Coordinated Changes in DNA Methylation in Antigen-Specific Memory CD4 T Cells

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Memory CD4$^+$ T cells are central regulators of both humoral and cellular immune responses. T cell differentiation results in specific changes in chromatin structure and DNA methylation of cytokine genes. Although the methylation status of a limited number of gene loci in T cells has been examined, the genome-wide DNA methylation status of memory CD4$^+$ T cells remains unexplored. To further elucidate the molecular signature of memory T cells, we conducted methylome and transcriptome analyses of memory CD4$^+$ T cells generated using T cells from TCR-transgenic mice. The resulting genome-wide DNA methylation profile revealed 1144 differentially methylated regions (DMRs) across the murine genome during the process of T cell differentiation, 552 of which were associated with gene loci. Interestingly, the majority of these DMRs were located in introns. These DMRs included genes such as CXCR6, Tbox21, Chsy1, and Cish, which are associated with cytokine production, homing to bone marrow, and immune responses. Methylation changes in memory T cells exposed to specific Ag appeared to regulate enhancer activity rather than promoter activity of immunologically relevant genes. In addition, methylation profiles differed between memory T cell subsets, demonstrating a link between T cell methylation status and T cell differentiation. By comparing DMRs between naive and Ag-specific memory T cells, this study provides new insights into the functional status of memory T cells. ***The Journal of Immunology*****, 2013, 190: 4076–4091.**

CD4$^+$ T cells are central regulators of both humoral and cellular immune responses. Activation of naive CD4$^+$ T cells by Ag induces cell proliferation, resulting in the formation of a large number of effector cells and, subsequently, a limited number of memory cells. Memory CD4$^+$ T cell populations are maintained by cytokine survival signals and homeostatic proliferation, such that they are able to respond rapidly to subsequent exposure to the same Ag (1, 2). Recently, it was reported that the first exposure of a naive T cell to Ag and cytokine signals results in specific changes in the cell's chromatin structure and in DNA methylation of the cell's cytokine genes (3–5).

Chromatin modifications are known to impose epigenetic controls on gene expression without changing DNA sequence (6). These modifications determine the level of cell type–specific gene transcription by modulating the accessibility of genes to transcription factors and the basal transcription apparatus. It is well known that epigenetic regulation is linked to gene repression of oncogenes and development-related genes (6, 7). Genes that are active (open) in a particular tissue or cell type have increased acetylation and methylation of their histones (e.g., H3K4 methylation), whereas genes that are inactive (closed) are characterized by highly condensed chromatin and decreased acetylation and methylation of their histones (e.g., H3K9 and H3K27 methylation). In addition, DNA methyltransferases establish and maintain the pattern of genomic DNA methylation of cytosines in CpG dinucleotides. DNA methylation status is generally considered to correlate inversely with transcriptional activity, with transcriptionally silent genes being highly methylated and transcriptionally active regions being relatively unmethylated (8, 9). DNA methylation is also associated with epigenetic gene regulation during embryogenesis, genomic imprinting, and X-chromosome inactivation (10, 11).

In the immune system, a lack of methylation at the appropriate loci in T and B lymphocytes is associated with transcription and rearrangement of Ig and TCR genes, as well as with cell lineage–specific expression of CD4, CD8, and CD21 (12–15). When naive T cells differentiate to Th1 cells, but not to Th2 cells, DNase hypersensitive sites appear in the IFN-γ gene (16). Furthermore, the IFN-γ gene is methylated to a lesser extent in human and

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; CGI, CpG island; DMR, differentially methylated region; GO, Gene Ontology Consortium Database; MSCC, methyl-sensitive cut counting; P/I, PMA/ionomycin; SAGE, serial analysis of gene expression; TAE, Tris–acetate–EDTA; Tg, transgenic; TSS, transcription start site.

murine Th1 and CD8 effector cells than in naive and Th2 cells. In contrast, the IL-4 and IL-5 genes are less methylated in Th2 cells than in Th1 cells. Treatment of T cells in vitro with drugs that inhibit histone deacetylases or DNA methylation increases IL-4 and IFN-γ expression. Moreover, naive T cells from conditional Dnmt1-knockout mice, which lack DNA (cytosine-5-)-methyltransferase 1, express substantially more IFN-γ and IL-4 after Ag activation, an effect that appears to be mediated, at least in part, by demethylation of the *cis*-regulatory element (17). Recently, it was demonstrated that demethylation of the FOXP3 locus is pivotal for differentiation of $CD4^+CD25^+$ regulatory T cells (18) and that the CpG regions of cell type–specific genes (e.g., IL2RA, CTLA4, and CD40LG) in conventional human $CD4^+$ T cells and regulatory T cells are differentially methylated (19). A limiting DNA methylation affects the proliferative potential of Ag-specific $CD8^+$ T cells with moderate effects on their differentiation to effector and memory $CD8^+$ T cells (20). Additionally, methyl-CpG–binding domain protein 2–deficient mice display reduced memory $CD8^+$ T cell differentiation following acute viral infection (21).

These findings indicate that DNA methylation is crucial for memory T cell development and cytokine production. However, in T cells, the DNA methylation status of only a limited number of genes has been examined. The genome-wide DNA methylation status of memory $CD4^+$ T cells derived from Ag-stimulated naive cells remains unexplored. In this study, we investigated the gene-expression profiles and genome-wide DNA methylation status of naive and Ag-specific memory $CD4^+$ murine T lymphocytes.

Materials and Methods

Mice

BALB/c mice were purchased from Clea (Tokyo, Japan). OVA-specific TCR-transgenic (Tg) mice (DO.11.10; OVA-specific TCR Tg × $RAG2^{-/-}$ mice) were maintained under specific pathogen–free conditions.

Reagents

The anti–CD4-Pacific Blue (RM4-5), anti-CD62L mAb (MEL-14), anti–CD25-PE (7D4), PE-conjugated anti-CD4 mAb (GK1.5-PE), anti–CD44-bio (IM7), anti–CD69-bio (H1.2F3), anti–CD127-bio (A7R34), IFN-γ–FITC (XMG1.2), anti–IL-4–Alexa Fluor 647 (11B11), anti–TNF-α–PE/Cy7 (MP6-XT22), anti-mouse TCR DO11.10-PerCP/Cy5.5 (KJ1-26), and streptavidin-allophycocyanin were purchased from BD Pharmingen and eBioscience.

Generation of effector and memory $CD4^+$ T cells

OVA-specific naive $CD4^+$ T cells were isolated from the spleens of DO11.10-Tg mice. To generate effector cells, naive $CD4^+$ T cells were stimulated with 1 μg/ml an OVA peptide (residues 323–339; ISQAVHAAHAEINEAGRD; synthesized by Sigma Genosys, Hokkaido, Japan) plus allophycocyanin for 5 d in vitro (22). Five million of these cells were transferred i.v. into normal syngeneic BALB/c recipient mice. In most experiments, 4 wk after effector cell transfer, $KJ1^+$ cells from the spleens of recipient mice were sorted by FACSVantage (BD Pharmingen) and used as memory $CD4^+$ T cells.

Assays for cytokine production

Naive effector and memory $KJ\text{-}1^+CD4^+$ T cells were restimulated with PMA (20 ng/ml)/ionomycin (1 μg/ml) (P/I) and brefeldin A (10 μg/ml) for 5 h. Cells were then fixed (Cytofix buffer; BD Pharmingen), permeabilized, stained intracellularly with anti–IFN-γ Ab, anti–IL-4 Ab, or anti–TNF-α Ab or its isotype control, and analyzed using a Gallios Flow Cytometer (Beckman Coulter).

Methyl-sensitive cut counting library construction

The integrity of cDNA was confirmed using an Agilent 2100 Bioanalyzer prior to construction of the methyl-sensitive cut counting (MSCC) libraries. The protocol for MSCC library construction was modified slightly from that described previously (23).

Adapters A1 (5′-TTTCCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATCCGAC-3′) and A2 (5′-CGGTCGGATCACCGACTGCCCATAGAGAGGAAAGCGGAGGCGTAGTGG-3′) contain a 5′ MmeI recognition site and a 5′-CG overhang; adapters B1 (5′-CGCCTTGGCCGTACAGCAGAGCTTACCGCAGAGAATGAGGAACCCGGGGCAG-3′) and B2 (5′-TTTCTGCCCCGGGTTCCTCATTCTCTGCGGTAAGCTCTGCTGTACGGCCAAGGCGNN-3′) contain a 3′-NN overhang and barcode (more information in Supplemental Table I), as described in the Applied Biosystems protocol. To construct the MSCC HpaII library, 1 μg genomic DNA isolated from $CD4^+$ T cells was mixed with 8 U HpaII (New England BioLabs) in 1× NEBuffer 1 in a 50-μl reaction volume and incubated at 37°C for 12 h. Another 8 U HpaII was added, and the mixture was incubated at 37°C for an additional 3 h. DNA was purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 12.5 μl dH_2O. This 12.5-μl DNA solution was mixed with 1.5 μl a mixture containing 5 μM adaptor A1, 5 μM adaptor A2, and 10 U T4 DNA ligase (Invitrogen) before incubation at 16°C for 12 h. DNA was again purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 8 μl dH_2O. This DNA was run on a 10% nondenaturing Tris–acetate–EDTA (TAE) polyacrylamide gel, and the 60–80-bp band was purified. After ethanol precipitation, the DNA pellet was resuspended in 70 μl a reaction mixture containing 14 U MmeI (New England BioLabs), 50 μM S-adenosyl methionine, and 1× NEBuffer 4. This mixture was incubated at 37°C for 12 h, after which DNA was again purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 13 μl dH_2O. This DNA solution was mixed with 1 μl each 5.8 μM adaptor B1 and B2 and 10 U T4 DNA ligase, and the mixture was incubated at 16°C for 12 h. DNA was again purified and resuspended in 20 μl dH_2O. This DNA solution was mixed with 10 U DNA polymerase I (New England BioLabs), 33 μM 2′-deoxynucleoside 5′-triphosphate, and 1× NEBuffer and incubated at 16°C for 30 min. DNA was again purified and resuspended in 8 μl dH_2O. This DNA was run on a 9% nondenaturing TAE polyacrylamide gel, and the 120–140-bp band was purified. The purified DNA was then amplified by PCR using the primers 5′-CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT-3′ and 5′-CTGCCCCGGGTTCCTCATTCTCT-3′. The 20-μl mixture for PCR contained 200 nM of each primer, 200 nM 2′-deoxynucleoside 5′-triphosphate, 1× PS buffer, and 1.25 U PrimeSTAR HS DNA polymerase (TaKaRa) and was run at 98°C for 30 s; 10 cycles at 98°C for 5 s, 62°C for 15 s, 72°C for 1 min; and then 72°C for 10 min. The PCR product was run on a 9% nondenaturing TAE polyacrylamide gel, and the 120–130-bp band was purified. The purified libraries were sequenced with the Applied Biosystems SOLiD4 system, following the manufacturer's protocol. The integrity of the cDNA was confirmed using an Agilent 2100 Bioanalyzer prior to construction of the MSCC libraries. A 1-ng sample of size-fractionated cDNA was used for sequencing reactions.

