On epigenetic regulation in atherosclerosis pathology

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Genetic and Epigenetic Regulation of CCR5 Transcription

Adapted with minor modification from:

Epigenetic control of CCR5 transcript levels in immune cells and modulation by small molecules inhibitors

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Abstract

The chemokine receptor CCR5 regulates trafficking of immune cells of the lymphoid and the myeloid lineage (such as monocytes, macrophages and immature dendritic cells) and microglia. Because of this, there is an increasing recognition of the important role of CCR5 in the pathology of (neuro-) inflammatory diseases such as atherosclerosis and multiple sclerosis. Expression of *CCR5* is under the control of a complexly organized promoter region upstream of the gene. The transcription factor cAMP-responsive element binding protein 1 (CREB-1) transactivates the *CCR5* P1 promoter. The cell-specific expression of *CCR5* however is realized by using various epigenetic marks providing a multivalent chromatin state particularly in monocytes. In this chapter the transcriptional regulation of *CCR5* is discussed, with a focus on the epigenetic peculiarities of *CCR5* transcription.

Introduction

The CC chemokine receptor 5 (CCR5) regulates trafficking of lymphoid cells such as memory/effector Th1 lymphocytes, or cells of the myeloid lineage (e.g. monocytes, macrophages, immature dendritic cells) and microglia. As such, CCR5 is implicated in the pathogenesis of various inflammatory diseases such as atherosclerosis and multiple sclerosis.[1–4] Furthermore, CCR5 also functions as a co-receptor for HIV-1.[5–7] Notably, CCR5 expression is markedly upregulated upon T cell activation,[8–11] which allows the activated T cells to migrate towards site(s) of inflammation.[8,12]

Upon encountering a pathogen, antigen presenting cells will present the antigenic peptide to resting naïve T cells which results in the generation and activation of antigen-specific T cells.[13,14] After activation, the T cells migrate to the site of inflammation, guided by chemokine receptors.[15] Similarly, circulating monocytes are also attracted to inflammatory sites by chemokine receptors, where they then can differentiate into e.g. macrophages or microglia.[16–18] Multiple sclerosis and atherosclerosis are greatly characterized by inflammatory lesions, consisting of T cells and macrophages or microglia.[19–21] The chemokine receptor CCR5 has been shown to be implicated in the pathogenesis of both of these diseases.[22–25]

Expression of CCR5 is under the control of a complexly organized promoter region upstream of the gene. The main transcriptional activity of the *CCR5* promoter region is contained within the downstream promoter P1.[9,11,26] A number of transcription factors have been shown to play a role in CCR5 transcriptional regulation (see Wierda et al.[27] and references therein). A graphical representation of the *CCR5* promoter organization and transcription factor binding sites is shown in figure 4–2. We have previously shown however that the transcription factor cAMP responsive element binding protein 1 (CREB-1) is the main transactivating factor for the *CCR5* P1 promoter.[26] However, considering the ubiquitous expression of CREB-1[28], we argued that epigenetic mechanisms are also involved in the cell type-specific regulation of *CCR5* transcription. In line with this notion is the observation that transient promoter-reporter studies in CCR5-deficient Jurkat T leukemia cells revealed that the *CCR5* promoter-reporter was activated upon transfection.[9] This observation infers that Jurkat T leukemia cells contain all the transcription factors required for *CCR5* transcription, and demonstrates that *CCR5* transcription is additionally controlled by epigenetic mechanisms.

Epigenetic mechanisms control the accessibility of DNA for transcription factors and are thought to form the basis for cell-to-cell inheritance of gene expression profiles.[29] Epigenetic mechanisms as such play an essential role in the regulation of gene transcription. Epigenetic modifications include methylation of DNA at CpG residues and posttranslational modifications of histone tails such as acetylation and methylation.[30] Together these modifications form a 'histone code' – like the genetic code – that controls transcription levels of genes.[31] Importantly, modifications to DNA and to histone tails have been shown to be functionally linked.[32]

Well-studied mechanisms that underlie gene repression by histone methylation involve tri-methylation of histone H3 at lysine 9 (H3K9Me3) and at lysine 27 (H3K27Me3), and of histone H4 at lysine 20 (H4K20Me3). These modifications are catalysed respectively by the lysine methyltransferases (KMTases) SUVAR39H1 (hKMT1A), Enhancer of Zeste homolog 2 (EZH2, hKMT6), a subunit of the Polycomb Repressive Complex 2 (PRC-2), and SUV4-20H1/H2 (hKMT5B/C).[33–36] The KMTase hSet1 and the MLL genes (hKMT2A/G) catalyses tri-methylation of K4-H3 (3MeK4H3) and this modification is associated with gene transcription.[36,37]

In this study we show that induction of CCR5 transcription – upon $CD4^+$ T cell activation – correlates with reduced levels of DNA methylation as well as changes in specific histone modifications within the *CCR5* promoter. To establish whether the found epigenetic profiles are T cell specific, we also determined the epigenetic profile in $CD14^+$ monocytes, being of the myeloid instead of the lymphoid lineage. It is shown that the CCR5 chromatin status in primary $CD14^+$ monocytes correlates with the intermediate transcription levels of *CCR5*. Furthermore, the T-lymphoblastic cell lines studied (Jurkat, Molt-4, HSB-2) do not express CCR5 and show a transcriptionally repressive chromatin environment. Moreover, we show that pharmacological interference in these epigenetic silencing mechanisms in the CCR5-deficient T leukemia cell lines results in the induction of CCR5 expression. Together, these data reveal that epigenetic mechanisms play a pivotal role in the control of *CCR5* transcription.

