inf-γ was suppressed even though SEB is a potent agent to induce inflammation. In contrast, the Ifn-γ

promoter in the SEB+vehicle activated lymphocytes had both H3K4me3 and H3K27me3. The bivalent modification of H3K4me3 and H3K27me3 in the promoter of Ifn-γ suggested that the expression of Ifn-γ can be quickly modulated according to the external signal. Similarly, TBX21, a Th1 specific transcription factor that controls the expression of Ifn-γ also had this bivalent modification in the SEB+vehicle treated sample. This kind of modification might be critical for a balanced immune response because prolonged expression of pro-inflammatory cytokines can have adverse effects on the host. Despite a significant difference in overall H3K9ac pattern in vehicle and THC treated cells, we did not find difference in the association of H3K9ac in these genes. The unexpected decrease of H3K9ac signal near the TSS of SEB+vehicle treated may indicate that SEB affects the function of enzymes that regulate histone acetylation and de-acetylation, and THC may partially relieve that effect. In future, we will use other antigens to activate the immune cells to determine whether the H3K9ac pattern in this experiment is unique to SEB stimulation.

In this study, we identified many genes with bivalent modification. H3K4me3 and H3K27me3 bivalent modification has been proposed to explain the plasticity of T cell differentiation, and genes with bivalent modification can be either expressed or silenced (25). However, our study also demonstrated that some genes are oppositely modified in SEB+vehicle and SEB+THC samples. For example, the promoter of Brca2 had active H3K4me3 marker in the SEB+vehicle treated sample but had suppressive H3K27me3 marker in the SEB+THC treated sample. While Cbx-1 had H3K27me3 in the SEB+vehicle treated sample, it had H3K4me3 in the SEB+THC treated sample. This suggested that the expression of these genes could be permanently altered by THC. Whether this is the case, however, needs further investigation.

It is known that many histone modifications can independently regulate gene expression. For example, in human CD8+ T cells, some active genes are associated with high levels of H3k4me3, while others are associated with H3K9ac (17). That may explain the lack of histone methylation markers in Rorc while its expression is down regulated by THC. It is possible that it is associated with other epigenetic modifications such as other histone acetylation markers and DNA methylation.

Long noncoding RNAs (lncRNAs) and miRNAs are parts of epigenetic regulation mechanism. *Bic* is an lncRNA whose expression is elevated in the activated T cells (51, 52). *Bic* can be further processed into miR-155. It has been shown that *Bic*/miR-155 is essential for immune function and mice with deficiency in *Bic*/miR-155 are immunodeficient (53). In a study of vulvar lichen sclerosus and lichen planus autoimmune disorders which are characterized by a strong Th1 response, the expression of *Bic*/mirR-155 was profoundly elevated (54). mirR-155 has also been shown to be over expressed in other autoimmune diseases and to enhance inflammatory T cell development (55). The altered histone methylation signal found in this study suggested that THC may also exert its function by regulating the expression of non-coding regulatory RNAs. Another example of histone methylation mediated miRNA expression is miR-212 and miR-132. These miRNAs play important roles in immune response, apoptosis and neuronal function. Expression of miR-212 enhances TRAIL –induced apoptosis, while inhibition of miR-212 renders cells resistant to TRAIL treatment (56). miR-132 has been indicated as an early response miRNA after viral infection and suggested as an innate immunity regulation miRNA (57). It has also been shown to potentiate anti-inflammatory signaling (58). Results from miR-212 and miR-132 knockout mice indicated that these miRNAs regulate synaptic transmission and plasticity (59). Altered histone methylation signal in their transcription start sites after THC treatment suggested that THC could exert a broad biological effect by modulating miRNA expression.

In this study we found that some genes have all four histone H3 methylations while others only have one type of methylation signal. It is unclear whether the regulation of genes with more epigenetic modifications has greater complexity than those with fewer modification signals. It is also not clear whether genes with two active markers such as H3K4me3 and H3K36me3 are more active than those with only one marker. It is also possible that the multiple modification signals

may come from different types of cells found in the lymph node.