An MspI control library was constructed in the same manner as the HpaII library, with the following exceptions: in the first step, 100 U MspI (New England BioLabs) was used instead of HpaII, and NEBuffer 2 was used instead of NEBuffer 1, and no amplification was performed following gel purification. All HpaII libraries were normalized to 5 million.

SOLiD BioScope software (version 1.3) was used to determine methyl-sensitive restriction enzyme scores and map MSCC sequence reads (20 bp from the MmeI restriction site) to the mouse genome assembly (NCBI37/mm9). A DNA-methylation score was defined as the sum of tag sequence hits (a plus-strand tag and a minus-strand tag) for each restriction enzyme site, in the absence of repetitive sites, and normalized to 10^6 reads by the specific enzyme. To avoid inaccurate identification of methylation sites, differentially methylated regions (DMRs) were defined as those with a change from 0 tags (high-methylation group) to >10 tags (low-methylation group).

Generation and sequencing of the 5′-serial analysis of gene expression library

A newly developed 5′-end mRNA collection method (24) has extended the range of the original 5′-end serial analysis of gene expression (SAGE) technique. This method initially profiles 25-nucleotide 5′-SAGE tags using a novel strategy that incorporates the oligo-capping method. The 5′-SAGE tags are then ligated directly to a linker for sequencing. The purified libraries were sequenced with a Solexa system, according to the manufacturer's protocol (Illumina). The integrity of the cDNA was confirmed using an Agilent 2100 Bioanalyzer prior to construction of 5′-SAGE libraries. A 1-ng sample of size-fractionated cDNA was used for sequencing reactions with the Illumina GA, performed according to the manufacturer's instructions. We assigned unique tags to RefSeq genes (University of California, Santa Cruz, http/hgdownload.cse.ucsc.edu/goldenPath/mm9/database/) when the start position of the tag was within 500 bp upstream of the transcription start site (TSS), based on RefSeq annotation.

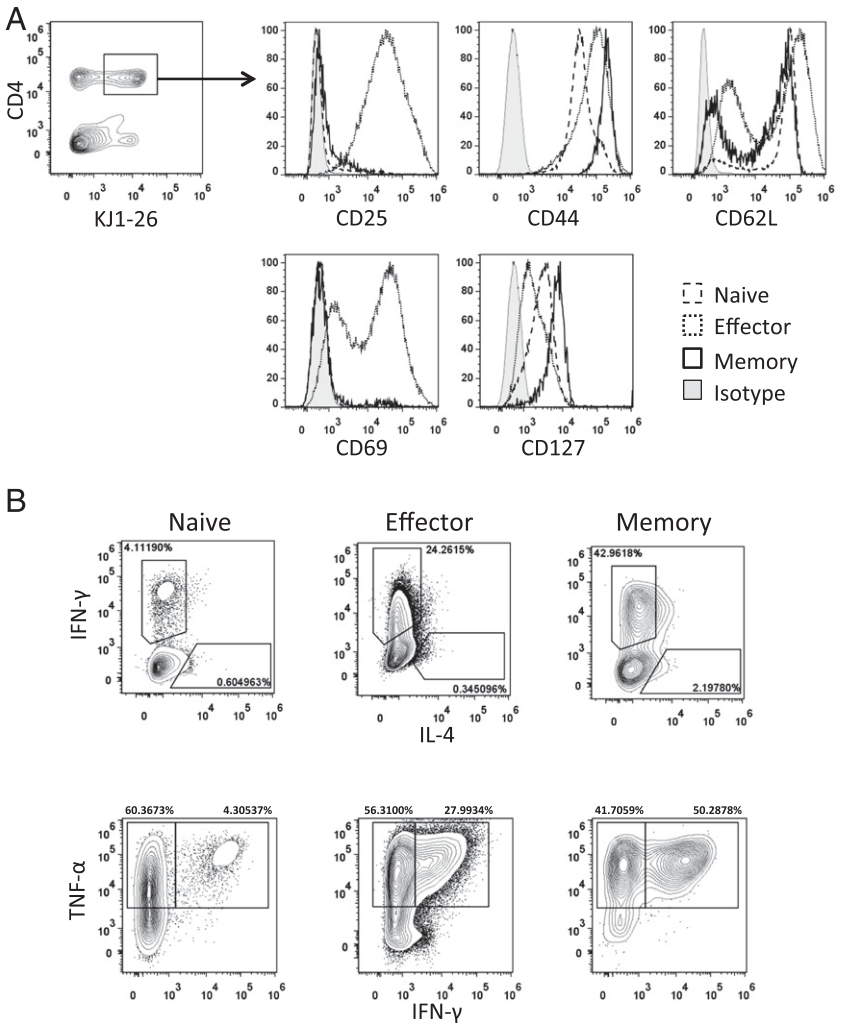

FIGURE 1. Surface marker and cytokine expression in naive and Ag-specific effector and memory CD4$^+$ T cells. Splenic CD4$^+$ T cells from DO11.10-Tg mice were stimulated with an OVA323–339 peptide plus APC for 5 d in vitro, resulting in Ag-specific effector cells, followed by transfer into normal syngeneic BALB/c recipient mice to generate memory cells. (**A**) Surface markers on CD4$^+$ T cells (double positive for KJ1 and CD4, *upper left panel*) were analyzed by flow cytometry. (**B**) IFN-γ, IL-4, and TNF-α production by naive, effector, and memory CD4 T cells was assessed by intracellular cytokine staining.

Real-time PCR

cDNA was prepared from total RNA samples using an Applied Biosystems (Foster City, CA) cDNA Archive Kit and random primers. The assay was run in triplicate for each RNA sample, in accordance with the manufacturer's recommendations, with each reaction containing 50 ng total cDNA (as total input RNA) per 20-μl reaction volume. The cycling conditions for SYBR Green dye I quantitative real-time PCR with 2× Applied Biosystems Universal Master Mix were 2 min at 50°C, 10 min at 95°C, followed by 40 rounds of 15 s at 95°C and 1 min at 60°C, with analysis by an Applied Biosystems 7500 PCR system. β-actin was used as the reference gene. Primer sequences are listed in Supplemental Table I. Data acquisition and analysis were performed using SDS 2.1 software in relative quantity mode, with each sample analyzed three times. After PCR, CT values were determined and used to calculate normalized $2^{-\Delta\Delta CT}$ values.

Luciferase reporter assay

Fragments of DMRs of the mouse *Nr1D1*, *Ptgir*, *Tnfsf4*, *Tbx21*, *Cish*, *Chsy1*, *Sdf4*, *Hps4*, *Sema4d*, *Mtss1*, *Klf7*, *Wdfy2*, *Nr5a1*, and *MapK1lip1* loci were amplified by PCR using genomic DNA as a template and the primers shown in Supplemental Table I. To generate a luciferase reporter vector on a CpG-free background, the 500–800-bp PCR product was inserted into the pCpGL-CMV/EF1 vector (a gift from Dr. M. Rehli and Dr. M. Klug) using the In-Fusion cloning system (Clontech), replacing the CMV enhancer with the DMR regions (19).

The luciferase reporter vector pCpGL-Cish-DMR/EF1 was methylated in vitro using methylase SssI (New England BioLabs), according to the manufacturer's instructions, followed by purification using a QIAquick PCR clean-up kit. In control samples using pCpGL-EF1 and pCpGL-Cish-DMR/EF1, the methyl-group donor S-adenosylmethionine was omitted. Successful methylation of the reporter plasmid containing the DMR was verified by reaction with the methylation-sensitive and methylation-resistant enzymes HpaII and MspI, respectively.

EL-4 T cells (5×10^6 cells) were transfected with 2.5 μg either methylated or unmethylated pCpGL-DMR/EF1 vector or using a control plasmid with no insert, in triplicate. Synthetic *Renilla* luciferase reporter vector (pRL-TK; Promega) was cotransfected (1.5 μg) and served as an internal control for efficiency. EL-4 cells were electroporated with a Bio-Rad Gene Pulser at 270 V and a capacitance of 975 μF. Twelve hours later, transfected cells were stimulated with PMA (50 ng/ml) and ionomycin (0.5 μg/ml) for 16 h. The cells were harvested, and luciferase activity was measured by the Dual Luciferase Assay system using an Orion L luminometer. Firefly raw light unit data were normalized to *Renilla* luciferase activity and expressed relative to the control vector with no insert.

Gene ontology

Gene ontology was estimated using GOstat software (25).

Table I. Genome-wide methylation sequencing summary for CD4$^+$ T cell DNA cut with HpaII or MspI restriction nuclease

Cell Type	Nuclease	No. of Hits in Genome	Unique Tags[a]	%
Naive	HpaII	9,902,632	5,074,880	51
	MspI	12,994,381	5,499,474	42
Effector	HpaII	9,349,718	6,039,406	65
	MspI	9,673,142	6,140,353	63
Memory	HpaII	13,582,273	7,055,612	52
	MspI	9,943,128	4,193,004	42
Total		65,445,274	34,002,729	52

Twenty-base pair MSCC tags were mapped in the genome.
[a]Number of tags in restriction sites for analysis of DNA methylation.

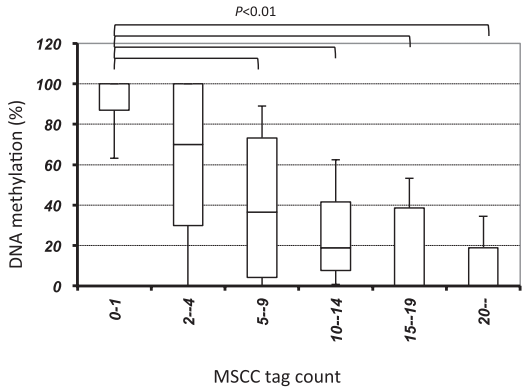

FIGURE 2. The relationship between MSCC tag counts and bisulfite sequencing data. To validate the methylation levels determined by MSCC, we designed primers targeting 130 profiled locations in bisulfite-treated DNA and performed PCR amplification and Sanger sequencing of the PCR product. Horizontal lines represent median methylation as determined by bisulfite sequencing, boxes represent the quartiles, and whiskers mark the 5th and 95th percentiles. $p < 0.01$, Kruskal–Wallis H test.