Materials & Methods

Cell culture and activation

Naïve human $CD4^+$ T cells were sorted from freshly isolated PBMC using a FACSAria Flow Cytometer (Becton Dickinson). Sorted cells were directly used for

chromatin immunoprecipitation (ChIP) analysis, RNA extraction and DNA isolation for bisulphite analysis. Naïve CD4+ T cells were also activated *in vitro* as described earlier.[38] In brief, naïve CD4+ T cells were stimulated with 1 µg/mL phytoheamagglutinin (PHA, Remel Europe Ltd.) and 20 U/mL IL-2 in the presence of irradiated allogeneic PBMCs (3000 Rad). After 11 days of culture, cells were restimulated the same way and after 12 days cells were harvested for ChIP analysis and bisulphite sequencing analysis. For RNA-extraction naïve CD4+ T cells were stimulated with anti-CD3 and anti-CD28 for 30 min. Thereafter CD4+ T cells were cultured for 48h in CFU-EC medium (Stemcell technologies). RNA was isolated with the RNA-Bee extraction method (see page 98).

The leukemic T cell lines Jurkat (Clone E6-1; American Type Culture Collection (ATCC)) and MOLT-4 (ATCC) were cultured in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (FCS; PAA), 100 IU/mL streptomycin, 100 IU/mL penicillin (both Lonza) and 2 mM L-glutamine (Gibco). The HSB-2 cell line was cultured in Iscove's modified Dulbecco's medium (IMDM; Lonza), supplemented with 10% heat-inactivated FCS, 100 IU/mL streptomycin, 100 IU/ml penicillin, and 2 mM L-glutamine.

To obtain CD14+ monocytes, PBMCs were freshly isolated from the blood of healthy volunteers by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare). Monocytes where enriched from the PBMC fraction by magnetic separation with CD14 magnetic beads (MACS; Miltenyi Biotec).

Flow cytometry

CCR5 expression on Jurkat, HSB-2, Molt-4 and primary T cells was determined by flow cytometry, using the mouse monoclonal antibody MC-5 (kind gift of Prof. M. Mack, University of Regensburg, Regensburg, Germany) and a PE-conjugated anti-mouse IgG secondary antibody (Becton Dickinson) and the appropriate controls. Data acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest programming. Data was analyzed using the FlowJo software package.

Bisulphite sequencing

Total genomic DNA was isolated from naïve and activated T cells, Jurkat T leukemia cells, and CD14+ monocytes. One µg of genomic DNA was used to bisulphite convert unmethylated CpGs using the EZ DNA Methylation kit (Zymo Research).

CCR5 promoter DNA was then amplified using primer sets for specific CpG containing regions (Table 4–1, Figure 4–2). PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel), cloned into pGEM-T easy vector (Promega), and individual clones were sequenced at the Leiden Genome Technology Center. Results of at least 10 individual clones are represented as pie charts for each CpG analyzed. The percentage of methylated clones is depicted in black.

Table 4–1: Primers used for ChIP, bisulphite sequencing and qPCR.

Gene	Promoter region	Region spanning, relative to CDS	Primer sequence, 5'-3'	Application
CCR5	B1	-3509 to -3090[a]	F: TGTTATTGAGTTTTGTTGTAGTATAGATA R: ACCAAACTTAAAACCTATCTTACCC	Bisulphite
	B3	-2625 to -2434[a]	F: TTTAGAAAAAGATGGGAAATTTGTT R: TCCTAAACTTCACATTAACCCTATATC	
	B4/5	-2210 to -1866[a]	F: TTAATAGATTTTGTGTAGTGGGATGAGTA R: CTCATCTCAAAAACTAACTAACAAAC	
		-2277 to -1932[a]	F: TGTGGGCTTTTGACTAGATGA R: TAGGGGAACGGATGTCTCAG	ChIP
		-47 to +188[b]	F: CTGAGACATCCGTTCCCCTA R: GCTCTTCAGCCTTTTGCAGT	qPCR
RPII		+3993 to +4172[c]	F: CAGGAGTGGATCCTGGAGAC R: GGAGCCATCAAAGGAGATGA	qPCR
CREB-1		+276 to +609 (isoform a)[d] +276 to +659 (isoform b)[e]	F: AACCAGCAGAGTGGAGATGCAGCT R: CTGTAGGAAGGCCTCCTTGAAAGA	semi-quantitative PCR
ICER		+150 to +750[f]	F: CAGATCCGAGCTCCTACTGC R: CAACTCGGCTCTCCAGACAT	semi-quantitative PCR

a: Based on accession number NC_000003.10
b: Based on accession number NM_000579.3
c: Based on accession number NM_000937.2
d: Based on accession number NM_004379.3
e: Based on accession number NM_134442.3
f: Based on accession number NM_182717.1

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described earlier.[26] One µg of cross-linked DNA was immunoprecipitated with antibodies (5 µg) directed to specific histone modifications (Table 4–2), or no antibody as background control. Quantitative PCR (qPCR) of the immune-precipitated chromatin was performed using the primer pairs shown in table 4–1.

Table 4–2: Antibodies used for ChIP.

Antibody reactivity	Manufacturer	Catalogue #:
H3Ac	Millipore	06-599
3MeK4H3	Cell Signalling Technology	97510
H3K9Me3	Abcam	ab8898
H4K20Me3	Abcam	ab9053
H3K27Me3	Millipore	07-449
CREB-1	Rockland	100-401-195; [62]
RNA pol II	Santa Cruz	sc899x

Zebularine, DZNep and MS275 treatment

For induction of expression of CCR5, Jurkat, HSB-2 and Molt-4 cells were exposed to 100 µM of Zebularine (V.E. Marquez) for 96 hours followed by an additional treatment with 2 µM of 3-Deazaneplanocin A (DZNep, V.E. Marquez) for 72 hours and 0.5 µM MS275 (Sigma-Aldrich) for 48 hours in IMDM (HSB-2) or RPMI-1640 (Jurkat and Molt-4) with supplements as described above.

