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Epigenetic modification of the human *CCR6* gene is associated with stable *CCR6* expression in T cells.

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Abstract

CCR6 is a chemokine receptor expressed on Th17 cells and regulatory T cells that is induced by T cell priming with certain cytokines, but how its expression and stability is regulated at the molecular level is largely unknown. Here, we identified and characterized a non-coding region of the human *CCR6* locus that displayed unmethylated CpG motifs (Differentially-Methylated-Region, DMR) selectively in *CCR6*⁺ lymphocytes. *CCR6* expression on circulating CD4⁺ T cells was stable upon cytokine-induced proliferation, but partially down-regulated upon TCR stimulation. However, *CCR6* down-regulation was mostly transient and the DMR within the *CCR6* locus remained demethylated. Notably, *in vitro* induction of *CCR6* expression with cytokines in TCR-activated naive CD4⁺ T cells was not associated with a demethylated DMR and resulted in unstable *CCR6* expression. Conversely, treatment with the DNA methylation inhibitor 5'-azacytidine induced demethylation of the DMR and led to increased and stable *CCR6* expression. Finally, when cloned into a reporter gene plasmid the DMR displayed transcriptional activity in memory T cells that was suppressed by DNA methylation. In summary, we have identified a non-coding region of the human *CCR6* gene with methylation-sensitive transcriptional activity in *CCR6*⁺ T cells that controls stable *CCR6* expression via epigenetic mechanisms.

Introduction

Specific patterns of chemokine receptor expression ensure an orchestrated migration of leukocytes throughout the body, where immune cells home to their target tissues both under steady-state and inflammatory conditions. The chemokine receptor *CCR6* is widely expressed on human blood and tissue leukocytes including subsets of dendritic cells, CD45RO⁺ effector/memory T cells, CD25^{high} regulatory T cells (Tregs), naive and memory B cells, NKT cells and NK cells¹⁻⁶. On memory T cells, *CCR6* is expressed on a population of auto-reactive IL-10-producing cells⁷ and subsets of skin- and mucosa-homing cells. Several studies have further shown that *CCR6* is consistently expressed on inflammatory IL-17-producing CD4⁺ T cells⁸⁻¹⁰ which are involved in a wide array of adverse inflammatory diseases, such as rheumatoid arthritis, psoriatic disease, inflammatory bowel disease or encephalopathies in mice and men¹⁰⁻¹⁴. More recently, *CCR6* was identified as a new risk factor for rheumatoid arthritis, and moreover, a nucleotide polymorphism within the *CCR6* locus was shown to be associated with susceptibility to rheumatoid arthritis, Graves' and Crohn's disease^{15,16}.

CCR6 was shown to play a pivotal role in directing inflammatory and regulatory cells to inflamed tissues or the gut, where the ligand of *CCR6* CCL20 is expressed^{13,17,18}. While most chemokine - chemokine receptor interactions are characterized by a high promiscuity, *CCR6* binds only CCL20 and beta-defensins¹.

It is widely assumed that the differentiation of T cells into specialized memory subsets also involves the acquisition and stable expression of homing- and chemokine receptor repertoires, allowing tissue- or inflammation-specific trafficking of these subsets¹⁹. Evidence has been provided in recent years that differentiation of T cells into distinct lineages with stable phenotypes and

functions involves epigenetic regulation of critical effector molecules²⁰⁻²² or lineage-specific transcription factors such as Foxp3 in Tregs²³⁻²⁶. Only few studies demonstrated that molecules involved in trafficking are subject to epigenetic regulation in T cells^{27,28} or cancer cells^{29,30}. In addition, a considerable plasticity in the expression of homing receptors has also been observed in some cases^{31,32}. Whether this also applies to the expression of CCR6 is not known. *In vitro*, CCR6 expression can be induced *de novo* on TCR-stimulated naive T cells by a cocktail of pro-inflammatory cytokines in combination with TGF- β ⁹.