In summary, we demonstrate the association between THC-mediated histone modifications and a switch from Th1 to Th2 response against bacterial superantigen. The precise mechanisms through which THC regulates histone methylation remains to be further addressed. In the current study, we examined the expression of some major histone methyltranferase, demethylase, acetyltransferase and deacetylase that are known to control these histone modifications (42, 43) and found that THC treatment failed to alter the expression of these enzymes, as determined by real time PCR. However, it is possible that the expression of other enzymes might be altered by THC. In addition, THC could modulate the functional activity of these enzymes. Some studies suggested that THC could act directly on the epigenetic modification machinery. For example, AEA, an endocannabinoid, has been shown to increase DNA methylation level in human keratinocytes through p38 (60). As for histone modification, it has been shown that agonists of cannabinoid receptors can increase the number of H3K9m3 positive glioma stem-like cells and this effect is blocked by CB antagonists (61). Interestingly, in 4 histone markers examined in this study, THC had the most profound effect on H3K9me3. Another example for the role of cannabinoids in histone modification is the association of increased overall histone H3 acetylation and decreased level of CB1 in Huntington's disease (62), suggesting that cannabinoid signaling could affect histone acetylation enzymes. Furthermore, THC has been shown to alter histone deacetylase 3 (HDAC3) in a dose-dependent manner (63). HDAC3 is a member of histone deacetylase family and along with other HDACs, is responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (64). Although we did not identify a significant change in the expression of Sirt1, the major deacetylase responsible for H3K9ac deacetylation in this study, we did observe a significant change in overall H3K9ac pattern after THC treatment (Fig 4b, c). Whether the expression and activity of other histone acetylation enzymes are altered by THC, needs further investigation. Another piece of evidence that suggests cannabinoids may directly regulate epigenetic modification comes from cannabinoid receptor knockout mice. In CB1 knockout mice, it has been shown that CB1 regulates chromatin remodeling during spermiogenesis (65).

As for THC-mediated alteration in histone methylation, currently there is no study which indicates that THC directly regulates the expression or activity of histone methyltransferases or demethylases. However, THC could indirectly regulate the activity of enzymes involved in histone methylation. For example, cannabinoids have been shown to down regulate PI3K/AKT signaling pathway (66, 67), a pathway also known to cause global alterations of H3K27me3 (68). On the other hand, some studies showed that administration of THC increases phosphorylation of AKT in mouse brain through CB1 (69). The discrepancy regarding the role of THC in AKT signaling may be due to the difference in cell type. Nonetheless, the effect of THC on AKT pathways may lead to regulation of histone methylation. AKT can phosphorylate EZH2 and suppress its methyltranferase activity, which results in a decrease of H3K27me3 (70). AKT also targets the association of histone with CBP, which regulates histone H3 acetylation (71). Additional studies are necessary to investigate whether the activity of EZH2 is altered by THC through AKT pathway.

THC may also indirectly regulate histone methylation through other pathways such as estrogen receptor (ER) pathway. It has been shown that histone demethylases LSD1 and KDM2A are required for the induction of ER signaling after E2 stimulation (72). On the other hand, histone demethylase, KDM4B, is induced in an ER-α dependent manner after E2 stimulation (73), indicating that the activation of ER pathway modulates histone methylation status. Many studies have shown that cannabinoid and estrogen pathways regulate each other. For example, some studies have suggested that both crude cannabis extract and THC inhibit the binding of estradiol to estradiol receptors *in vivo* (74, 75). Recent studies showed that some estrogen receptor modulators can bind to cannabinoid receptors (76, 77). These results have raised the possibility that THC could regulate histone methylation through ER signaling. Thus, the current study opens new avenues to investigate the epigenetic pathways through which THC regulates the immune response. Because

histone modifications can occur at many sites and at different levels, additional studies are necessary to address this because the current study focused on only certain histone markers. Secondly, the regulation of enzymes involved in histone modifications is very complex and thus, further investigations are necessary.