RNA isolation and (quantitative) RT-PCR

Total RNA was isolated using the RNA-Bee extraction method (TelTest) from naïve and activated $CD4^+$ T cells, from $CD14^+$ monocytes and from Jurkat, HSB-2 and Molt-4 cells prior to and after treatment with Zebularine, DZNep and MS275. From 1µg of RNA, cDNA was synthesized using 250ng random hexamers (Promega) and Superscript III reverse transcriptase (Invitrogen).

CCR5 and *RNA polymerase II (RPII)* transcripts were quantified on an iCycler IQ system (BioRad Laboratories) using the IQ SYBR Green Supermix (BioRad Laboratories). Relative transcript levels of *CCR5* were calculated with the comparative Ct method (or ΔΔCt method) and related to *RPII* transcript levels. The induced levels of *CCR5*, after treatment of Jurkat, Molt-4 and HSB-2 cells with Zebularine, DZNep and MS275, are also depicted relative to the *CCR5* expression level in *in vitro* activated primary T cells. The primers used in the qPCR reactions are shown in table 4–1.

CREB-1 and *inducible cAMP early repressor* (*ICER*), the inducible isoform of *cAMP-responsive element modulator* (*CREM*) transcripts were analyzed in triplicate by semi-quantitative PCR as previously described.[26] PCR products were separated

by gel electrophoresis on a 1.5% agarose gel, run at 90V for 45min, and visualized by ethidium bromide staining. Densitrometric analysis was performed in ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Results

DNA methylation patterns of the CCR5 P1 promoter.

Using flow cytometry we found that only a few naïve primary CD4⁺ T lymphocytes express low levels of CCR5 at the cell surface, whereas CCR5 cell surface expression is markedly upregulated after *in vitro* activation of these cells (Figure 4–1A). The CCR5 cell surface expression pattern of activated CD4⁺ T cells is accompanied by relatively high levels of *CCR5* transcripts (Figure 4–1B). In naïve T cells

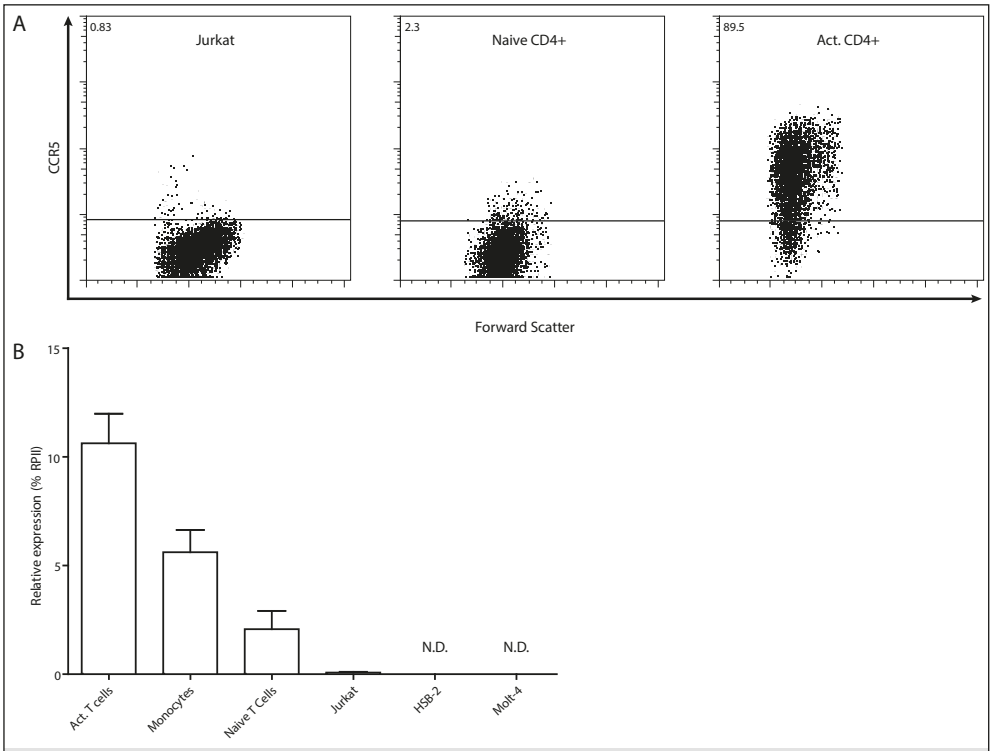

Figure 4–1. (A) Cell surface expression of CCR5 in Jurkat T leukaemia cells, naïve and activated CD4⁺ T cells as determined by flow cytometry. Numbers indicate percentage of CCR5 positive cells. (B) Relative transcript levels of *CCR5* in various cells types. Numbers indicate expression percentage relative to *RNA polymerase II* (*RPII*). N.D. indicates transcript levels were below the detection threshold.

CCR5 transcripts were detected at low levels (Figure 4–1B). Myeloid cells, such as monocytes express CCR5 at low to intermediate levels.[39] When compared with activated and naïve CD4⁺ T cells, CD14⁺ monocytes indeed show intermediate levels of *CCR5* transcripts (Figure 4–1B). In contrast, most established tumour T cell lines completely lack CCR5 surface expression, including the human CD4⁺ leukemic T cell lines Jurkat, Molt-4 and HSB-2 (Figure 4–1A and Figure 4–5A). Furthermore, these leukemic T cell lines show only very low or undetectable CCR5 transcript levels (Figure 4–1B).