Bisulfite sequencing

Bisulfite sequencing was performed to verify SOLiD data. Bisulfite modification of genomic DNA was performed using the EpiTect Bisulfite Kit (QIAGEN). We used Methyl Primer Express software (Applied Biosystems) to design primers. Bisulfite-treated DNA was amplified by PCR. The PCR products were cloned into the pCR2.1-TOPO vector and transformed into One Shot TOP10 Competent Cells (Invitrogen). At least 24 clones were sequenced using an ABI3730 Sequencer. The data were analyzed using QUMA, a quantification tool for methylation analysis (Riken Institute of Physical and Chemical Research, Yokohama, Japan).

Statistical analysis

Comparisons of each 5′-end tag were performed using Z-test statistics (24).

Accession number

5′-end and MSCC tags have been deposited in the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP007816.

Results

Isolation of Ag-specific memory CD4$^+$ T cells

To characterize memory T cells using methylome and transcriptome analysis, we generated memory CD4$^+$ T cells from DO11.10 OVA-specific TCR-Tg mice. Splenic CD4$^+$ T cells from the DO11.10-Tg mice were stimulated with an OVA323–339 peptide plus allophycocyanin for 5 d in vitro and then transferred i.v. into normal syngeneic BALB/c recipient mice. The transferred DO11.10-Tg T cells were monitored by staining with a clonotypic KJ1 mAb. At the time of transfer, cell surface marker expression was CD44^{high} CD127$^+$ CD25$^+$ CD69$^+$ and CD62L$^+$, but by 4 wk after cell transfer the activation markers CD25 and CD69 were no longer expressed (Fig. 1A). These observations support the development of effector and memory T cell phenotypes, respectively. To confirm the functional status of these cells, cytokine-production profiles of naive and Ag-stimulated effector and memory cell populations were investigated. Within effector and memory T cell populations, 24 and 43%, respectively, expressed IFN-γ but not IL-4, within which 28 and 50% of cells coexpressed TNF-α (Fig. 1B).

DNA-methylation profiling in memory T cells

In this study, we used a recently developed MSCC method (23) that enables high-throughput, genome-wide identification of methylated CpG sites by SOLiD sequencing. Using the HpaII restriction nuclease, which recognizes unmethylated CCGG, most short-sequence tag fragments at HpaII cleavage sites can be uniquely mapped to genome locations. Methylation-sensitive restriction enzymes typically have a recognition site that contains a CpG dinucleotide, and cleavage is blocked if that site is methylated. Sites with many reads are inferred to have low methylation levels, whereas sites with few or no reads are inferred to have high methylation levels. The murine genome contains 1,594,139 CCGG sites, of which 1,130,065 (71%) can be uniquely mapped. Although each restriction enzyme site can generate two library tags, we considered the sum of tag sequences for each restriction enzyme site. A total of 619,060 sites (55%) was located within the promoter and gene body regions of unique genes, and 11% of these were within CpG islands (CGIs). A control library was also constructed by replacing HpaII with MspI, a methylation-insensitive isoschizomer of HpaII. The tags cut with MspI were used for determining zero-tag count or nonhit sites, because no tag from a HpaII library may correspond to a fully methylated site or false negative.

Using the SOLiD platform, ~65 million reads of methylation tags from naive, effector, and memory CD4$^+$ T cell genomes cut with HpaII or MspI were aligned to the mouse genome, with at most two mismatches, to allow for sequencing errors and single nucleotide polymorphisms. Thirty-four million (52%) of these tags were aligned to unique sites after repetitive sequences were excluded (Table I). These MSCC data were analyzed for the methylation levels of individual sites based on bisulfite sequencing. When MSCC tag counts and DNA methylation for randomly selected HapII sites were compared, the number of MSCC methylation tags correlated with the methylation levels derived from bisulfite data, consistent with results reported previously (23) (Fig. 2). Therefore, we defined three categories of methylation sites: low or hypo (median methylation <20%), intermediate (>20 to <80%), and high or hyper (>80%). A total of 65 and 64% of unique CpG sites in naive and memory CD4$^+$ T cells, respectively, was hypermethylated, whereas 13% in both naive and memory cells had low methylation. Around TSSs, 28 and 31% of sites in naive and memory cells, respectively, were hypermethylated, whereas 45 and 41%, respectively, had low methylation. In addition, only 28 and 30% of CGIs in naive and memory cells, respectively, were methylated.

Comparison of CpG methylation between naive and memory T cells

To observe changes in DNA methylation during T cell differentiation, the methylation status of CpG sites in gene-associated

No. of DMRs	No. of sites	*P-value*	Position
52	96,011	3.05E−7	Promoter+1st exon
54	84,749	2.00E−04	Exon (2nd−)
446	438,300	9.03E−01	Intron
592	511,005	1.06E−05	Inter−genic regions
1144	1,130,065		Total

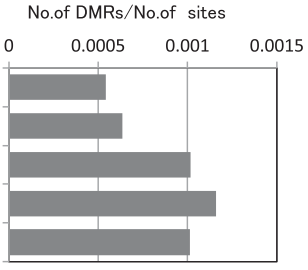

FIGURE 3. DMRs in DNA from naive and memory CD4$^+$ T cells. DMRs were classified based on their location in promoter (up to 500 bp from a TSS, based on RefSeq annotation), exon, intron, and intergenic regions based on their position relative to known genes. The number of sites represents defined HpaII restriction sites. The p values were calculated using the Fisher exact test.

Table II. Methylation of the 5′-region of naive and memory CD4$^+$ T cell genes with a DMR in an intron

No. of Tags		
Naive cells	Memory cells	No. of Genes (%)
Hypomethylation (≥10)	Hypomethylation (≥10)	273 (87.5)
Hypomethylation (≥10)	Hypermethylation (≤2)	0 (0)
Hypermethylation (≤2)	Hypomethylation (≥10)	1 (0.3)
Hypermethylation (≤2)	Hypermethylation (≤2)	29 (9.3)
Obscure methylation		9 (2.9)
Total		312 (100)

regions (the gene body including 500 bp upstream from the TSS) was compared between naive and memory T cells. When a DMR was defined as a change from 0 to >10 tags at sites cut by MspI, 1144 sites were identified as DMRs during T cell differentiation (Supplemental Table II). Fifty-one percent (552) of these DMRs were in gene-associated regions, and 467 sites associated with 437 genes were unmethylated in memory cells. In contrast, 85 sites associated with 84 genes were methylated in memory cells. The remaining 49% of the DMRs were in intergenic regions. Fig. 3 shows the DMR positions in the genome. The number of DMRs in the 5′-region (500 bp upstream from the TSS and first exon) was significantly lower than in other regions. Many DMRs were located in introns, with a few in CGIs. Our data indicated that DNA methylation in gene-promoter regions did not always correspond to a repressive epigenetic event in CD4$^+$ T cells. It is well known that the region upstream of a gene, including the promoter, is important for gene expression. Thus, we examined the DNA methylation status of gene-upstream regions (promoter and first exon) for DMRs. Others investigators reported a correlation between the methylation status of adjacent CpG sites and a high incidence of short-range comethylation (26, 27). Eighty-eight percent of genes with DMRs showed hypomethylation in their promoter/first exon in naive and memory T cells (Table II). CpG methylation of the first intron and second exon of *Cish* and of the first intron of *Tbx21*, but not of the promoter regions, was different between naive and memory T cells (Fig. 4). The results of MSCC analysis of a series of DMRs was consistent with bisulfite sequencing data. These data suggest that DNA methylation in the gene body (introns and after second exons) may be characteristic of the memory cell phenotype. To identify the function of genes differentially methylated between naive and memory T cells, genes with DMRs were classified using the Gene Ontology Consortium database (GO) (Table III). Genes associated with cell communication, signal transduction, and intracellular signaling pathways tended to be hypomethylated in memory T cells. In contrast, genes associated with development processes and biological regulation tended to be hypomethylated in naive T cells.

DNA methylation and gene expression in memory T cells

To investigate the relationship between gene expression and changes in CpG methylation in DMRs, we analyzed the gene expression of naive cells, in vitro–activated effector cells, and memory CD4$^+$ T cells using the Illumina/Solexa sequencing system. More than 12 million 25-base 5′-SAGE tags were obtained from the three libraries and matched to sequences in the