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ACKNOWLEDGMENTS This study is supported in part by NIH grants P01AT003961, R01AT006888, R01ES019313, R01MH094755, P20GM103641 and VA Merit Award BX001357.

Figure Legends:

Figure 1. Effect of THC on lymph node cell proliferation and Th 1 and Th2 subpopulations
C57BL/6J mice were treated with THC or vehicle as described in Methods on day 0 and 1, and two hours later, 10μg of staphylococcal enterotoxin B (SEB) was injected in each foot pad. Three days after SEB challenge, draining popliteal lymph nodes SEB+Vehicle or SEB+THC treated mice (n=3) were harvested and cells analyzed. **a**) Total cells in 2 popliteal lymph nodes in each mouse. **b)** Cells were gated by $CD4^+$ and analyzed by FACS for the expression of Ki67. **c,d,e,f**) Based on flow cytometric analysis as described in Methods, cell number of various CD4+ T cell subpopulations expressing IFN-γ, TBX21, IL-4 or GATA3 were depicted. **g, h**) overall frequency and mean fluorescence intensity (MFI) of Ki67, CD4 and IFN-γ triple positive cell. **i, j)** overall frequency and MFI of Ki67,CD4 and IL-4 triple positive cell. *P* values were determined by Student's t-test.

Figure 2. Genome-wide histone H3 methylation level in lymph node cells
a-e) C57BL/6J mice were treated with SEB+THC or SEB+vehicle as described in Fig 1. The LN cells were studied for genome-wide histone H3 methylation and acetylation as described in Methods. ChIP-Seq signal density is color-coded. The outer circle is the SEB+vehicle treated sample and the inner circle is SEB+THC treated sample. **f**) Correlation of overall signal of these histone markers. Heat map was generated by DiffBind. **g)** Relative mRNA abundance of histone-lysine N-methyltransferase MLL (H3K4me3), EZH2 (H3K27me3), SETD2 (H3K36me3), SUV39H1(H3K9me3), Lysine-specific demethylase KDM5B (H3K4me3), KDM6A (H3K27me3), KDM4A (H3K9me3 and H3K36me3), histone acetyltransferase KAT2A (H3K9ac) and NAD-dependent deacetylase SIRT1 (H3K9ac) as determined by real-time PCR. The amount in the vehicle treated sample was set as 1.

Figure 3. Histone H3 methylation regions in activated lymph node cells
C57BL/6J mice were treated with SEB+THC or SEB+vehicle as described in Fig 1. The LN cells were studied for histone H3 methylation and acetylation regions. **a**) Venn diagrams of the overlap and unique regions of histone marker between the SEB+vehicle(veh) and SEB+THC(THC) treated lymph node cells. **b**) Representative ChIP-Seq result displayed in UCSC genome browser.

Figure 4. Distribution of histone methylation signal among genomic features
C57BL/6J mice were treated with SEB+THC or SEB+vehicle as described in Fig 1. The LN cells were studied for histone markers as described in Methods. **a**) The percentage of methylation signal located in the promoter regions (3kb upstream of TSS), gene body (intron and exon) and intergenic region. **b**, **c**) The relative enrichment profile of each histone methylation near the TSS, within the transcript and near the TTS in the SEB+vehicle treated (**b**) and SEB+THC treated (**c**) lymph node cells.

Figure 5. Genes associated with histone methylation signal in lymph node cells
C57BL/6J mice were treated with SEB+THC or SEB+vehicle as described in Fig 1. The LN cells were studied for genes associated with histone markers. **a**) Venn diagrams of overlap and unique genes associated with each histone marker as well as H3K4me3/H3K27me3 bivalent modification in the SEB+vehicle (veh) and SEB+THC(THC) treated samples. **b**) Classification of these genes according to their cellular function.