Evaluating the role of epigenetic mechanisms in the regulation of *CCR5* expression we first assessed the CpG methylation status of three subregions regions of the *CCR5* downstream promoter P1 (Figure 4–2). The most downstream subregion (B4/5), which is known to be transactivated by CREB-1,[26] appears to be mostly unmethylated and displays only marginal differences in DNA methylation between the various cell types (Figure 4–2). The upstream subregions B1 and B3 display remarkable differences in DNA methylation status. In activated T cells, the CpG residues in these subregions of the P1 promoter display low levels of DNA methylation. In monocytes, which express intermediate levels of CCR5, the promoter subregions B1 and B3 are highly methylated, while the B4/5 region displays low levels of DNA methylation (Figure 4–2). By contrast, in naïve CD4⁺ T cells these subregions are mainly methylated and almost completely methylated in Jurkat T cells. Together, these data reveal that the intermediate, low and lack of *CCR5* transcription levels, in monocytes, unstimulated CD4⁺ T cells and in Jurkat T leukemia cells

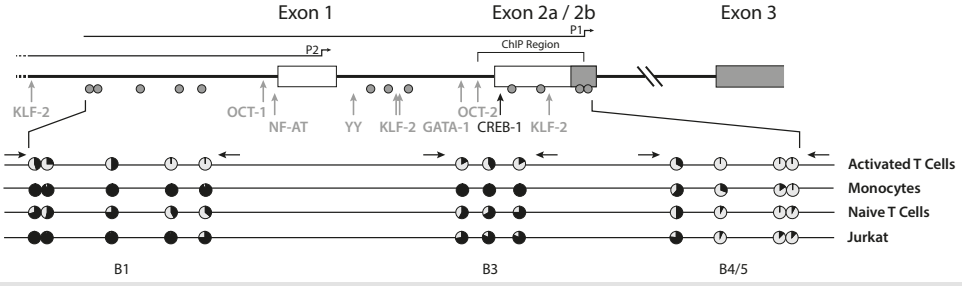

Figure 4–2. Methylation analysis of the *CCR5* promoter of several cell types. Each circle represents a single CpG residue. The percentage of clones methylated at a specific residue is indicated by the black colour. The distance between each circle represents the relative distance between CpG residues on the genomic sequence. Horizontal arrows indicate the relative position of primers used to amplify bisulphite modified DNA. The CREB-1 binding site most likely involved in *CCR5* transactivation is indicated with a vertical arrow.[26] Other transcription factors that have been attributed to *CCR5* transcription regulation are indicated with grey arrows. The region investigated by chromatin immunoprecipitation is annotated as "ChIP region".

respectively, are associated with high levels of DNA methylation in the subregions B1 and B3 of the P1 promoter but not in the B4/5 subregion.

Histone modifications of the CCR5 P1 promoter

Next we determined the association of specific histone acetylation and methylation modifications within chromatin of the *CCR5* P1 promoter by chromatin immunoprecipitation (ChIP) (Figure 4–2 and Figure 4–3A–C). CCR5 expressing, activated, $CD4^+$ T cells display relative high levels of H3Ac (Figure 4–3A). Interestingly, monocytes display H3Ac levels in chromatin of the *CCR5* P1 promoter, which are similar to activated T cells (Figure 4–3A). This is in contrast to the non-CCR5 expressing naïve T cells and Jurkat T cells, which display markedly lower levels of H3Ac in *CCR5* P1 chromatin.

CCR5-expressing activated T cells display relatively high levels of the permissive 3MeK4H3 mark in *CCR5* P1 chromatin. Interestingly, naïve T cells expressing low levels of CCR5 show similar levels of the permissive 3MeK4H3 mark (Figure 4–3A). In contrast, CCR5-deficient Jurkat T cells display low levels of the permissive 3MeK4H3 modification (Figure 4–3A).

The repressive marks H3K9Me3 and H3K27Me3 are only present at very low levels in chromatin of low CCR5-expressing naïve T cells (Figure 4–3B). In contrast, the repressive mark H4K20Me3 is highly enriched at the *CCR5* P1 promoter region

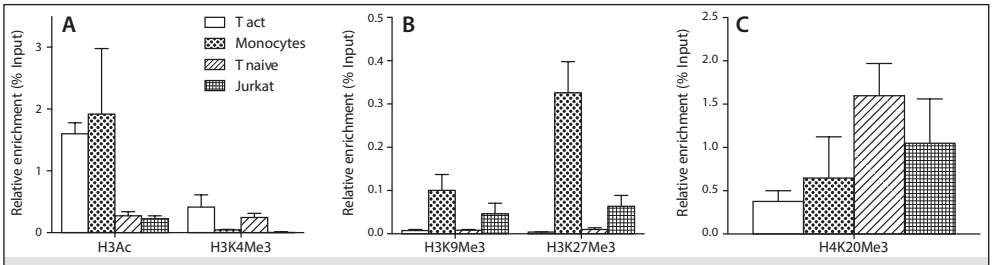

Figure 4–3. Chromatin environment at the *CCR5* promoter as determined by ChIP analysis. CCR5 expressing activated $CD4^+$ T cells clearly show higher levels of transcriptionally permissive chromatin marks H3Ac and H3K4Me3 (A) whereas there is an opposite association with transcriptionally repressive chromatin marks H3K9Me3, H3K27Me3 (B) and H4K20Me3 (C). These non-permissive marks are clearly present in higher amounts in CCR5 non-expressing cells (Jurkat T leukaemia cells, naïve $CD4^+$ T cells) versus expressing activated $CD4^+$ T cells. Naïve T cells and $CD14^+$ monocytes show a poised chromatin state, encompassed by both transcriptionally permissive and non-permissive marks. Whereas naïve T cells show relatively low levels of H3Ac, monocytes have high levels of H3Ac.

of naïve T cells (Figure 4–3C). The presence of both an activating mark (3MeK4H3) and a repressive mark (H4K20Me3) indicates a bivalent, so-called 'poised' state of the *CCR5* promoter chromatin of naïve CD4$^+$ T cells.