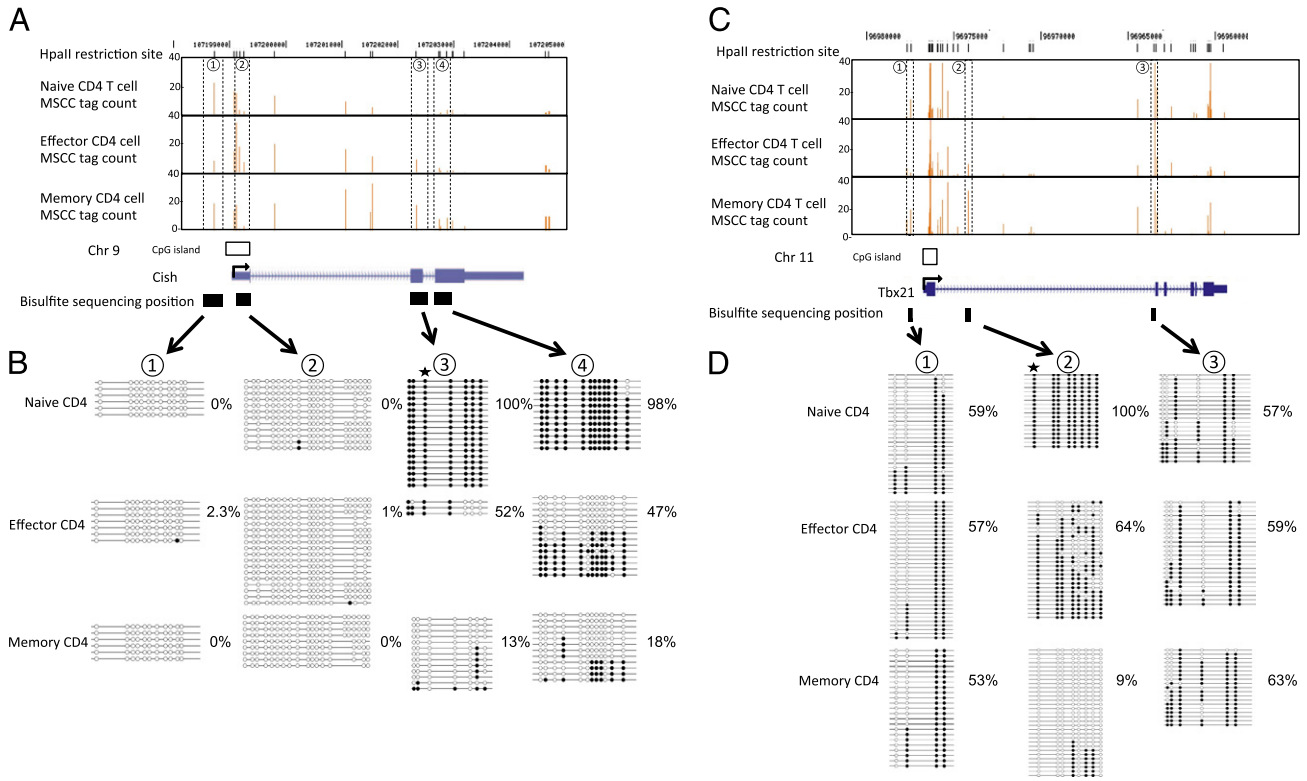

FIGURE 4. DMRs in the *Cish* and *Tbx21* loci of naive, effector, and memory T cells. Genomic organization of the mouse *Cish* (**A**) and *Tbx21* (**C**) loci, showing transcription start sites (arrows), single CGI (boxes), and exons (light blue). MSCC analysis of naive, effector, and memory T cells was across the 5′-end of each loci. Each vertical line (brown) represents a mean normalized tag from the MSCC analysis at the genomic location (listed on the *x*-axis) within the *Cish* and *Tbx21* loci on chromosomes 9 and 11, respectively (University of California, Santa Cruz genome browser). Results of genomic bisulfite sequencing for *Cish* (**B**) and *Tbx21* (**D**). Each row of circles represents an individual clone sequenced in the analysis after bisulfite treatment and PCR. Open circles indicate CpG sites at which no DNA methylation was detected. Filled circles indicate CpG sites that were methylated. Stars indicate the position of restriction sites detected by MSCC. Percentage values indicate the DNA methylation ratio of each region, as measured by bisulfite sequencing.

Table III. Gene ontology of DMR-associated genes

Best GO	Category	Count	Total	p Value[a]
Hypomethylated in memory T cells				
GO:0007154	Cell communication	119	5560	3.49E−18
GO:0007165	Signal transduction	111	5142	2.65E−17
GO:0007242	Intracellular signaling pathway	55	1965	4.94E−14
GO:0007267	Cell–cell signaling	25	640	5.25E−11
GO:0032502	Developmental process	69	3347	1.20E−08
GO:0007275	Multicellular organismal development	53	2299	1.20E−08
GO:0032501	Multicellular organismal process	75	3822	2.33E−08
GO:0048731	System development	39	1605	5.67E−07
GO:0065007	Biological regulation	109	6731	7.44E−07
GO:0050789	Regulation of biological process	101	6140	1.22E−06
GO:0007215	Glutamate signaling pathway	6	21	3.93E−06
GO:0048519	Negative regulation of biological process	30	1182	7.67E−06
GO:0048856	Anatomical structure development	43	2005	8.94E−06
GO:0009966	Regulation of signal transduction	23	800	8.94E−06
GO:0048523	Negative regulation of cellular process	29	1137	8.96E−06
Hypomethylated in memory T cells				
GO:0032502	Developmental process	19	3347	4.57E−06
GO:0065007	Biological regulation	29	6731	4.57E−06
GO:0050789	Regulation of biological process	27	6140	6.68E−06
GO:0016070	RNA metabolic process	21	4155	8.43E−06

[a]Each category was based on a p value $< 1.0E-05$.

murine genome (Table IV). Seventy-four percent of unique mapped tags were associated with RefSeq cDNA sequences, corresponding to ~12,000–14,000 different protein-coding genes in this cell type (Supplemental Table III). The expression level of 1256 genes was significantly different between naive and effector cells, whereas 259 genes were expressed significantly differently between naive and memory cells ($p < 0.001$, >10-fold difference). The 30 genes with the largest relative difference between effector and naive cells and between memory and naive cells are listed in Table V.

When gene-expression levels and DMRs were compared between naive and memory CD4 T cells, 24 DMRs were associated with increased expression of genes (e.g., CXCR6, Tbox21, Chsy1, and Cish) in memory cells compared with naive cells (>10 tags and >4-fold difference) (Table VI). In contrast, 27 DMRs were associated with decreased expression of other genes (e.g., Maff, Ephb6, and Trpm2). Classification using GO revealed that these genes are related to signal transduction, cell communication, and immune responses. These findings indicate that key genes relating to the memory phenotype undergo variable changes in DNA methylation during $CD4^+$ T cell differentiation.

The relationship between DNA methylation and enhancer activity

To examine the functional implications of these DMRs, we constructed a luciferase reporter vector consisting of the EF1 promoter and sequences derived from the DMR in the introns of 15 genes, which positively and negatively correlated with gene expression. Transient transfections were performed in untreated or P/I-treated EL-4 T cells using unmethylated (CpG) or in vitro SssI-methylated (mCpG) reporter plasmids. The transcriptional activity of the luciferase reporter construct containing the DMR of *Ptgir*, *Tnfsf4*, *Tbx21*, *Cish*, *Chsy1*, *IL7r*, and *Acot7* genes was 2-fold greater than that of the empty control vector (pCpGL-EF1) (Fig. 5). For these genes, transcriptional activation was reduced following in vitro methylation of the CpGs in the corresponding DMRs, demonstrating a suppressive effect of methylation on enhancer function. In contrast, for the luciferase reporter constructs containing the DMR of seven of the eight genes that showed reduced expression in memory cells compared with naive cells, transcriptional activity was unchanged relative to the empty control vector. Further validation confirmed that MSCC tag counts correlated with bisulfite-sequencing data for these genes. For example, DMRs in *Klf7* and *Mapk1ip1* had higher MSCC counts in memory cells (indicating less DNA methylation) but higher expression levels in naive cells (Fig. 6). Thus, although these DMRs may possess an alternative function, such as inhibition of silencer binding to the gene region, they do not influence enhancer activity.

DNA methylation status in T cell subsets

We next investigated DNA methylation in effector $CD4^+$ T cells. Effector $CD4^+$ T cells were isolated 5 d after Ag stimulation for gene-expression analysis. Interestingly, DMR methylation in effector cells followed different kinetics during differentiation compared with naive and memory cells. DMRs were classified into six distinct groups by DNA-methylation analysis (Table VII). Twenty-seven percent of DMRs were hypermethylated in naive and effector cells but were hypomethylated in memory cells

Table IV. Summary of $CD4^+$ T cell sequencing

Cell Type	Sequenced Tags	Unique Tags	Mapped Tags (one locus)	Tags in RefSeq	Gene No.	Gene No. (>1 copy)
$CD4^+$ naive T cells	12,088,592	7,883,186	4,122,853	3,382,975	14,064	8,715
$CD4^+$ effector T cells	8,660,468	4,547,959	4,449,231	2,790,122	12,877	8,756
$CD4^+$ memory T cells	11,442,151	6,258,543	3,916,175	3,179,174	13,384	8,138

Unique tags were aligned to a position unambiguously. Unique tags in TSSs were the number of unique tags mapped to regions within 500 bases of the representative TSSs of genes in the RefSeq database. Unique tags were categorized into three groups based on the number of mismatches in individual alignments. Effector T cells were generated from $CD4^+$ T cells from DO11.10-Tg mice stimulated with an OVA peptide plus allophycocyanin conditions for 5 d in vitro. Memory CD4 T cells were isolated from spleen and lymph node at 4 wk after cell transfer. 1 copy = 20 tags/3 million tags, because human cells are predicted to contain 300,000 mRNA molecules.

Table V. Gene-expression profile of effector and memory CD4$^+$ T cells compared with naive CD4$^+$ T cells