Figure 6 Histone methylation pattern and gene expression in lymph node cells
C57BL/6J mice were treated with SEB+THC or SEB+vehicle as described in Fig 1. The LN cells were studied for histone methylation pattern and gene methylation as described in Methods. **a**, **b**) Alteration of histone methylation in the promoter region of genes from cells exposed to SEB+vehicle (vehicle) or SEB+THC (THC). **c**) Example of genes with opposite histone methylation in SEB+vehicle (vehicle) or SEB+THC (THC) treated cells. **d**) Relative mRNA abundance of selected genes as determined by real-

time PCR. The amount in the vehicle treated sample was set as 1. **e**) Example of potential genes and miRNAs whose expression might be regulated by THC.

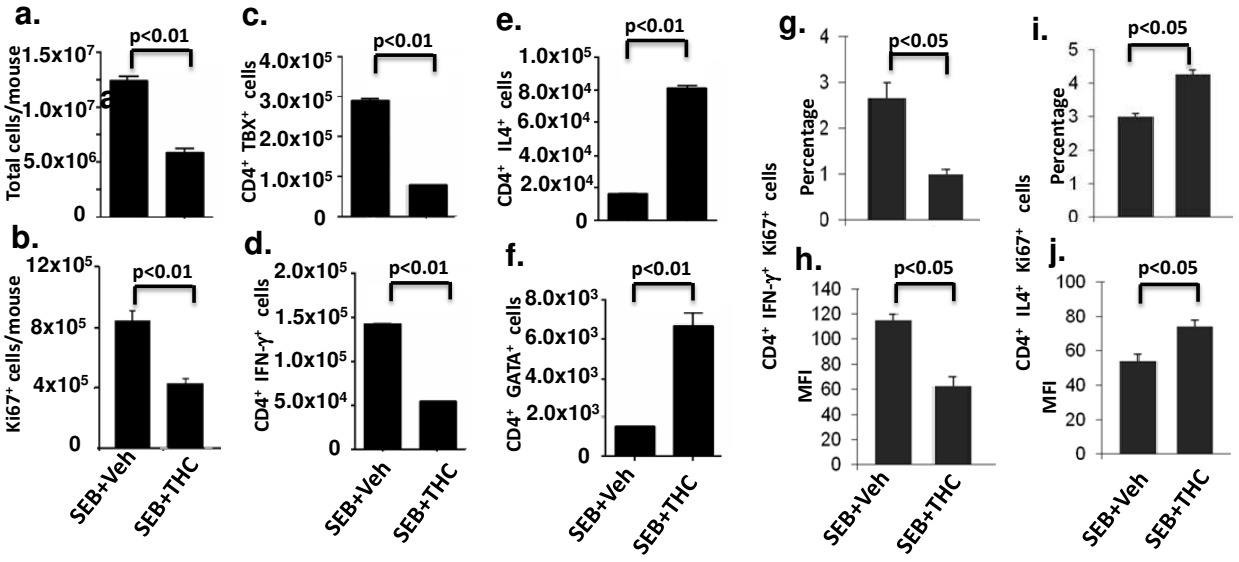


Figure 1. Effect of THC on lymph node cell proliferation and Th 1 and Th2 subpopulations