The transcriptional regulation of CCR6 is not well understood; a region with promoter activity has been identified in the mouse *CCR6* gene³³, and over-expression of the transcription factor ROR γ t, the master regulator of Th17 cells, leads to CCR6 expression on human and murine T cells^{10,34}. However, whether stable CCR6 expression on human T cells is controlled by epigenetic mechanisms has not been studied so far. We therefore investigated whether epigenetic mechanisms including DNA methylation of the *CCR6* locus contribute to the regulation of stable CCR6 expression in human T cells. We could identify a non-coding region in the *CCR6* gene, harboring transcriptional activity in primary T cells and being differentially methylated in human CCR6⁻ and CCR6⁺ T cells. These observations and the inducing effect of the DNA-methylation inhibitor 5'-azacytidine suggests that epigenetic mechanisms are involved in the regulation of stable CCR6 expression and the imprinting of distinct homing properties in human memory T cells.

Material and Methods

Cells, antibodies and flow cytometry

Peripheral blood mononuclear cells (PBMCs) from Buffy coats (DRK Blutspendedienst) were separated with a Ficoll-Hypaque gradient (Sigma-Aldrich). Cell surface antigens were analyzed by single-parameter or multi-parameter fluorescence-activated cell sorter (FACS) analysis using the following monoclonal antibodies: PE-anti-CCR6 (11A9) and Alexa700-anti-CD4 (RPA-T4) (both from BD Biosciences), APC-anti-CD25 (BC96; eBioscience), PE-Cy5-anti-CD8 (B9.11), PE-Cy5-anti-CD56 (N901) and FITC-anti-CD25 (B1.49.9, all Beckman Coulter). Antibodies generated in house (DRFZ): FITC-anti-CD45RA (4G11), Alexa405-anti-CD4 (TT1) and Alexa405-anti-CD3 (OKT3). In some experiments, CCR6 expression was detected by indirect immunofluorescence using biotinylated CCR6 (11A9, BD Biosciences) followed by staining with APC-conjugated streptavidin (SouthernBiotech). Stains were performed in Phosphate buffered saline containing 0.5 % bovine serum albumin to block unspecific binding. A FACSCanto II (BD Biosciences) was used for data acquisition and analysis was performed using the FlowJo software (Treestar).

T-cell isolation and FACS[®] sorting

Total CD4⁺ and CD8⁺ T cells were enriched from PBMCs using anti-CD4 or anti-CD8 magnetic beads, respectively, and the autoMACS separation system

according to the manufacturer's instructions (Miltenyi Biotec). After subsequent staining with anti-CD4, anti-CD8, anti-CD25, anti-CD45RA and anti-CCR6 the following cell populations were sorted on a FACS Aria[®] or FACSDiva[®] cell sorter (BD Biosciences): naive CD4⁺ T cells (CD4⁺CD25⁻CD45RA⁺), CD4⁺ Tregs (CD4⁺CD25^{high}), CCR6⁻ naive CD4⁺ T cells, CCR6⁻ memory CD4⁺ T cells (CD4⁺CD25⁻CD45RA⁻), CCR6⁺ memory CD4⁺ T cells, CCR6⁻ naive CD8⁺ T cells (CD8⁺CD25⁻CD45RA⁺), CCR6⁻ memory CD8⁺ T cells (CD8⁺CD25⁻CD45RA⁻) and CCR6⁺ memory CD8⁺ T cells. Upon re-analysis, sorted cells routinely showed >95 % purity. *Ex vivo* isolated cells were either used for methylation analyses or for *in vitro* cell cultures (see below).

T-cell culture of naive and memory CD4⁺ T cells

Cells were cultured in complete RPMI 1640 Glutamax medium (Invitrogen) containing 5 % human serum (complete medium [CM]). For stimulation of naive CD4⁺ T cells, 1x10⁵ cells were cultured in CM supplemented with 20 ng/ml IL-2 (R&D Systems) and 2 µg/ml neutralizing antibodies against IL-4, IL-12 and IFN-γ (neutral conditions, antibodies from BD Biosciences) in flat-bottom microtiter plates and 1x10⁵ magnetic beads coated with anti-CD3 and anti-CD28 (Dynabeads, Invitrogen) for four days followed by transfer in CM containing 1000 U/ml IL-2 (Proleukin, Chiron). For induction of CCR6 on naive CD4⁺ cells, the following cytokines were added at the beginning of the culture: 10 ng/ml TGF-β, 10 ng/ml IL-6, 10 ng/ml IL-1, 10 ng/ml TNF-α (all R&D). In some experiments, stimulation of cells was repeated up to two times under the same conditions. For induction of CCR6 on naive CD4⁺ T cells with the methylation inhibitor 5' Azacytidine (Aza, Sigma-Aldrich) 5 µM Aza was added after 48 hours of culture.