Naive T Cells Effector > Naive	No. of Tags in Effector T Cells	No. of Tags in Memory T Cells	RefSeq	Description
0	54,848	4	NM_008630	Metallothionein 2
2	27,314	161	NM_139198	Placenta-specific 8
1	2,483	7	NM_011340	Serine or cysteine proteinase inhibitor clade
0	1,837	66	NM_001111099	Cyclin-dependent kinase inhibitor 1A P21
1	1,620	19	NM_145158	Elastin microfibril interfacer 2
0	1,354	5	NM_013542	Granzyme B
1	1,100	185	NM_008519	Leukotriene B4 receptor 1
5	6,117	7	NM_009375	Thyroglobulin
1	931	5	NM_133662	Immediate early response 3
1	904	1	NM_053095	IL 24
0	895	20	NM_021397	Repressor of GATA
2	1,461	17	NM_007796	CTL-associated protein 2
7	5,661	3819	NM_026820	IFN-induced transmembrane protein 1
11	7,979	94	NM_010370	Granzyme A
0	713	4	NM_001080815	Gastric inhibitory polypeptide receptor
0	543	21	NM_008147	gp49 A
1	448	9	NM_133720	Cysteinyl leukotriene receptor 2
2	879	3	NM_009150	Selenium binding protein 1
50	22,626	82	NM_011401	Solute carrier family 2 facilitated glucose
0	453	2	NM_147776	von Willebrand factor A domain–related protein
3	1,202	81	NM_011498	Basic helix-loop-helix domain containing class
2	724	0	NM_008156	GPI specific
0	348	1	NM_178241	IL-8 receptor α
63	21,938	26	NM_013602	Metallothionein 1
39	13,419	53	NM_001077508	TNF receptor superfamily
1	299	38	NM_008337	IFN γ
0	326	4	NM_001004174	Hypothetical protein LOC433470
0	325	0	NM_207279	Phosphatidylinositol-specific phospholipase C X
0	322	21	NM_013532	Leukocyte Ig-like receptor
0	319	0	NM_009137	Chemokine C-C motif ligand 22
Effector < Naive				
1517	0	159	NM_009777	Complement component 1 q subcomponent, B chain
665	0	93	NM_007574	Complement component 1 q subcomponent, C chain
590	0	39	NM_007572	Complement component 1 q subcomponent, A chain
426	0	43	NM_001083955	Hemoglobin α adult chain 2
407	0	384	NM_011703	Vasoactive intestinal peptide receptor 1
3535	10	1,617	NM_008052	Deltex 1 homolog
2037	7	121	NM_001042605	CD74 Ag isoform 1
306	0	4	NM_019577	Chemokine C-C motif ligand 24
289	0	14	NM_007995	Ficolin A
313	1	13	NM_001080934	Solute carrier family 16 monocarboxylic acid
219	0	5	NM_001037859	Colony stimulating factor 1 receptor
178	0	139	NM_033596	Cistone cluster 2 H4
146	1	14	NM_011414	Secretory leukocyte peptidase inhibitor
387	3	302	NM_013832	RAS protein activator like 1 GAP1 like
120	0	19	NM_133209	Paired immunoglobulin-like type 2 receptor β
117	0	9	NM_008220	Hemoglobin β adult major chain
96	1	33	NM_025806	Hypothetical protein LOC66857
78	0	102	NM_145227	2′-5′ oligoadenylate synthetase 2
78	0	163	NM_178185	Histone cluster 1 H2ao
78	0	283	NM_001033813	Hypothetical protein LOC619310
85	1	7	NM_008076	γ-aminobutyric acid GABA-C receptor
74	0	3	NM_177686	C-type lectin domain family 12 member a
79	1	3	NM_016704	Complement component 6
71	1	6	NM_009913	Chemokine C-C motif receptor 9
64	0	11	NM_138673	Stabilin-2
64	0	9	NM_001024932	Paired immunoglobulin-like type 2 receptor β 2
69	1	5	NM_011518	Spleen tyrosine kinase
523	9	28	NM_009525	Wingless-related MMTV integration site 5B
59	0	4	NM_009721	Na+/K+ -ATPase β 1 subunit
1615	28	734	NM_010494	ICAM 2
Memory > Naive				
7	5,661	3819	NM_026820	IFN-induced transmembrane protein 1
2	13	931	NM_001099217	Lymphocyte Ag 6 complex locus C2
15	59	3884	NM_010741	Lymphocyte Ag 6 complex locus C
1	1,100	185	NM_008519	Leukotriene B4 receptor 1
2	122	360	NM_015789	Dickkopf-like 1
1	5	163	NM_010553	IL 18 receptor accessory protein
0	309	179	NM_031395	Synaptotagmin-like 3 isoform a
1	2	146	NM_009915	Chemokine C-C motif receptor 2

(*Table continues*)

Table V. (*Continued*)

No. of Tags in				
Naive T Cells Effector > Naive	Effector T Cells	Memory T Cells	RefSeq	Description
12	641	1,661	NM_011313	S100 calcium binding protein A6 calcyclin
0	73	129	NM_177716	Hypothetical protein LOC239650
23	4,403	2,963	NM_030694	IFN-induced transmembrane protein 2
209	317	22,679	NM_013653	Chemokine C-C motif ligand 5
1	114	88	NM_146064	Acyl-CoA:cholesterol acyltransferase 2
1	3	84	NM_133643	EDAR ectodysplasin-A receptor–associated death
2	27,314	161	NM_139198	Placenta-specific 8
3	16	224	NM_013599	Matrix metallopeptidase 9
1	53	70	NM_030712	Chemokine C-X-C motif receptor 6
4	72	268	NM_011311	S100 calcium binding protein A4
3	140	186	NM_019507	T-box 21
4	1,051	238	NM_024253	NK cell group 7 sequence
0	1,837	66	NM_001111099	Cyclin-dependent kinase inhibitor 1A P21
1	4	59	NM_016685	Cartilage oligomeric matrix protein
12	34	815	NM_009910	Chemokine C-X-C motif receptor 3
1	0	50	NM_016958	Keratin 14
1	41	44	NM_008967	PG I receptor IP
132	729	5,823	NM_001013384	Podocan-like 1
0	31	43	NM_010177	Fas ligand TNF superfamily member 6
1	299	38	NM_008337	IFN γ
1	1	37	NM_010730	Annexin A1
1	4	36	NM_018734	Guanylate nucleotide binding protein 4
Naive > Memory				
817	114	8	NM_207231	ADP-ribosylation-like factor 12 protein
82	37	1	NM_175274	Tweety 3
306	0	4	NM_019577	Chemokine C-C motif ligand 24
75	74	0	NM_010358	Gst μ 1
61	52	1	NM_011129	Septin 4
53	63	1	NM_027406	Aldehyde dehydrogenase 1 family member l1
51	1	1	NM_029162	Zinc finger protein 509
51	0	1	NM_008694	Neutrophilic granule protein
51	1	1	NM_153510	Paired immunoglobulin-like type 2 receptor α
51	24	1	NM_007405	Adenylate cyclase 6
48	37	1	NM_011984	Homer homolog 3
148	5	3	NM_009238	SRY-box containing gene 4
46	12	1	NM_013569	Voltage-gated potassium channel subfamily H,
44	10	1	NM_026629	Hypothetical protein LOC68235
43	25	1	NM_011692	Von Hippel-Lindau binding protein 1
219	0	5	NM_001037859	Colony stimulating factor 1 receptor
115	22	3	NM_008538	Myristoylated alanine rich protein kinase C
36	22	1	NM_177758	Zinc finger and SCAN domains 20
36	0	1	NM_013612	Solute carrier family 11 proton-coupled
36	7	1	NM_009223	Stannin
72	18	2	NM_001033929	Threonine synthase-like 2
36	53	0	NM_011232	RAD1 homolog
94	18	3	NM_011639	Thyroid receptor-interacting protein 6
30	4	1	NM_133921	Ovarian zinc finger protein
30	110	1	NM_020006	CDC42 effector protein Rho GTPase binding 4
33	16	0	NM_009372	TG-interacting factor
29	1	1	NM_013667	Solute carrier family 22 member 2
28	14	1	NM_030557	Myoneurin
177	25	6	NM_001081127	A disintegrin-like and metallopeptidase
31	103	0	NM_033612	Elastase 1 pancreatic

The 30 genes with the largest relative differences between effector and naive cells and between memory and naive cells are listed. The total number of tags from naive (3,382,975), effector (2,790,122), and memory (3,179,174) cells was normalized to 3,000,000.

(pattern 1). For example, the extent of DNA methylation in the DMR of *CXCR6* was 92% in naive cells, 80% in effector cells, and 6% in memory T cells (Supplemental Fig. 1). Moreover, 43% of DMRs were hypermethylated in the naive phase, intermediately methylated in the effector phase, and hypomethylated in the memory phase (pattern 2). In *Cish*, for example, DNA methylation in the DMR in the second exon was 100% in naive cells, 52% in effector cells, and 13% in memory cells. An additional 17% of DMRs were hypermethylated in naive cells, intermediately methylated in effector cells, and hypomethylated in memory cells (pattern 3). GO classifications for each DMR methylation pattern revealed that genes in pattern 1 mostly fell into GO categories related to cell communication and signal transduction, whereas genes in pattern 3 aligned with GO categories related to negative regulation of cellular processes (Table VIII). These data indicate that the timing of methylation changes during T cell differentiation is regulated independently for each gene.

It is well known that central and effector memory T cells are distinct in their differentiation status. Therefore, we also investigated the DNA methylation status of selected DMRs in subpopulations of central and effector memory $CD4^+$ T cells from an untreated conventional BALB/c mouse. These DMRs were different across various T cell subsets, reinforcing the finding that the methylation status of T cell subsets reflects T cell differentiation (Fig. 7).

Table VI. Correlation between DNA methylation and gene expression in naive, effector, and memory CD4$^+$ T cells

Restriction Site	Chr	No. of Nucleotides from Nearest TSS	Symbol	Description	RefSeq	Position	Distance from Nearest CGI (bp)	DNA Methylation Score[a]						Gene Expression				
								Naive Cell HpaII	Naive Cell MspI	Effector Cell HpaII	Effector Cell MspI	Memory Cell HpaII	Memory Cell MspI	N4/M4 Fold	M4/N4 Fold	Naive CD4	Effector CD4	Memory CD4
123716994	Chr9	1,392	Cxcr6	Chemokine C-X-C motif receptor 6	NM_030712	Intron1	43,618	0	5	2	7	18	4	0	78	1	53	70
96974152	Chr11	2,440	Tbx21	T-box 21	NM_019507	Intron1	−1,727	0	2	8	8	30	5	0	69	3	140	186
17493213	Chr7	1,375	Ptgir	PG I receptor IP	NM_008967	Intron1	−876	0	10	5	6	11	6	0	49	1	41	44
73291652	Chr7	37,252	Chsy1	Carbohydrate chondroitin synthase 1	NM_001081163	Intron2	−37,769	0	10	8	9	48	6	0	20	16	127	326
9453075	Chr15	6,536	Il7r	IL 7 receptor precursor	NM_008372	Intron2	383,054	0	4	6	1	13	5	0	17	29	198	498
151561336	Chr4	9,094	Acot7	Acyl-CoA thioesterase 7	NM_133348	Intron1	−9,631	0	5	5	6	12	4	0	14	66	289	949
107202323	Chr9	3,304	Cish	Cytokine inducible SH2-containing protein	NM_009895	Exon_2/3	−3,446	0	1	8	9	17	5	0	10	53	5018	528
107201507	Chr9	2,488	Cish	Cytokine inducible SH2-containing protein	NM_009895	Intron1	−2,630	0	2	0	0	12	1	0	10	53	5018	528
112879061	Chr6	17,427	Srgap3	SLIT-ROBO Rho GTPase activating protein 3	NM_080448	Intron1	−117,067	0	16	10	10	20	14	0	9	5	241	46
41404992	Chr19	54,614	Pik3ap1	Phosphoinositide-3-kinase adaptor protein 1	NM_031376	Intron3	−45,447	0	4	4	2	17	4	0	5	5	14	28
41404611	Chr19	54,995	Pik3ap1	Phosphoinositide-3-kinase adaptor protein 1	NM_031376	Intron3	−45,828	0	11	9	15	11	9	0	5	5	14	28
43981837	Chr4	11,264	Glipr2	GLI pathogenesis–related 2	NM_027450	Intron3	−11,607	0	6	1	2	32	2	0	5	59	795	285
52379040	Chr2	153,059	Cacnb4	Calcium channel voltage–dependent, β 4	NM_001037099	Intron2	99,263	0	7	7	10	34	6	0	4	6	3	26
60189952	Chr2	31,367	Ly75	Lymphocyte Ag 75	NM_013825	Intron11	−31,177	0	1	4	2	12	5	0	4	12	56	47
29371127	Chr17	3,606	Cpne5	Copine V	NM_153166	Intron1	−3,256	0	10	3	11	11	8	0	4	4	37	18
44355013	Chr17	29,493	Clic5	Chloride intracellular channel 5	NM_172621	Intron1	−29,555	0	7	10	5	52	9	0	4	4	8	14
28393931	Chr2	25,081	Ralgds	Ral guanine nucleotide dissociation stimulator	NM_009058	Intron1	−4,975	0	8	13	15	10	1	0	4	8	81	32
155388145	Chr4	342	Tnfrsf4	TNF receptor superfamily	NM_011659	Intron1	20,771	0	5	21	6	22	3	0	4	394	5522	1455
79745901	Chr17	8,528	Cdc42ep3	CDC42 effector protein Rho GTPase binding 3	NM_026514	Intron1	−7,859	0	9	3	14	15	3	0	4	5	2	19
27822267	Chr2	80,063	Col5a1	Procollagen type V, α 1	NM_015734	Intron16	−80,584	0	16	3	9	11	10	0	4	4	1	12