Activated CD4$^+$ T cells show a twofold higher *CCR5* transcription level as compared to monocytes. Assessing the chromatin status of CD14$^+$ monocytes, we observe the presence of relative high levels of the repressive marks H3K9Me3 and H3K27Me3 in the monocytic *CCR5* P1 promoter (Figure 4–3B). Conversely, the repressive mark H4K20Me3 is only slightly enriched in monocytes as compared to activated T cells (Figure 4–3C). Furthermore, hardly any of the permissive 3MeK4H3 mark could be detected, yet monocytes show high levels of H3Ac in the *CCR5* promoter (Figure 4–3A). This indicates that also monocytes display a chromatin state in which repressive and permissive histone modification marks co-exist. Compared to naïve CD4$^+$ T cells however the chromatin state of CD14$^+$ monocytes, is markedly different, permitting transcription of *CCR5*.

CCR5-deficient Jurkat T cells show relative high levels of the repressive H3K9Me3 and H3K27Me3 histone marks, when compared with naïve and activated T cells (Figure 4–3B). Like naïve T cells, Jurkat T leukemia cells also show higher levels of the repressive H4K20Me3 modification when compared to activated T cells (Figure 4–3C). The presence of these repressive marks in the absence of activating histone modifications clearly shows a repressive chromatin conformation encompassing the *CCR5* P1 promoter in Jurkat T cells.

Taken together, these data show that there is a differential pattern of chromatin conformation of the *CCR5* P1 promoter region in the different cell populations investigated in this study. Our observations also indicate that the *CCR5* transcription profiles could not be explained by a single epigenetic modification, but rather the sum of modifications appears to determine the level of *CCR5* transcripts in the various cell types investigated.

Re-expression of CCR5 through pharmacologic interference in epigenetic mechanisms in Jurkat, Molt-4 and HSB-2 T cell lines

To show that DNA methylation, and histone acetylation/methylation mechanisms control *CCR5* transcription, we aimed to induce *CCR5* transcription in non–CCR5-expressing cells through pharmacologic interference in the catalytic activities of the various enzymes involved in these epigenetic regulatory processes. Figure 4–4 presents a schematic overview of the working mechanisms of the agents used for

this purpose. Zebularine is a potent inhibitor of DNA-methylation showing much lower toxicity then the widely used inhibitor 5-Aza-dC.[40,41] First recognized as an inhibitor with specificity for the KMTase EZH2, DZNep is now regarded as a more general lysine methyltransferase inhibitor, with a high affinity for the enzymes that triple-methylate K20H4 and K27H3 (Miranda *et al.* (2009),[42] Tan *et al.* (2007)[43] and own observations). Finally, MS275 is a potent inhibitor of histone deacetylase activities (HDACs), with high affinity for the class I HDACs 1 and 3.[44]

Originally we found that inhibition of DNA-methylation by 5-Aza-dC treatment resulted in only a modest and time-dependent induction of CCR5 mRNA expression levels in Jurkat cells (results not shown). However, combining inhibition of DNA and histone methylation by inclusion of DZNep resulted in a clear synergistic induction of CCR5 mRNA expression, whereas inhibition of histone methylation alone was found only marginally effective (results not shown). Additional treatment with the HDAC inhibitor MS275 mainly potentiated the effect obtained by the other inhibitors (results not shown).

We therefore combined all of the above-mentioned inhibitors to induce CCR5 expression in Jurkat, Molt-4 and HSB-2 T leukemia cells and included Zebularine rather than 5-Aza-dC for the aforementioned reasons. After treatment with Zebularine, in combination with DZNep and MS275, 67.7% of Jurkat cells are CCR5

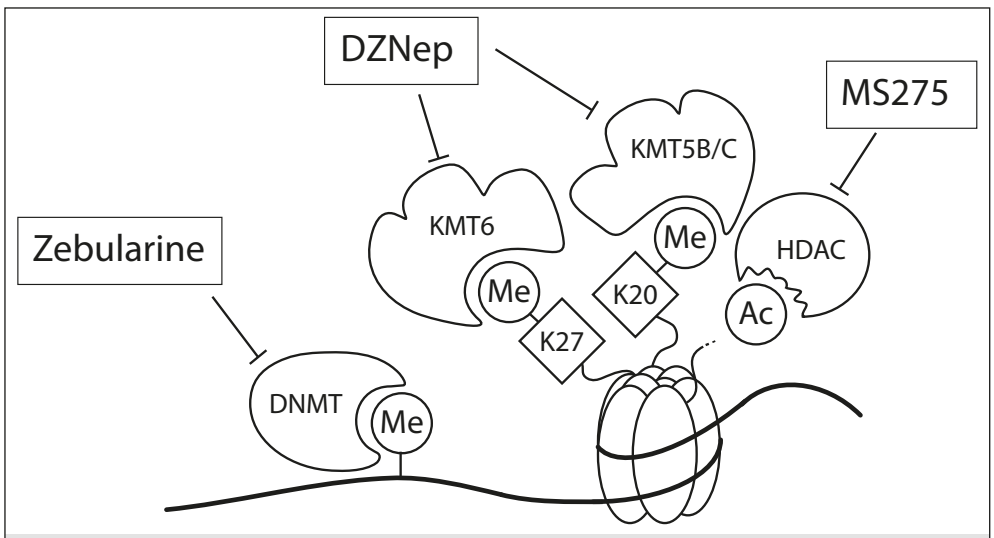

Figure 4–4. Schematic representation of the working mechanism for the pharmacological intervention in *CCR5* transcription. Zebularine inhibits DNMTs, whereas MS275 inhibits HDACs. DZNep is a more general KMTase inhibitor.[42]