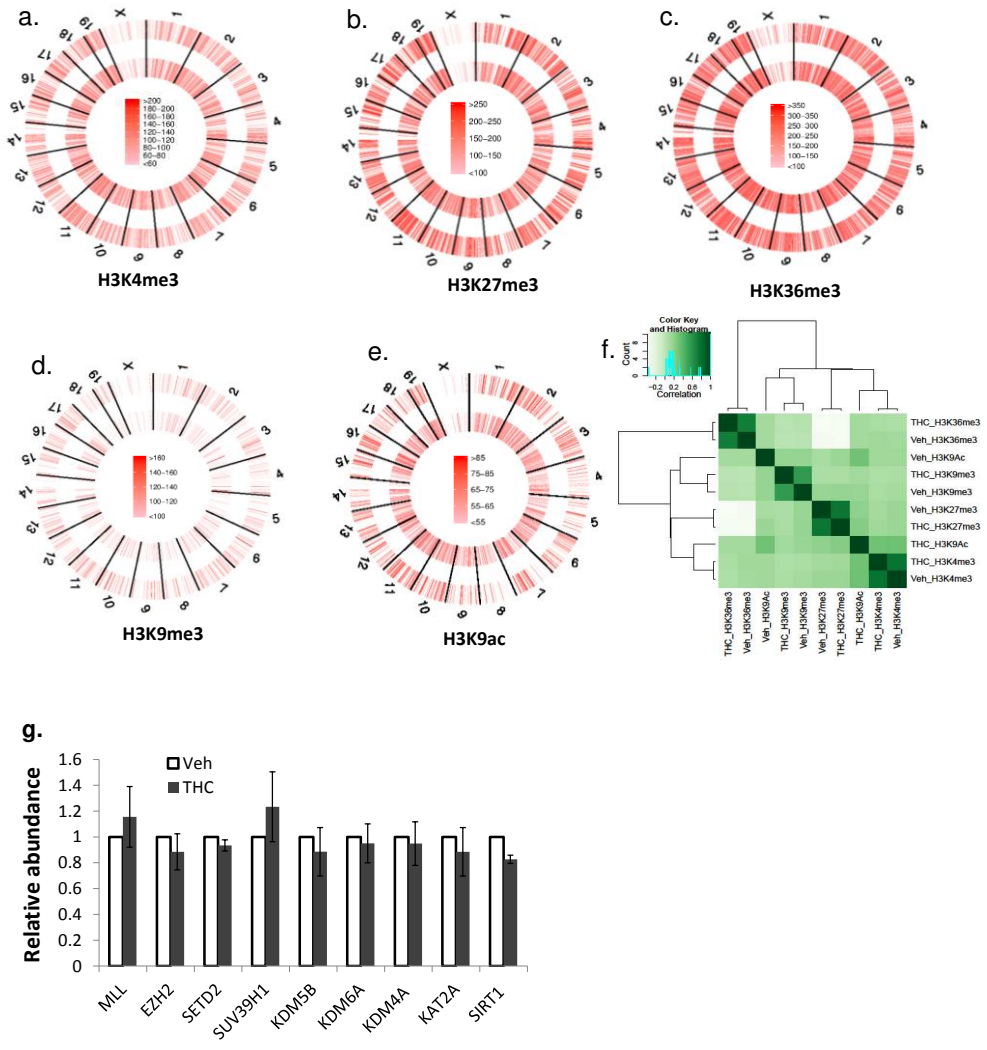
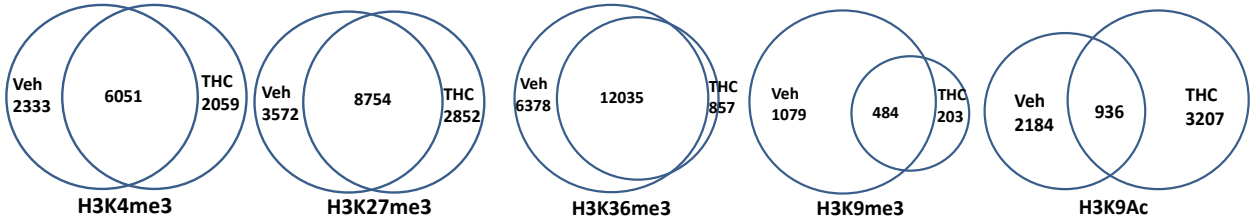


Figure 2. Genome-wide histone H3 methylation level in lymph node cells

a.