Memory CD4⁺CCR6⁺ and CD4⁺CCR6⁻ cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and cultured either in CM containing magnetic beads as described above or 10 ng/ml IL-7 and 10 ng/ml IL-15 (R&D Systems). After four days only the cultures containing Dynabeads were transferred in CM containing 1000 U/ml IL-2 (Proleukin, Chiron). At the end of the cultures, cells were analyzed by flow cytometry and used for methylation analyses (see below).

Cloning of luciferase reporter plasmids and *in vitro* methylation

An extended version of the differentially methylated region of the human *CCR6* locus was amplified by PCR using genomic DNA as a template and the following primers: (A) 5'-AGTCATGCATCTCTCAGATGGAGGAGGGAC-3', (B) 5'-TCAGACTAGTAGAGGCCGTGTGAGTTAAAGAC-3'. For generation of a luciferase reporter vector in a CpG-free background, the 1073bp PCR product was inserted into the pCpGL-CMV/EF1 vector^{35,36} from which the CMV promoter was removed. Sequencing of the cloned region revealed a 100 % identity with the *CCR6* region sequence of the *Homo sapiens* genome stored at Ensembl. A reporter plasmid without insert served as control.

The luciferase reporter vector pCpGL-CCR6-DMR/EF1 was methylated *in vitro* using the methylase SssI (New England Biolabs) according to the manufacturer's recommendations, followed by purification using the QIAquick PCR clean-up kit (Qiagen). In control samples, using pCpGL-EF1 and pCpGL-CCR6-DMR/EF1, the methyl-group donor S-adenosylmethionine was omitted. Successful methylation was verified by restriction enzyme treatment of the reporter plasmid containing the CCR6 DMR with the methylation-sensitive and methylation-insensitive enzymes *HpaII* and *MspI* (New England Biolabs), respectively.

Transfection and luciferase reporter assays

Cells from 4 different donors were sorted for CD4⁺ memory CCR6⁺ and CCR6⁻ populations and activated using anti-CD3/CD28 Dynabeads (Invitrogen) for 60 hours. Magnetic beads were removed and 5 x 10⁶ of pooled cells were transfected using 2.5 µg of either methylated or non-methylated pCpGL-CCR6-DMR/EF1 vector or insert-less control plasmid in triplicates. Synthetic renilla luciferase reporter vector (pRL-TK; Promega) was co-transfected (1.5 µg) and served as an internal control for transfection efficiency. Nucleofection was performed according to Amaxa technique using the primary Human T Cell Nucleofector Kit (Lonza). Cells were immediately resuspended for culture in prewarmed RPMI1640 medium containing IL-2 (Proleukin, Chiron) (w/o penicillin/streptomycin). After 48 hours cells were harvested and luciferase activity was measured on Orion L luminometer using the Dual Luciferase Assay system (Promega). Firefly RLU data was normalized to renilla luciferase activity and x-fold induction was calculated by comparison to insert-less control vector.

Methylation analysis

Genomic DNA was isolated from sorted T cell subsets using the DNeasy tissue kit (Qiagen) following the protocol for cultured animal cells. Bisulfite treatment of genomic DNA was performed as previously described³⁷. PCR was performed in a final volume of 25 µL containing 1× PCR Buffer, 1 U Taq DNA polymerase (Qiagen), 200 µM dNTP, 12.5 pmol of each primer, and 7 ng bisulfite-treated genomic DNA at 95°C for 15 min, and 40 cycles of 95°C for 1 min, 55°C for 45 s and 72°C for 1 min, and a final extension step of 10 min at 72°C. PCR products were purified using ExoSAP-IT (USB Corp.) and sequenced applying the PCR primers and the ABI Big Dye Terminator v1.1-chemistry (Applied Biosystems) followed by capillary electrophoresis on an ABI 3100 genetic analyzer. AB1 files were interpreted using ESME³⁸.

Primers used for the reactions are given in Supplemental Fig. 2.

***In silico* analyses**

For prediction of putative transcription factor binding sites the tool MatInspector (Genomatix) was used.

Statistics

Data are expressed as the mean $\pm$ SD. Differences between groups were assessed using the Wilcoxon rank test as indicated. P values < 0.05 were considered significant and the level of significance was indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

A non-coding region in the *CCR6* locus displays differential methylation that correlates with *CCR6* expression.