positive as determined by flow cytometry (Figure 4–5A). In untreated Jurkat cells, only 0.83% of the cells stain positive for CCR5 (Figure 4–1A). Correspondingly, after treatment the levels of *CCR5* transcripts found in Jurkat T cells increased to 43% of the *CCR5* transcript levels found in activated CD4⁺ T cells (Figure 4–5B). HSB-2 and Molt-4 were more refractory to this combined epigenetic treatment, however still 49.4% and 18.2% of the cells respectively were expressing CCR5 at the cell surface after treatment (Figure 4–5A), whereas transcript levels were 20% and 4.8% relative to activated T cell transcript levels in HSB-2 and Molt-4 respectively (Figure 4–5B).

Next we evaluated the effect of the epigenetic drug treatment on the expression characteristics of CREB-1 and ICER in Jurkat cells by semi-quantitative RT-PCR as we have previously explored.[26] ICER, the inducible cAMP early repressor, which is induced by forskolin, competes with CREB-1 for DNA binding. We and more recently also others have shown that induction of ICER by forskolin treatment indeed reduces CCR5 expression.[26,45] In figure 4–6 it is shown that pharmalogical induction of CCR5 expression did neither result in the induction of CREB-1, nor in a reduction

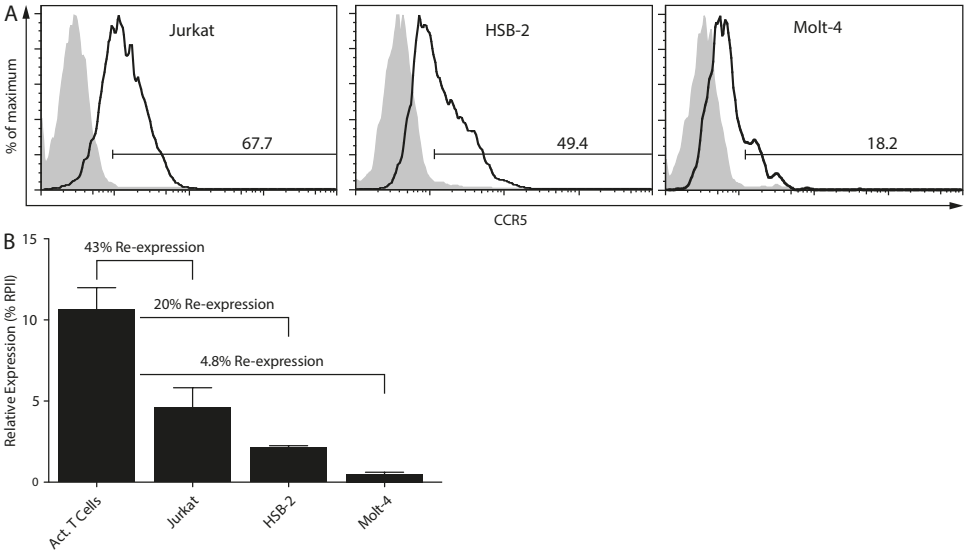

Figure 4–5. (A) Restoration of CCR5 expression in Jurkat, HSB-2 and Molt- 4 T leukaemia cell lines following exposure of cells to Zebularine, DZNep and MS275 determined by flow cytometry. Numbers indicate the percentage of CCR5 positive cells; filled histograms represent the non-treated cells and open histograms the treated cells. (B) Levels of *CCR5* transcripts in treated Jurkat, HSB-2 and Molt-4 T leukaemia cells relative to *CCR5* transcript levels in activated CD4⁺ T cells. The transcript level data of activated T cells are the same as shown in figure 4–1B.

of ICER in Jurkat T cells. Notably, when compared with naïve or activated $CD4^+$ T cells, Jurkat cells do express CREB-1, but hardly any ICER could be detected. In contrast, naïve T cells show low levels of CREB-1, with relatively high levels of ICER. Upon activation, the levels of ICER are reduced while on the other hand CREB-1 levels are induced (Figure 4–6). These observations indicate that in Jurkat T cells induction of CCR5 expression most likely is not resulting from alterations in the interplay of CREB-1 and ICER.