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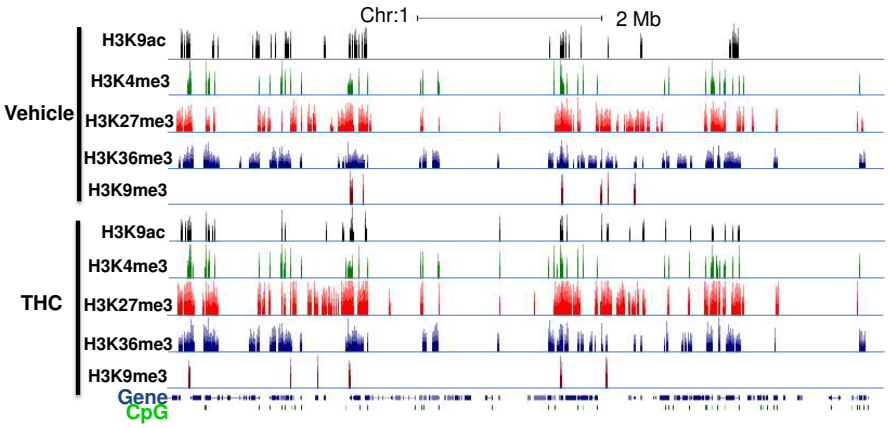
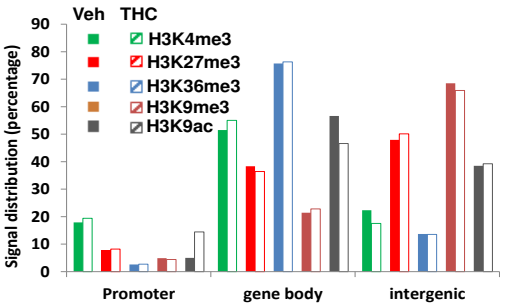


Figure 3. Histone H3 methylation regions in activated lymph node cells

a.



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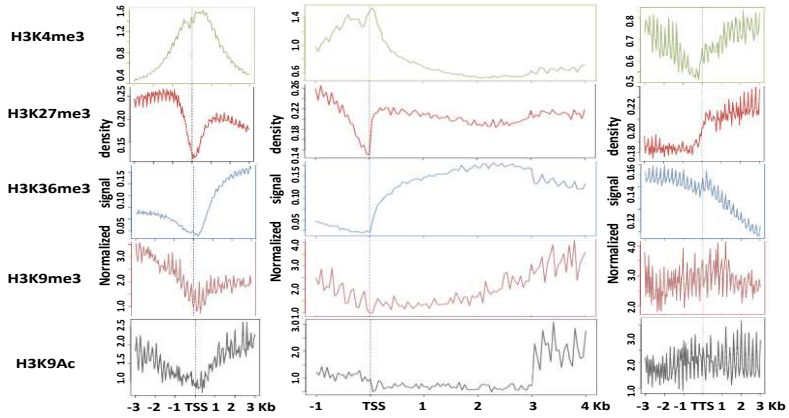


c.

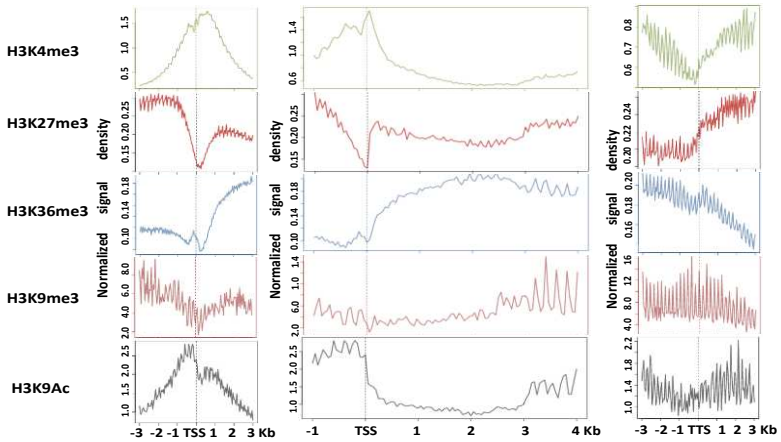
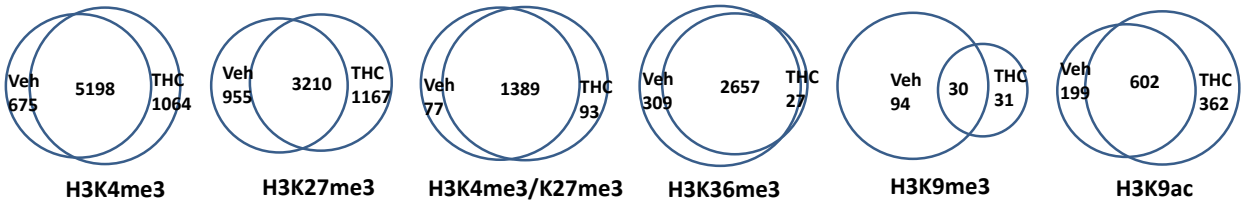