We have previously conducted a screen for epigenetically regulated genes in human Tregs by comparing DNA-methylation patterns of conventional naive $CD4^+$ T cells and $CD25^{\text{high}}CD4^+$ Tregs using the differential methylation hybridization (DMH) technique²⁵. In this DMH screen, a CpG-rich region within the *CCR6* locus turned out to be fully methylated in naive $CD4^+$ T cells and demethylated in Tregs (data not shown). The identified differentially methylated region (DMR) is located upstream of two reported *CCR6* transcripts and overlaps with a putative *CCR6* promoter³⁹ in a region displaying substantial (30-70%) homology between man and mouse (Fig. 1A, and Supplemental Fig. 2). To validate the DMH data, we here performed bisulfite sequencing using genomic DNA from conventional naive $CD45RA^+CD4^+$ T cells and $CD25^{\text{high}}CD4^+$ Tregs isolated from human peripheral blood. In accordance with the DMH data, $CD25^{\text{high}}CD4^+$ Tregs showed a largely demethylated DMR (average CpG methylation rate 23.8 %), whereas conventional naive T cells were almost fully methylated (average methylation rate 81 %, Fig. 1B, and Supplemental Fig. 2).

In addition to Tregs⁶, expression of *CCR6* has been reported for both $CD4^+$ and $CD8^+$ memory T cells^{5,7,9}. In order to understand whether the observed demethylation of the *CCR6* locus is confined to the fraction of cells that express *CCR6*, *ex vivo* $CD4^+$ and $CD8^+$ T cell subsets were sorted according to *CCR6* and *CD45RA* expression (Fig. 2A) and analyzed by bisulfite sequencing of the *CCR6* locus. Both, naive $CD45RA^+CD4^+$ T cells lacking *CCR6* expression as well as $CCR6^-CD45RA^-CD4^+$ memory T cells were strongly methylated, whereas *CCR6*-expressing $CD4^+$ memory T cells turned out to be almost completely demethylated in the analyzed DMR (Fig. 2B). Although $CD8^+$ T cells displayed an overall lower level of DNA methylation, $CCR6^+CD8^+$ memory T cells clearly displayed a less methylated DMR when compared to $CCR6^-CD8^+$ memory T cells (Fig. 2B). In accordance with these data, peripheral blood leukocyte subsets lacking *CCR6* expression, such as resting $CD14^+$ monocytes and $CD15^+$ granulocytes^{1,40,41}, displayed an almost completely methylated DMR (average methylation >84 %), whereas *CCR6*-expressing $CD56^+$ NK and NKT cells^{2,4} and mature B cells³ were fully demethylated at this site (data not shown). Together, our data suggest that *CCR6* expression correlates with demethylation of the DMR in human leukocytes.

CCR6⁺ T cells stably express CCR6 upon cell division and maintain a demethylated DMR.

In order to investigate if both CCR6 expression on CD4⁺ memory T cells and the corresponding methylation pattern of the DMR were stable upon cell division, sorted CCR6⁺CD25⁻CD45RA⁻CD4⁺ T cells were labeled with CFSE and either stimulated with cytokines that mediate homeostatic turnover of memory cells, or by triggering via the TCR. Consistent with previous reports⁴², memory T cells proliferated in the presence of homeostatic cytokines such as IL-7 and IL-15, and all proliferating cells maintained high levels of CCR6 expression even after multiple cell divisions (Fig. 3A). TCR stimulation resulted in a down-regulation of CCR6 expression as has been reported⁴³, which was inhibited by TGF- β (Fig. 3A). However, under all stimulation conditions tested the methylation status of the DMR remained unchanged and showed a comparable low level of methylation (Fig. 3B and data not shown). Importantly, TCR-induced down-regulation of CCR6 expression was mostly transient, since a majority (50-80% among 4 donors) of FACS-purified CCR6⁻/_{low} cells that had lost surface CCR6 expression upon TCR stimulation rapidly re-expressed CCR6 after culture in the absence of TCR stimulation (Fig. 3C). Thus, demethylation of the DMR is linked to long-term stability of CCR6 expression.