(*Table continues*)

Table VI. (*Continued*)

Restriction Site	Chr	No. of Nucleotides from Nearest TSS	Symbol	Description	RefSeq	Position	Distance from Nearest CGI (bp)	DNA Methylation Score[a]						Gene Expression				
								Naive Cell HpaII	Naive Cell MspI	Effector Cell HpaII	Effector Cell MspI	Memory Cell HpaII	Memory Cell MspI	N4/M4 Fold	M4/N4 Fold	Naive CD4	Effector CD4	Memory CD4
41565822	Chr6	10,256	Ephb6	Eph receptor B6	NM_007680	Intron6	−10,660	0	10	7	5	10	11	12	0	207	11	17
77375561	Chr10	54,639	Trpm2	Transient receptor potential cation channel	NM_138301	Intron29	10,730	0	5	12	8	10	3	11	0	21	2	2
58854000	Chr15	59,542	Mtss1	Actin monomer-binding protein	NM_144800	Intron3	−58,831	0	4	15	6	21	3	9	0	179	60	19
79178744	Chr15	637	Maff	V-maf musculoaponeurotic fibrosarcoma oncogene	NM_010755	Intron1	−1,005	0	2	0	1	16	1	9	0	25	122	3
38564748	Chr2	5,312	Nr5a1	Nuclear receptor subfamily 5 group A, member 1	NM_139051	Intron3	−1,049	0	9	1	0	10	2	9	0	17	13	2
75013736	Chr12	4,720	Hif1a	Hypoxia inducible factor 1 α subunit	NM_010431	Intron1	−5,274	0	10	11	7	21	4	8	0	59	276	8
49653434	Chr2	10,229	2310010M24Rik	Hypothetical protein LOC71897	NM_027990	Intron1	−10,474	0	7	2	10	13	7	7	0	51	11	8
124129324	Chr5	4,974	Clip1	Restin	NM_019765	Intron1	−4,195	0	1	8	7	10	2	7	0	289	61	44
124474362	Chr5	7,905	Vps37b	Vacuolar protein sorting 37B	NM_177876	Intron1	−7,571	0	15	0	6	10	10	6	0	2537	1085	394
63517774	Chr14	61,260	Wdfy2	WD repeat and FYVE domain containing 2	NM_175546	Intron2	−61,692	0	4	35	7	25	2	6	0	30	17	5
56872366	Chr18	4,840	Lmnb1	Lamin B1	NM_010721	Intron1	−5,420	0	5	1	6	18	2	6	0	73	53	12
3378068	Chr10	179,872	Oprm1	Opioid receptor μ 1	NM_001039652	Intron3	243,866	0	7	3	14	14	6	5	0	30	3	6
5574804	Chr10	159,689	Esr1	Estrogen receptor 1 α	NM_007956	Intron3	−58,813	0	16	1	9	11	13	5	0	46	7	9
66089658	Chr17	32,361	Rab31	Rab31-like	NM_133685	Intron1	−31,962	0	3	5	4	23	5	5	0	19	27	4
54300515	Chr13	13,018	Hrh2	Histamine receptor H 2 isoform 1	NM_001010973	Intron1	−13,119	0	6	1	10	14	0	5	0	32	4	7
71926521	Chr12	21	Frmd6	FERM domain containing 6	NM_028127	Exon_1/ 14_first exon	0	0	4	8	0	14	1	4	0	38	31	9
58574849	Chr6	28,184	Abcg2	ATP-binding cassette subfamily G, member 2	NM_011920	Intron1	15,477	0	6	7	1	10	0	4	0	39	103	9
88790629	Chr12	5,768	1810035L17Rik	Hypothetical protein LOC380773	NM_026958	Intron3	−6,286	0	4	6	2	15	9	4	0	34	130	9
64135805	Chr1	32,156	Klf7	Kruppel-like factor 7 ubiquitous	NM_033563	Intron1	−32,381	0	6	4	11	17	1	4	0	108	48	27
21359913	Chr2	70,720	Gpr158	G protein–coupled receptor 158	NM_001004761	Intron2	−69,515	0	8	0	3	13	10	4	0	11	3	3
13609664	Chr8	67,921	Rasa3	RAS p21 protein activator 3	NM_009025	intron3	−67,326	0	8	5	2	14	6	4	0	570	349	157
24828918	Chr8	48,754	Zmat4	Zinc finger matrin type 4	NM_177086	intron1	−48,830	0	10	3	14	11	16	4	0	40	5	11

(*Table continues*)

Table VI. (*Continued*)

Restriction Site	Chr	No. of Nucleotides from Nearest TSS	Symbol	Description	RefSeq	Position	Distance from Nearest CGI (bp)	DNA Methylation Score[a]						Gene Expression				
								Naive Cell HpaII	Naive Cell MspI	Effector Cell HpaII	Effector Cell MspI	Memory Cell HpaII	Memory Cell MspI	N4/M4 Fold	M4/N4 Fold	Naive CD4	Effector CD4	Memory CD4
146036806	Chr7	1,142	Mapk1ip1	MAPK-interacting and spindle-stabilizing	NM_001045483	intron1	−827	0	7	3	5	11	5	4	0	233	152	66
96960335	Chr11	16,257	Tbx21	T-box 21	NM_019507	Exon_6/6_lastExon	−15,544	19	1	1	0	0	2	0	69	3	140	186
94729332	Chr1	1,070	Gpc1	Glypican 1	NM_016696	Intron1	0	16	2	0	0	0	1	0	21	11	22	227
128915487	Chr4	10,198	C77080	Hypothetical protein LOC97130	NM_001033189	Intron1	−581	12	3	1	1	0	2	0	6	3	3	15
148238956	Chr4	60,456	Casz1	Castor homolog 1 zinc finger	NM_027195	Intron2	26,131	10	17	1	16	0	7	0	4	10	13	36
120328900	Chr2	39,292	Capn3	Calpain 3 isoform a	NM_007601	Exon_21/24	60,309	15	7	2	6	0	13	22	0	61	9	3
35990963	Chr18	1,492	Cxxc5	CXXC finger 5	NM_133687	Intron1	0	28	4	9	2	0	1	13	0	12	22	1
126838794	Chr8	83,501	Galnt2	UDP-*N*-acetyl-α-D-galactosamine: polypeptide	NM_139272	Intron3	−83,718	16	5	0	2	0	1	8	0	128	55	16
63546881	Chr14	90,367	Wdfy2	WD repeat and FYVE domain containing 2	NM_175546	Intron4	−90,799	20	1	1	9	0	6	6	0	30	17	5

The category was represented using the criteria of DMRs (changing from 0 to >10 tags at the sites able to be digested by MspI between naive and memory CD4 T cells) and gene expression (memory or naive; >10 tags and >4-fold difference). Each number of gene-expression tags from naive (3,382,975), effector (2,790,122), and memory (3,179,174) cells was normalized to 3,000,000.

[a]DNA methylation score is described in *Materials and Methods*.

Gene	MCSS Tag No.		Expression Tag No.	
	Naive	Memory	Naive	Memory
Ptgir	0	45	12	44
Tnfrsf4	0	22	394	1455
Tbx21	0	30	3	186
Cish	0	12	53	528
Chsy1	0	48	16	326
IL7r	0	13	29	498
Acot7	0	12	66	949
RASA3	0	14	570	157
Maff	0	16	25	3
Hrh2	0	14	32	7
Mtss1	0	21	179	19
Klf7	0	17	108	27
Wdfy2	0	25	30	5
Nr5a1	0	10	17	2
Mapk1ip1	0	11	122	22

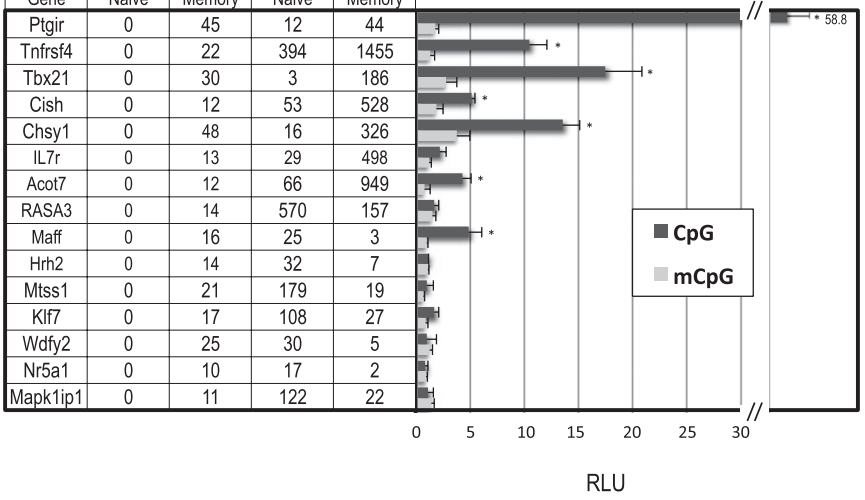

FIGURE 5. Transcriptional activity of a luciferase reporter gene in unmethylated and methylated DMR sequences from the introns of 15 genes. Transient transfections were performed with a control plasmid (pCpGL-EF1 promoter) or pCpGL-EF-DMR in P/I-treated EL-4 T cells using unmethylated (CpG) or in vitro SssI methylated (mCpG) reporter plasmids. Firefly raw light unit (RLU) data were normalized to *Renilla* luciferase activity relative to the control vector with no insert. $*p < 0.05$, unmethylated versus methylated plasmids, paired Student *t* test.