Figure 4. Distribution of histone methylation signal among genomic features

a.



b.

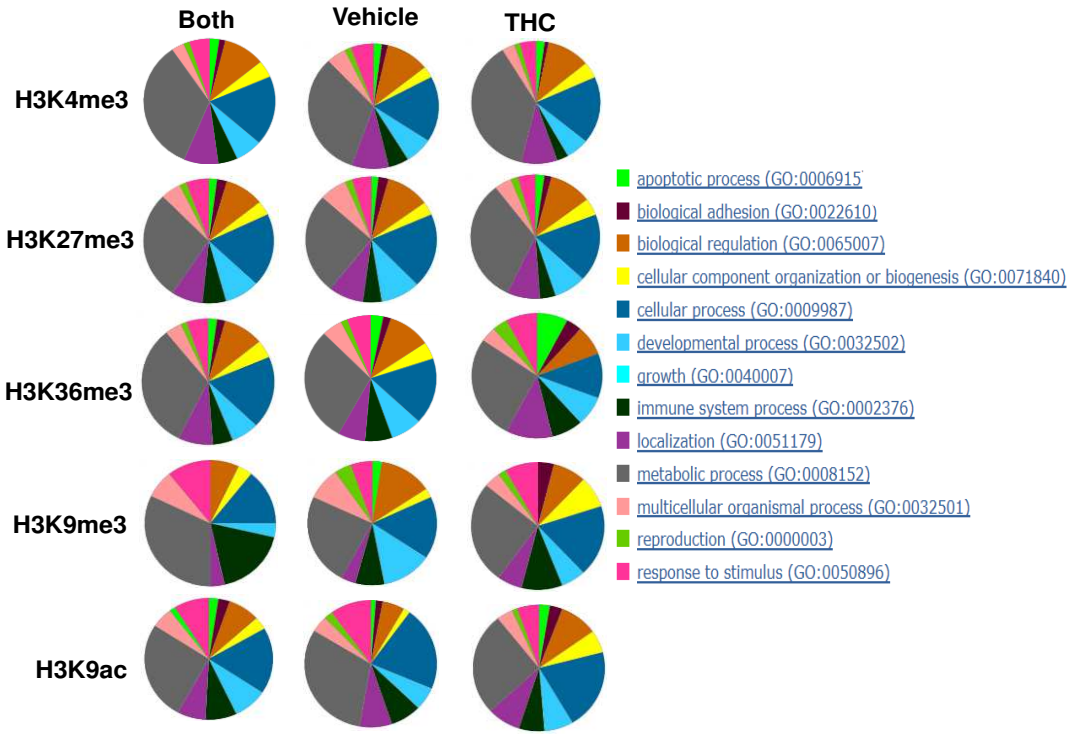


Figure 5. Genes associated with histone methylation signal in lymph node cells

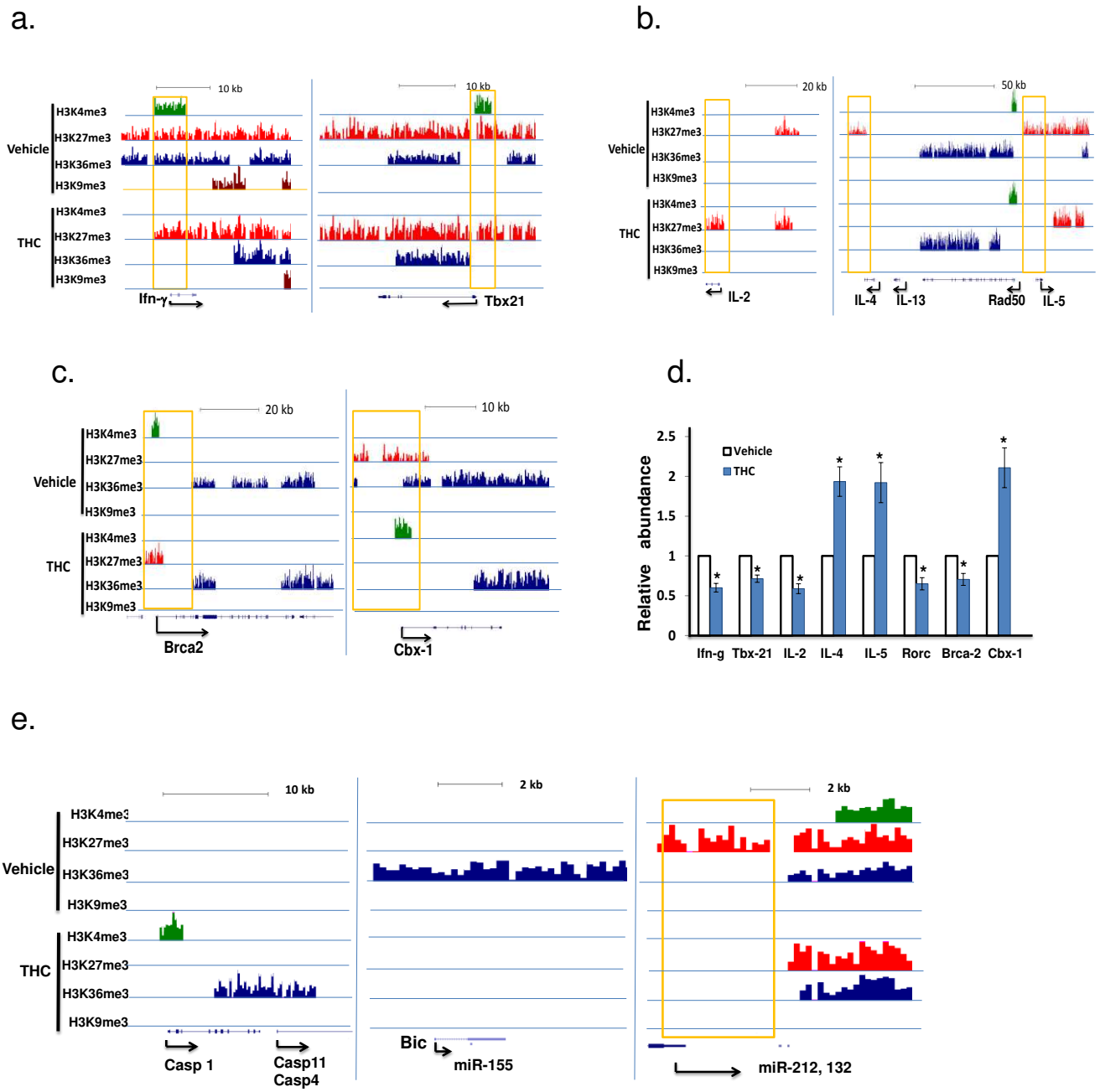
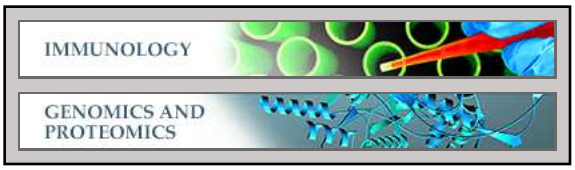


Figure 6 Histone methylation pattern and gene expression in lymph node cells

Immunology:

Histone modifications are associated with Delta(9)-tetrahydrocannabinol-mediated alterations in antigen-specific T cell responses



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J. Biol. Chem. published online May 19, 2014