***In vitro* induction of CCR6 expression is unstable and does not lead to demethylation of the DMR.**

CCR6 expression can be induced *in vitro* on a fraction of naive CD4⁺ T cells upon activation in the presence of a cocktail of cytokines including TNF- α , IL-6, IL-1 and TGF- β ⁹. We asked whether this *de novo* induction of CCR6 expression is associated with a demethylation of the DMR.

Naive CD45RA⁺CCR6⁻CD4⁺ T cells were stimulated with anti-CD3/anti-CD28 Dynabeads[®] in the presence of the inflammatory cytokine-cocktail and TGF- β . At day 6 of *in vitro* culture, a significantly higher fraction of T cells expressed CCR6 when compared to T cells stimulated in the absence of exogenous cytokines (Fig. 4A) while IL-17 producing capacities were not acquired under these conditions (data not shown). However, both cell populations were almost completely methylated within the DMR, showing no difference to unstimulated naive T cells (Fig. 4A and 2B). Even after sorting into CCR6⁻ and CCR6⁺ cells, no selective demethylation of the DMR could be observed within *de novo* induced CCR6⁺ cells (Fig. 4B). Although repetitive stimulation in the presence of the cytokine cocktail further increased the frequency of CCR6⁺ cells, demethylation of the DMR was still not detectable (Fig. 4C and 4D), suggesting that inflammatory cytokines raise the expression rate, but do not induce a demethylation of the CCR6 locus. In contrast to *ex vivo* isolated CD4⁺ memory T cells, CCR6 *de novo* induction in naive T cells by TCR stimulation in the presence of inflammatory cytokines led only to transient CCR6 expression, and virtually all cells lost CCR6 expression upon prolonged *in vitro* culture in the absence of the inducing cytokines (Suppl. Fig. 1). Thus, the failure of cytokines to induce demethylation of the DMR *in vitro* is associated with unstable CCR6 expression.

Inhibition of DNA methylation leads to stable CCR6 expression in TCR-stimulated naive T cells.

The DNA methylation status can be pharmacologically manipulated by induction of DNA replication in the presence of DNA-methyltransferase inhibitors such as 5'-azacytidine (Aza). To further investigate whether demethylation of the DMR is involved in the stabilization of CCR6 expression, we activated naive CD4⁺ T cells with anti-CD3 and anti-CD28 Dynabeads® in the presence of Aza. Strikingly, Aza-treatment induced CCR6 expression in a significant fraction of cells even in the absence of exogenous cytokines (Fig. 5A, left panel). Combined treatment with cytokines and Aza resulted in an increase in CCR6 induction when compared to cells treated with cytokines alone. In addition, in these Aza plus cytokine-treated cells a clear demethylation of the DMR could be detected (Fig. 5A, right panel). Interestingly, when Aza-induced CCR6⁺ cells were sorted and restimulated under neutral conditions in the absence of Aza, a remarkable stability of CCR6 expression was observed in these cells, suggesting that demethylation of the *CCR6* locus controls stable chemokine receptor expression in T cells (Fig. 5B).

The differentially methylated element of the *CCR6* locus mediates transcriptional activity.

The differential methylated region of the *CCR6* locus starts 444/446 bases upstream of two predicted *CCR6* transcripts (Fig. 1A) and partially overlaps with an *in silico* predicted promoter region. However, prediction strength was weak and a promoter-typical transcriptional start site was absent in the analyzed region. A number of putative binding sites for transcriptional regulators such as ETS1, GATA, NFAT, CREB and others could be identified (Genomatix; applying a matrix similarity > 0.9; Suppl. Fig. 2).

To analyze the role of the DMR for the transcriptional regulation of *CCR6* expression, we cloned this element into a CpG-free luciferase reporter vector, (kindly supplied by M. Rehli, Regensburg) which contains a human EF1 promoter and allows the detection of transcriptionally active enhancer elements as well as its dependency on DNA methylation³⁵. To include further possibly important sequences, we extended the region into both directions to cover a total of 1072 bp (Supplemental Fig. 2); The CpGs in the extended region displayed the same differential methylation pattern (Supplemental Fig. 2) as found for the DMR (Fig. 2). Upon transfection of either CCR6⁺ or CCR6⁻ memory CD4⁺ T cells isolated from peripheral blood, transcriptional activity of the luciferase reporter construct containing the extended DMR was increased 6-21 fold compared to the empty control vector (pCpGL-EF1) in CCR6⁺ cells and 5-10 fold in CCR6⁻ cells (Fig. 6). The notion of an enhancer function of the extended DMR is furthermore supported by the presence of an activating histon modification mark (H3K4monomethyl) typical for enhancers in this region, as to be found in the database of the "ENCODE" project for a lymphoblastoid cell line⁴⁴.