We also investigated whether the pharmalogical induction of CCR5 expression was associated with alterations in the histone acetylation/methylation profile and recruitment of CREB-1 and RNA polymerase II in CCR5 promoter chromatin. As shown in figure 4–7A there is a clear increase in the H3Ac mark (associated with gene expression) after treatment, whereas histone marks associated with gene repression appear to be more resistant to the treatment. Shown in figure 4–7B is that the permissive *CCR5* chromatin structure in activated T cells (Figure 4–3) results in increased recruitment of CREB-1 and RNA polymerase II into CCR5 promoter chromatin when compared with naïve T cells. Similarly, the induction of CCR5 expression after epigenetic treatment of Jurkat cells is also accompanied by an

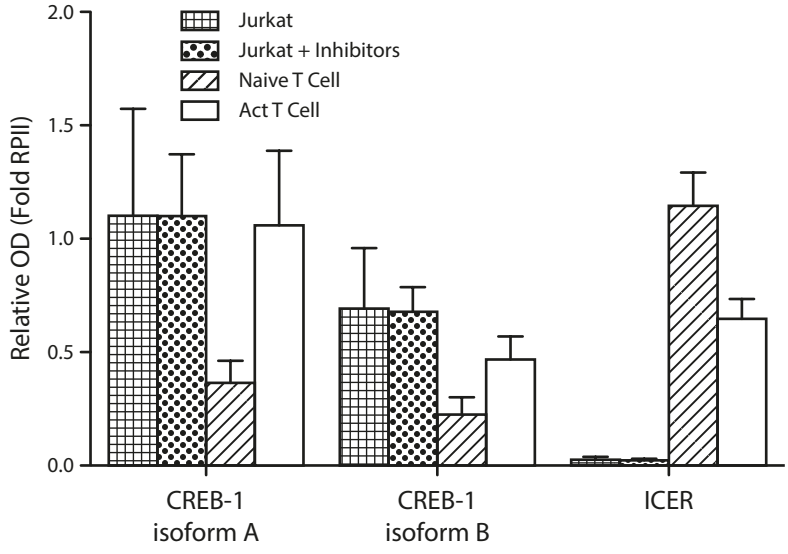

Figure 4–6. Semi-quantitative RT-PCR for CREB-1 isoforms and ICER were performed in triplicate. Activated T cells show higher levels of both CREB-1 isoforms, when compared to naïve T cells, whereas naïve T cells show higher levels of ICER then activated T cells. Jurkat T leukaemia cells show virtually undetectable levels of ICER. Treatment of Jurkat cells with Zebularine, DZNep and MS275 does not influence CREB-1 or ICER transcript levels.

increase in the recruitment of CREB-1 and RNA polymerase II into CCR5 promoter chromatin (Figure 4–7B). Together, the pharmalogical inhibition of the activities of the various epigenetic enzymes that account for the repressive chromatin state of CCR5 in Jurkat T cells has resulted in a shift into a more open chromatin structure.

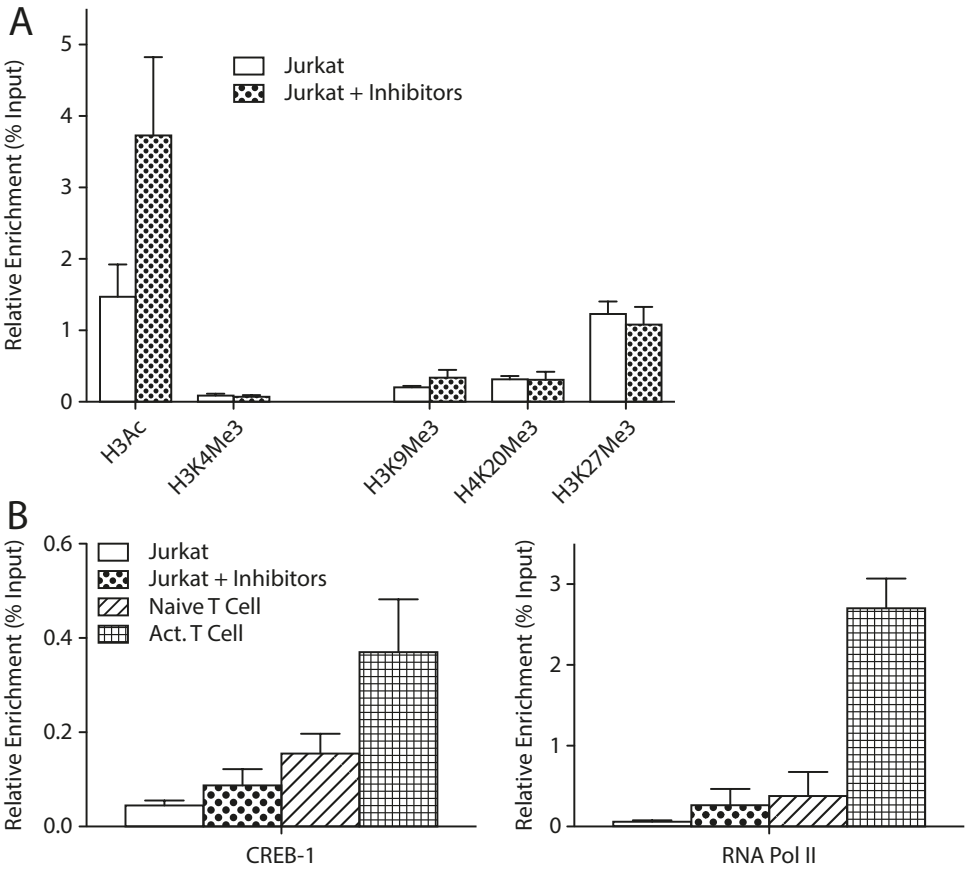

Figure 4–7. (A) ChIP analysis of histone modification at the *CCR5* promoter in Jurkat cells, after treatment with Zebularine, DZNep and MS275. The treatment of Jurkat cells results in an increase of H3Ac at the *CCR5* promoter. Repressive marks at the *CCR5* chromatin are not influenced much by the treatment, although a minor decrease in H3K27Me3 can be noted. (B) ChIP analysis of the *CCR5* promoter for CREB-1 and RNA polymerase II after treatment with SMIs in Jurkat, compared to both naïve and activated T cells. Treatment of Jurkat cells with Zebularine, DZNep and MS275 slightly increases CREB-1 in chromatin of the *CCR5* promoter. In both naïve and activated T cells higher levels of chromatin-associated CREB-1 can be found. Compared to naïve T cells, there is an increase of CREB-1 in activated T cells. Treatment of Jurkat cells with SMIs increases RNA polymerase II recruitment to the *CCR5* promoter to levels similar of naïve T cells. In comparison to activated T cells, the levels of RNA polymerase II in the *CCR5* promoter region of treated Jurkat cells are modest.