Discussion

Following activation with Ag, naive T cells differentiate into short-lived effector T cells and long-lived memory T cells. However, the molecular mechanisms behind the generation and maintenance of memory CD4$^+$ T cells remain unclear. To address this problem, we studied changes in epigenetic modification and gene expression in Ag-specific CD4$^+$ T cells using massive parallel DNA sequencing.

Phenotypically, both naive and memory T cell subsets are made up of small resting cells with upregulated IL-7R expression, which is necessary for their survival in vivo. Effector and memory T cells exhibit increased expression of adhesion markers (e.g., CD44 and LFA-1) and decreased expression of the lymph node homing receptor CD62L (28). This expression pattern was confirmed in the current study. Furthermore, our analyses indicated that, compared with naive CD4$^+$ T cells, the genes that were upregulated in memory CD4$^+$ T cells (e.g., IL-7R, Bcl2, Bcl2l1, and Cdkn1a and the chemokine-related genes CCL5, CCR2, CXCR6, and CXCR3) were related to cytokine production and development and maintenance of the memory phase. Expression of the Th1 genes IFN-γ, Tbox21, and IL18RAP also increased in memory CD4$^+$ T cells. In addition, the expression of several other genes [i.e., IFN-induced *trans*-membrane protein 1 (IFITM1) (29), Dkkl1 (30), and Il18rap (31)], which are related to proliferative capacity and Th1-type immunological reactions, increased in memory CD4$^+$ T cells compared with naive T cells.

It is well known that gene expression involves activation of transcription factors and/or epigenetic changes in the genome. CpG dinucleotides upstream of genes that are active in a particular tissue or cell type are less methylated, whereas inactive genes are surrounded by highly condensed chromatin and have densely methylated upstream CpG dinucleotides. A useful technique for gauging gene accessibility in the chromatin context is to monitor sensitivity of the relevant DNA sequences to digestion with DNaseI in intact nuclei (32). In general, genome sites encoding genes located in active chromatin that are actively transcribed or that have the potential to be transcribed upon stimulation are more sensitive to DNase I digestion than are sites encoding genes in inactive or closed chromatin. In this study, we used the recently developed MSCC method that enables cost-effective, high-throughput, genome-wide identification of methylated CpG sites. We identi-

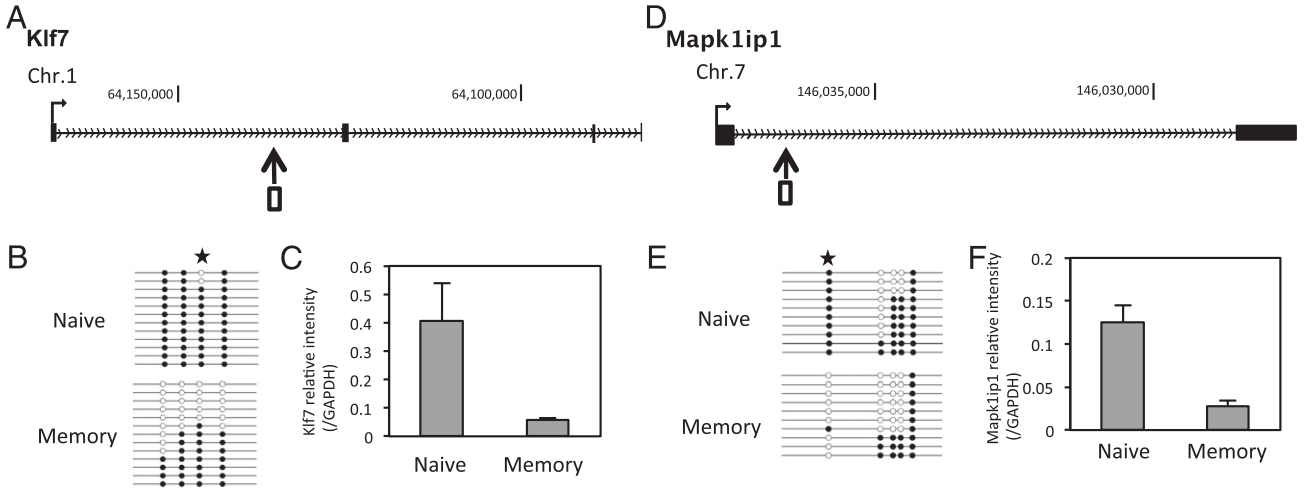

FIGURE 6. DMRs in the *Mapk1ip1* and *Klf7* loci of naive and memory T cells. Genomic organization of the mouse *Klf7* (**A**) and *Mapk1ip1* (**D**) loci showing transcription start sites (→), exons (black boxes), DMRs that were detected by MSCC (↑), and bisulfite sequencing positions (white boxes). (**B** and **E**) Results of genomic bisulfite sequencing, where each row of circles represents an individual clone sequenced following bisulfite treatment and PCR. Open circles indicate CpG sites at which no DNA methylation was detected. Stars indicate the position of restriction sites detected by MSCC. Filled circles indicate CpG sites that were methylated. (**C** and **F**) Downregulated gene expression in memory CD4 T cells measured by quantitative real-time PCR. RT-PCR was performed as described in *Materials and Methods*.

Table VII. DNA methylation status of DMRs in naive, effector, and memory $CD4^+$ T cells

Pattern	DNA Methylation Status			No. of DMR (%)
	Naive	Effector	Memory	
1	High	High	Low	314 (27%)
2	High	Int	Low	495 (43%)
3	High	Low	Low	198 (17%)
4	Low	Low	High	25 (2%)
5	Low	Int	High	42 (4%)
6	Low	High	High	70 (6%)
	Total			1144 (100%)

High, High methylation status (≤2); Int, intermediate methylation status (3–9 tags); Low, low methylation status (>9 tags).

fied 1,144 regions in the mouse genome that were differentially methylated in the process of T cell differentiation. All of these DMRs were in gene body sites without CGIs, highlighting the fact that DNA methylation can occur at sites other than CGIs. Irizarry et al. (33) reported that methylation of CGI shores that exist in close proximity (~2 kb) to CGIs is closely associated with transcriptional inactivation. Most tissue-specific DNA methylation seems not to occur within CGI, but rather at CGI shores. However, our data demonstrate that most DMRs in naive and memory $CD4^+$ T cells are not associated with CGI or CGI shores. Furthermore, most DMRs in naive and memory $CD4^+$ T cells were located in gene bodies, rather than in the promoter regions, as is the case for tumor cells.

Of the DMRs identified in naive and memory $CD4^+$ T cells, 51 were potentially associated with gene expression. Gene body methylation is common in ubiquitously expressed genes and is correlated with gene expression (23). Furthermore, intergenic methylation recently was reported to play a major role in regulating cell context–specific alternative promoters in gene bodies (34). In contrast, several groups (19, 35, 36) reported that, in human and mouse regulatory T cells, the majority of DMRs are located at promoter-distal sites and that many of these regions display DNA methylation-dependent enhancer activity in reporter gene assays. Tsuji-Takayama et al. (37) demonstrated that production of IL-10 in regulatory T cells was enhanced by IL-2 through a STAT5-responsive intron enhancer in the IL-10 locus. However, Lai et al. (38) reported that DNA methylation in an

Table VIII. GOs classified by methylation state of DMRs in effector cells

GO Hyper(N)-Hyper(E)-Hypo(M)	Genes	Count	Total	p Value
GO:0007154	Cell communication	25	5560	0.00507
GO:0007165	Signal transduction	23	5142	0.00772
GO:0016477	Cell migration	5	233	0.00772
GO:0006928	Cell motility	6	383	0.00772
GO:0051674	Localization of cell	6	383	0.00772
GO:0022610	Biological adhesion	9	960	0.00772
GO:0007155	Cell adhesion	9	960	0.00772
Hyper(N)-Int(E)-Hypo(M)				
GO:0007154	Cell communication	74	5560	6.09E−12
GO:0007165	Signal transduction	69	5142	2.71E−10
GO:0007242	Intracellular signal transduction	33	1965	2.85E−07
GO:0007275	Multicellular organismal development	33	2299	6.72E−05
GO:0007267	Cell–cell signaling	17	640	8.05E−05
GO:0032502	Developmental process	42	3347	0.000126
GO:0051179	Localization	51	4481	0.000243
GO:0007215	Glutamate signaling pathway	4	21	0.00075
GO:0032501	Multicellular organismal process	44	3822	0.000793
GO:0009966	Regulation of signal transduction	17	800	0.000793
GO:0048731	System development	23	1605	0.00236
GO:0051234	Establishment localization	45	4135	0.00298
GO:0006810	Transport	44	4035	0.00327
GO:0050789	Regulation of biological process	60	6140	0.00428
GO:0007268	Synaptic transmission	9	290	0.00434
GO:0048856	Anatomical structure development	26	2005	0.00472
GO:0065007	Biological regulation	64	6731	0.00472
Hyper(N)-Hypo(E)-Hypo(M)				
GO:0048523	Negative regulation of cellular process	13	1137	0.000127
GO:0048519	Negative regulation of biological process	13	1182	0.000127
GO:0050794	Regulation of cellular process	26	5704	0.000748
GO:0065007	Biological regulation	28	6731	0.00227
GO:0050789	Regulation of biological process	26	6140	0.00289
GO:0018212	Peptidyl-tyrosine modification	3	44	0.0064
GO:0007242	Intracellular signal transduction	13	1965	0.0072
GO:0007165	Signal transduction	22	5142	0.00765
GO:0007154	Cell communication	23	5560	0.00893
Hypo(N)-Int(E)-Hyper(M)				
GO:0007275	Multicellular organismal development	9	2299	0.00989
GO:0032501	Multicellular organismal process	11	3822	0.00989
Hypo(N)-Hypo(E)-Hyper(M)				
None				
Hypo(N)-Hyper(E)-Hyper(M)				
None				

GOs with a p value < 0.01 are shown.

E, Effector T cells; Hyper, hypermethylation status (more than nine tags); Hypo, hypomethylation status (two or fewer tags); Int, intermediate methylation status (three to nine tags); M, memory T cells; N, naive T cells.