Strikingly, transcriptional activation was completely lost after in vitro methylation of the CpG's in the extended DMR, demonstrating that the methylation status critically affects its enhancer function. These findings demonstrate that the extended DMR of the *CCR6* locus displays methylation-sensitive enhancer activity independently of TCR-mediated signals, suggesting that this element and its demethylation is functionally involved in the maintenance of stable *CCR6* expression in memory T cells.

Discussion

Differential expression of distinct chemokine receptors on immune cells ensures a coordinated temporal-spatial distribution of various functional subsets and differentiation stages of effector and regulatory cells to and within lymphoid and non-lymphoid tissues. Some of the migratory phenotypes, become apparently permanently imprinted upon differentiation^{28,45} and allow a selective delivery of memory populations to specific compartments of the body ("homing"). How stable homing phenotypes are acquired and maintained in the progeny of T and B cells is not well understood. Epigenetic mechanisms, notably the methylation/demethylation of CpG-rich DNA regions involved in transcriptional regulation, are ideally suited to provide a long-term memory of phenotypic changes due to the imprinting of "heritable" methylation signatures^{22,46}. We here investigated whether differential DNA methylation might be involved in the acquisition of stable *CCR6* expression in primary human T cells.

A region within the *CCR6* locus that was differentially methylated between Tregs and naïve CD4⁺ T cells was initially identified by a DMH approach²⁵. This DMR was now verified by bisulfite sequencing of sorted human blood leukocyte subsets. The observed methylation patterns matched largely the expression of *CCR6* in various leukocyte subsets, where *CCR6*-expressing cells such as CD56⁺ NK and NKT cells^{2,4}, certain CD4⁺ and CD8⁺ T cell subsets^{47,48}, mature B cells³ and CD4⁺ Tregs⁶ displayed a partially or even completely demethylated DMR, whereas the locus was completely methylated in resting CD14⁺ monocytes and CD15⁺ granulocytes lacking *CCR6* expression^{1,40,41}. Among CD4⁺ and CD8⁺ memory T cells, demethylation of the DMR was confined to those subsets expressing *CCR6* on their surface, suggesting a role for this genetic element and its methylation status in *CCR6* regulation.

Demethylation of essential regulatory DNA regions is usually regarded as a signature for a stable, heritable expression of the regulated gene, as we could recently exemplify for the Treg-specific transcription factor *Foxp3*²⁴. As *CCR6* expression can be either transient or permanent, we investigated in the present study whether such an epigenetic imprinting also occurs at the DMR in the *CCR6* locus.

Like many other receptors for inflammatory chemokines, *CCR6* is expressed on a large proportion of circulating memory T cells, but is absent from naïve T cells^{5,47}, suggesting that *CCR6* expression is acquired during T cell priming. Along this line, *CCR6* expression can be induced upon priming of naïve *CCR6*⁺CD4⁺ T cells using a cytokine cocktail containing IL-1, IL-6, TGF- β and

TNF- α ^{9,47}. Importantly, T cells that had acquired CCR6 expression *in vivo* displayed a demethylated DMR and showed stable CCR6 expression in dividing cells in the absence of TCR stimulation. The methylation status in memory cells was independent from the cytokine profile, as we detected demethylation of the DMR in both IL-17-secreting and non-secreting CCR6⁺ T cells (unpublished data). Although CCR6 was down-regulated on memory cells upon TCR triggering, this down-regulation was mostly transient and did not lead to re-methylation of the DMR. The observed CCR6 down-regulation under these conditions might even be due to receptor recycling rather than transcriptional down-regulation. In contrast, *in vitro* induction of CCR6 on naive T cells using cytokines was neither stable nor associated with demethylation of the DMR. Even though the percentage of CCR6⁺ cells increased with repetitive stimulations, CCR6 expression remained unstable and the DMR fully methylated. A similar observation was recently made for the Treg-specific transcription factor Foxp3, where the Treg-specific demethylated enhancer region in the *Foxp3* locus was only found to be demethylated in *ex vivo* isolated stable Tregs, but not in *in vitro* induced Tregs which only transiently expressed Foxp3⁴⁹. Even repetitive stimulations with the inducing cytokine TGF- β were neither sufficient to convey stability of Foxp3 expression nor demethylation of the enhancer region. In other cases, however, repetitive stimulations under permissive conditions has been shown to achieve stability of certain phenotypes, such as selectin-ligand expression in CD4⁺ T cells⁴⁵ and cytokine production in polarized Th1 and Th2 cells⁵⁰. Together, our present data suggest that specific, so far unknown signals are required to imprint a stable CCR6 expression profile in CD4⁺ T cells by demethylation of the DMR in the *CCR6* locus.