This is accompanied by an increase in promoter association of the transcription factor CREB-1 and recruitment of RNA polymerase II.

Discussion

This study reveals that epigenetic mechanisms involving DNA methylation, histone acetylation and methylation modifications all contribute to the transcriptional regulation of CCR5 expression. In CCR5-deficient T leukemia cells we show that the promoter region is mainly characterized by repressive histone marks in the presence of methylated DNA. In CCR5-expressing activated T cells this region is mainly associated with activating histone marks and low levels of DNA methylation. Interestingly, the B4/5 region in the CCR5 promoter, which was previously attributed to CREB–1-mediated transactivation is mostly unmethylated both in Jurkat and activated T cells.

Intermediate or low CCR5-expressing monocytes and naïve T cells respectively are characterized by both repressive histone methylation marks and permissive histone acetylation marks. In naïve T cells an intermediate level of DNA methylation accompanies these histone modifications. However, in monocytes the level of DNA methylation is markedly higher as compared to naïve T cells, with the B4/5 region in a mostly unmethylated state in both cell types. Together, the cell types investigated here show that the B4/5 region is mostly unmethylated, irrespective of CCR5 transcription. This suggests that the B1 and B3 regions could contribute to the transcriptional regulation of CCR5 as has been argued previously.[26,46,47]

Notably, monocytes and naïve $CD4^+$ T cells represent a poised state recognized by the presence of both repressive and permissive histone marks. Considering the various histone triple-methylation modifications investigated, we conclude that acetylation of histone H3 is essential for CCR5 expression as is illustrated in naïve T cells and in monocytes. The dominant role of histone modifications is further underscored by the fact that monocytes show high levels of DNA methylation. Although DNA methylation is usually interpreted as a repressive chromatin mark, this study as well as some recent other studies show that DNA methylation in the absence of repressive histone marks permits active gene transcription.[48–54] This is also in line with previous studies showing that the presence of the H3K27Me3 histone modification correlated with lack of transcription despite absence of DNA methylation.[50,55,56] Interestingly, the monocyte population presented in this study shows transcription in presence of DNA methylation, H3K27Me3, H3K9Me3 and H3Ac, but notably low levels of H4K20Me3. This underscores – as has been previously

noted[48] – that not all epigenetic histone marks contribute equally to a specific chromatin status. Rather, the sum of epigenetic modifications, or "epigenetic profile", is more important than individual modifications to allow gene transcription.

The role of epigenetic regulatory mechanisms in the control of CCR5 transcription is also underscored by the pharmacological interference in the identified components of epigenetic regulation. Since the epigenetic modifications were observed in both DNA and in histones encompassing the CCR5 promoter, we combined the various inhibitors to induce re-expression. This intervention resulted in the re-expression of CCR5 in Jurkat, HSB-2 and Molt-4 T leukemia cells, albeit that the levels of re-expression differ between the cell lines investigated and were never on par with activated T cells. Although the individual epigenetic inhibitors allowed marginal induction of CCR5 transcripts (data not shown), combination of inhibitors induced much higher transcription levels.

Changing the DNA methylation status through pharmacological disruption with Zebularine requires incorporation of Zebularine into the DNA.[40,41] Demethylation through usage of Zebularine thus requires replication of DNA and therefore proliferation of cells. Jurkat, HSB-2 and Molt-4 cell lines show different doubling times. The difference in re-expression levels of CCR5 after combined epigenetic therapy can therefore be explained by this difference in cell doubling times. Furthermore the relative toxicity of MS275 and DZNep may lower the proliferative capacity of the cells, thereby influencing the efficacy of Zebularine treatment. Especially since DNA methylation and histone modifications are intimately linked,[32,57] this may result in a situation where 100% re-expression of the gene of interest might prove to be a challenge. Yet despite these drawbacks, interference in the epigenetic machinery still results in a dramatic rise of CCR5 transcripts in T leukemia cells.

Together, these data strongly indicate that histone acetylation and methylation modification mechanisms contribute to the transcriptional control of CCR5. In addition, we show that chromatin in a bivalent state allows for the fine-tuning of transcription levels, as has been shown before for other genes.[32,58] Moreover, our data suggest that epigenetic deregulation could be one of the mechanisms leading to enhanced CCR5 expression as observed in a variety of inflammatory conditions. Although we demonstrate in this study the re-expression of CCR5, it could be envisioned that the use of lysine acetyltransferase inhibitors (e.g. curcumin or garcinol[59,60]) may have the opposite effect. As such, CCR5-mediated trafficking of lymphoid and myeloid cells is a possible target for pharmacological intervention.

Interference in these deregulated epigenetic processes may therefore be a promising therapy for the treatment of inflammatory diseases.

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Authorship Contributions

RJW, HFK, MCJAvE, AB, JCvL, SC, and SBG performed experiments. RJW and HFK wrote the paper. VEM provided essential reagents. JWJ, PHAQ, and PJvdE critically discussed and reviewed the paper. PJvdE supervised the project.

Conflict-of-interest disclosure

The authors confirm that there are no conflicts of interest.

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