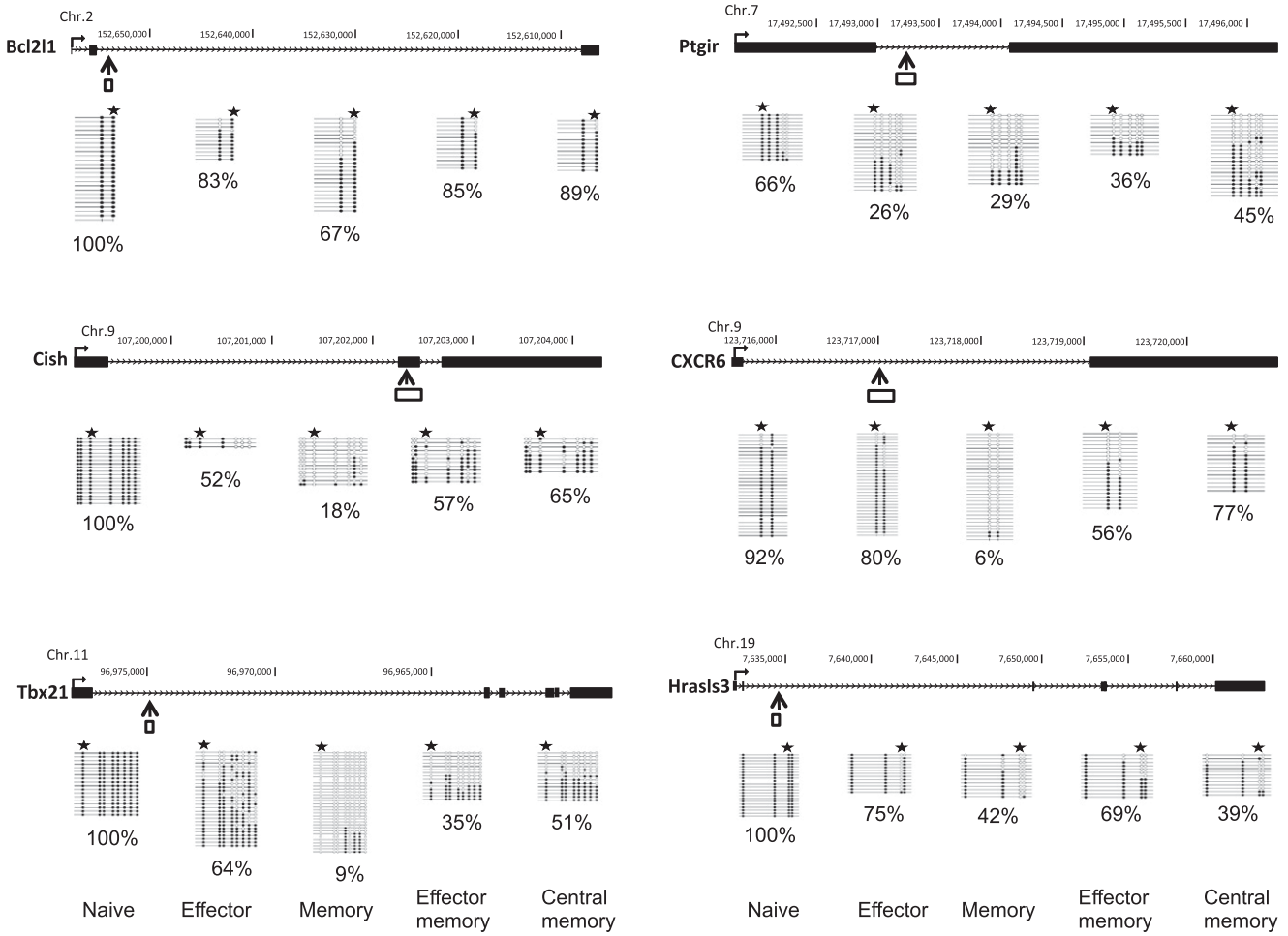

FIGURE 7. DNA methylation status of selected DMRs in subpopulations of central and effector memory CD4$^+$ T cells. CD62L$^+$ CCR7$^+$ and CD62L$^-$ CCR7$^-$ CD4 T cells from BALB/c mice were isolated to represent "central memory" and "effector memory" T cells, respectively. Genomic organization of the mouse *Cish*, *Hrasls3*, *Tbx21*, *CXCR6*, *Bcl2l1*, and *Ptgir* loci, showing transcription start sites (→), exons (black box), DMRs that were detected by MSCC (↑), and bisulfite sequencing position (white box). Graphs show results of genomic bisulfite sequencing, where each row of circles represents an individual clone sequenced in the analysis after bisulfite treatment and PCR. Open circles indicate CpG sites at which no DNA methylation was detected. Filled circles indicate CpG sites that were methylated. Stars indicate the position of restriction sites detected by MSCC. Percentage values indicate the DNA methylation ratio of each region, as measured by bisulfite sequencing.

intron can prevent enhancer-blocking transcription factor–mediated silencing. We used a reporter assay to examine the 51 gene-expression–associated DMRs and obtained results consistent with earlier reports. When loci containing DMRs were cloned into the reporter gene plasmid, the DMRs possessed enhancer activity in naive T cells in which DNA methylation was suppressed. Like previous studies, our results revealed different enhancer activities for different DMRs. It was reported that, compared with normal control cells, the DNA methylation of gene promoter regions differed in CD4$^+$ T cells in patients with rheumatoid arthritis (39), subacute cutaneous lupus erythematosus (40), and systemic lupus erythematosus (41). Together, these results suggest that, in the normal immune state, these DMRs are associated with enhancer activity rather than with promoter activity.

Genes associated with the 51 gene-expression–associated DMRs in naive and memory CD4$^+$ T cells were functionally categorized as relating to signal transduction, cell communication, and immune responses. As predicted, IL-7R, Bcl2l1, Tbox21, and CXCR6 genes were associated with changes in DNA methylation. Kim et al. (42) reported that DNA methylation is involved in regulating IL-7R expression in T cells. They found that IL-7Rα high CD8 T cells had stronger cell signaling and survival responses to IL-7 compared with IL-7Rα low CD8 T cells. Together with these findings, our results indicate that DNA methylation of the IL-7R gene in CD4$^+$ T cells may be a key mechanism for modifying IL-7–mediated T cell development and survival. In addition, in the current study, expression of Tbx21, as well as of the Th1-related gene Ptgir, was also correlated with DNA methylation. Lymph node cells from sensitized Ptgir($^{-/-}$) mice show reduced IFN-γ production and a smaller T-bet($^+$) subset compared with control mice (43).

There were also several genes relating to memory CD4$^+$ T cells homing to bone marrow (BM) that were associated with changes in DNA methylation. Tokoyada et al. (44) reported that >80% of Ly-6C^{hi}CD44^{hi}CD62L$^-$ memory CD4 T lymphocytes reside in the BM of adult mice and associate with IL-7–expressing VCAM-1 stroma cells. Our results demonstrate that Ly-6C is expressed more highly in memory CD4$^+$ T cells than in naive CD4$^+$ T cells. Because IL-7 is the main cytokine required for CD4$^+$ T cell survival (45), the BM is predicted to function as a survival niche for memory CD4$^+$ T cells. Thus, in the memory phase of immunity, memory Th cells are maintained in BM as resting, but highly reactive, cells in niches defined by IL-7–expressing stroma cells. In addition, when gene expression between CD44^{hi}CD62L$^-$CD4$^+$ T cells from the spleen and BM were compared, CD24, CD122, CXCR6, and CCR2 levels on CD44^{hi}CD62L$^-$CD4$^+$ T cells from the BM were higher than on the same cells from the spleen (45).

Our data also reveal upregulation of gene expression and unmethylation of *CXCR6* in the memory phase, suggesting that the unmethylation of DNA in gene body regions may be related to the homing of $CD44^{hi}CD62L^{-}CD4^{+}$ T cells to the BM.

In memory $CD4^{+}$ T cells, the genes Chsy1 and Itgb1 were linked to changes in DNA methylation in introns. Chsy1 synthesizes chondroitin sulfate and regulates many biological processes, including cell proliferation, recognition, and extracellular matrix deposition. Yin (46) showed that Chsy1 is the most prominent secreted protein in myeloma cell–osteoclast coculture conditioned medium and that Chsy1 activates Notch2 signaling in myeloma cells in the BM microenvironment. Therefore, Chsy1 may play an important role in cell–cell interactions, such as those between T cells and osteoclasts in the BM microenvironment. In contrast, Itgb1 is critical for maintenance of Ag-specific $CD4^{+}$ T cells in the BM (47). Therefore, DNA methylation in gene body regions is likely to play an important role in $CD4^{+}$ T cell homing to BM.

The expression of Cish was also associated with changes in DNA methylation in gene body regions. Cish is a member of the SOCS family, which was discovered as a negative regulator of cytokine signaling. However, in CD4 promoter-driven Cish-Tg mice, elevated Cish expression promotes T cell proliferation and survival after TCR activation relative to T cells in control mice (48). Moreover, Nakajima et al. (49) showed that expression of both Cish mRNA and protein is significantly increased in allergen-stimulated $CD4^{+}$ T cells from hen egg–allergic patients relative to patients not allergic to hen eggs. In addition, Khor et al. (50) identified a panel of Cish single nucleotide polymorphisms associated with increased susceptibility to infectious diseases, such as bacteremia, malaria, and tuberculosis. Thus, Cish expression caused by demethylation within the *Cish* locus in memory T cells may play a role in some infectious and allergic diseases.

In the current study, differences in methylated regions between naive and memory $CD4^{+}$ T cells did not always correlate with gene expression. The promoter and enhancer regions of differentially expressed genes were unmethylated, even in naive $CD4^{+}$ T cells. Therefore, gene expression in the naive phase is likely to be regulated primarily by the activation of transcription factors. However, changes in the DNA methylation of unsynchronized genes may prepare T cells for rapid responses following secondary stimulation via TCR signaling or other stimuli, such as inflammatory cytokines, bacteria, and viruses.

Variable DNA methylation of the enhancers of genes related to T cell development and survival represents a novel mechanism underlying the regulation of gene expression in memory $CD4^{+}$ T cells. In this study, we demonstrated the important role that methylation and demethylation of DNA in exons and introns play in regulating gene-expression patterns in Ag-specific memory $CD4^{+}$ T cells.

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Disclosures

The authors have no financial conflicts of interest.

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