Our hypothesis that the methylation status of a regulatory gene element in the *CCR6* locus dictates long-term stability of CCR6 expression was further corroborated by experiments involving pharmacological DNA hypomethylation. Application of the DNA methyltransferase inhibitor 5'-azacytidine during T cell stimulation not only increased CCR6 expression, even in absence of exogenous CCR6-inducing cytokines, but also resulted in CCR6⁺ cells that showed stable CCR6 expression upon *in vitro* expansion, a finding that was previously also observed for Foxp3 expression in *in vitro* induced Tregs⁴⁹.

To understand the function of the DMR in transcriptional regulation of CCR6, we cloned an extended version of this element in a human EF1 promoter/luciferase reporter gene construct. To cover a larger part of the conserved region, we extended the initially identified region downstream up to the beginning of the predicted CCR6 transcripts. This resulted in a strong increase in transcriptional activity of the reporter, suggesting that the element is contributing to transcriptional regulation, e.g. by acting as an enhancer. A number of putative transcription factor binding sites were predicted for this region, although only one was conserved between mouse and man. Some of them, such as ETS1 or CREB, have been reported to bind in a methylation-dependent manner to their target sequences^{36,51}. Strikingly, the only transcription factor binding site conserved between mouse and man in the extended DMR under the criteria used here predicts binding of ETS1.

As to be expected, transcriptional activity was highest in CCR6⁺ memory cells providing a permissive milieu for gene activity. Use of a reporter vector devoid of CpG motifs (³⁵) allowed us to test the consequence of DNA methylation for the activity of the extended CCR6 DMR. Consistent with the hypothesis that differential methylation of this region is decisive for the capacity to express stably CCR6, the methylated form of the extended DMR was not able to mediate transcriptional activity, even in the permissive CCR6⁺ cells, similar to what has been found in case of Foxp3 ³⁶.

In conclusion, we here provide for the first time experimental evidence that epigenetic mechanisms are not only regulating the transcriptional activity of a chemokine receptor but moreover might play a key role in the imprinting of a permanent expression pattern, thus endowing differentiated memory T cells with topographical memory and shaping their long-term migratory behavior.

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Authorship

Svenja Steinfeld: performed research, analyzed data, and wrote the paper; Stefan Floess: performed research, analyzed data, and wrote the paper; Dirk Engelbert, Barbara Haeringer: performed research, analyzed data; Udo Baron: performed research; Laura Rivino: performed research; Bodo Steckel: performed research; Andreas Gruetzkau: designed research, performed research; Sven Olek: designed research; Jens Geginat: designed research and wrote the paper; Jochen Huehn: designed research and wrote the paper; Alf Hamann: designed research and wrote the paper.

Conflict of interest disclosure

Udo Baron and Sven Olek are employees and owners of Epiontis GmbH, who owns CCR-6 methylation patent applications. The other authors disclosed no potential conflicts of interest.

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Figure legends

Figure 1: A non-coding region in the *CCR6* gene displays differential methylation in human PBMCs.

A) Localization of the CpG-rich region within the human *CCR6* locus. The exon structure of the putative *CCR6* transcripts, obtained from the Ensembl database (GRCh37), were plotted together with a homology chart (human-mouse; Vista), predicted promoter regions (Genomatix) and the identified differentially methylated region (DMR) in the *CCR6* locus. The grey colored boxes in the *CCR6* transcripts represent non-coding exon regions, whereas the black boxes contain coding regions. B) DNA methylation pattern for *CCR6* were analyzed in sorted $CD4^+CD45RA^+$ and $CD4^+CD25^{high}$ T cells from pooled PBMC (five donors). Each column represents one blood cell subset with each row representing a single CpG site. The numbers indicate the position of the CpG motif as indicated in suppl. Fig.2. DNA methylation was measured by bisulfite sequencing. CpG methylation levels are color-coded according to a scale ranging from yellow (0 % methylation) to blue (100 % methylation).

Figure 2: Demethylation of the *CCR6* region correlates with *CCR6* expression in human $CD4^+$ and $CD8^+$ T cells.

A) Representative FACS-staining of *CCR6* and *CD45RA* on $CD4^+CD25^-$ and $CD8^+CD25^-$ lymphocytes. B) $CD4^+$ and $CD8^+$ cells were isolated from PBMC and sorted into $CCR6^-CD45RA^+$ naive and $CCR6^-$ and $CCR6^+CD45RA^-$ memory T cells resulting in subsets with a purity >95 %. Methylation analyses of sorted subsets are shown for one representative donor. The degree of methylation is color-coded according to the scale in Fig. 1B. Graphs below show the mean of methylation from all CpG sites from five to six donors for $CD4^+$ T cells and three donors for $CD8^+$ T cells, respectively.

Figure 3: Circulating $CCR6^+$ T cells stably express *CCR6* upon *in vitro* expansion and maintain a demethylated DMR.

A) CFSE-labeled $CCR6^+CD4^+$ memory T cells were cultured for six days in medium containing recombinant human IL-7 and IL-15 or anti-CD3/anti-CD28 Dynabeads[®] with or without TGF- β and reanalyzed for *CCR6* expression. One representative donor out of four is shown. B) *Ex vivo* isolated $CCR6^+CD4^+$ memory T cells were stimulated with anti-CD3/anti-CD28 Dynabeads[®] in the absence or presence of TGF- β . After six days, methylation analysis of the DMR was assessed. C) *Ex vivo* isolated $CCR6^+CD4^+$ memory T cells were stimulated with anti-CD3/anti-CD28 Dynabeads[®], sorted on day 6 in $CCR6^-$ and $CCR6^+$ cells and cultured for additional three days in medium containing IL-2. Data are representative for two donors.

Figure 4: *In vitro* induced *CCR6* expression is unstable and not associated with a demethylated DMR.

A) Naive CD4⁺ T cells were stimulated with anti-CD3/anti-CD28 Dynabeads[®] without (white bar) or with pro-inflammatory cytokines and TGF- β (black bar) for four days, followed by culture in IL-2. After six to seven days, cells were analyzed for CCR6 expression (n=21, left graph). The methylation profile of CD4⁺ T cells cultured with (+) and without (-) cytokines is shown in the right graph (n=2). B) Cells stimulated with cytokines as in A were sorted into CCR6⁺ and CCR6⁻ cells (shown for one representative donor) and analyzed for methylation of the DMR. C) Naive CD4⁺ T cells were stimulated and cultured as in A. At day six, a fraction of the cultured cells were stimulated again under the same conditions. Cells were analyzed for CCR6 expression (one representative donor). D) Methylation analysis of the CCR6 DMR (one representative donor, left panel) and FACS analysis of CCR6 expression in cells cultured as described in C (n=11, right panel).

Figure 5: Inhibition of DNA methylation leads to stable CCR6 expression.

A) Naive CD4⁺ T cells were stimulated with or without pro-inflammatory cytokines and TGF- β . After 48 hours, Aza was added to the media for another 48 hours. Expression of CCR6 (n>18, left panel) and methylation of the DMR (n=3, right panel) was analyzed on day 6. B) Naive CD4⁺ T cells were stimulated with Aza and without cytokines as in A. After six days, cells were sorted according to their CCR6 expression and restimulated under neutral conditions in the absence of Aza for additional five days followed by reanalysis of CCR6 expression. Data shown are representative for two independently performed experiments.

Figure 6: The CCR6 DMR mediates transcriptional activity.

Activated CCR6⁺ and CCR6⁻ CD45RA⁻CD4⁺ memory T cells were transfected with control plasmid (pCpGL-EF1 Promoter) or pCpGL-EF1-CCR6-DMR, where the extended DMR was cloned into the pCpGL-EF1-promoter plasmid. The relative luciferase light units were normalized to Renilla luciferase activity. Values for the pCpGL-EF1-promoter plasmid were set to 1 to obtain the x-fold activation. Shown is the mean luciferase activity of one representative out of three experiments with cells pooled from four donors each.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6