CCR5 in multiple sclerosis : expression, regulation and modulation by statins

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CCR5 transcriptional regulation

CC chemokine receptor 5 (CCR5) gene promoter activation by the cyclic AMP response element binding (CREB) transcription factor

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Abstract

The chemokine receptor CCR5 is implicated in the pathogenesis of various inflammatory diseases such as multiple sclerosis (MS), atherosclerosis, transplant rejection and autoimmunity. In previous studies, we have shown that MS lesions are characterized by enhanced expression of transcription factors associated with stress-responses, i.e. IRF-1, NF-κB and CREB-1, which modulate expression of both classes of major histocompatibility complex (MHC) molecules. The expression of MHC-I and MHC-II molecules greatly overlaps with the expression of CCR5 in MS lesions. Therefore, we investigated whether these factors are also involved in the transcriptional regulation of CCR5. Using in vitro assays, we determined that neither IRF-1 nor NF-κB is involved in the activation of the CCR5 promoter. This is corroborated by the finding that these factors are not involved in the induction of endogenous CCR5 transcription in various cell types. In contrast, we show that CCR5 expression is regulated by the cAMP/CREB pathway and that interference in this pathway affects endogenous CCR5 transcription. From this, we conclude that the cAMP/CREB pathway is involved in the regulation of CCR5 transcription and that, given the ubiquitous nature of CREB-1 expression, additional regulatory mechanisms must contribute to cell type-specific expression of CCR5.

Introduction

Chemoattractant cytokines (CC) or chemokines are secreted proteins that mediate the migration of leukocytes towards sites of inflammation. Leukocyte trafficking is initiated following activation by chemokines through binding to specific chemokine receptors on their cell surface. The CC chemokine receptor 5 (CCR5) regulates trafficking and effector functions of memory/effector T-lymphocytes, macrophages, and immature dendritic cells (DCs). Interactions between chemokines and chemokine receptors are promiscuous, i.e. most chemokines activate more than one receptor and most chemokine receptors can bind several chemokines [1]. It is well established that CCR5 binds the chemokines CCL5 (RANTES), CCL3 (MIP-1α) and CCL4 (MIP-1β) to fulfill its role in the regulation of differentiation and anatomical distribution of leukocytes to meet with local requirements for an adequate immune response against pathogens. Because of its important immune regulatory role, CCR5 is also implicated in the pathogenesis of various inflammatory diseases such as atherosclerosis, transplant rejection and autoimmunity, and neurodegenerative diseases [2-6]. In addition, CCR5 also serves as a coreceptor for viral entry of HIV-1 [7,8]. CCR5 is mainly expressed on a subset of T-lymphocytes, monocytes, macrophages, DCs and microglia.

Multiple Sclerosis (MS) is a demyelinating disease with inflammatory aspects that are mediated by infiltrating leukocytes, resulting in extensive inflammation and demyelination of the central nervous system [9]. Enhanced expression of CCR5 has been noted in MS affected central nervous system (CNS) tissue when compared with normal appearing white matter (NAMW) of patients and with control brain tissue of non-MS patients [10,11]. In particular increased expression of CCR5 has been detected on reactive microglia near the edges of active demyelinating MS lesions and on phagocytic (foamy) macrophages present inside these lesions [10,11]. In addition, expression of CCR5 has been found on reactive astrocytes in a number of MS cases [10].

In earlier studies, we have found that expression of MHC-II and MHC-I is enhanced in MS lesions [12]. This enhanced expression is mainly found on activated microglia and foamy macrophages, but also on astrocytes. We have shown that this enhanced expression is due to a concomitant increase in the expression of transcription factors controlling expression of both classes of MHC molecules. These include MHC-specific transcription factors and general factors, such as IRF-1, NF-κB and CREB-1 [12,13]. The fact that the latter group can be activated by a variety of stresses including viral and bacterial infection, inflammation and tissue damage [14-16] suggests that a general state of cell activation is present in MS lesions.

Because the expression of CCR5 on CNS resident cell resembles to a great extent the expression of both classes of MHC molecules found in MS lesions, we investigated whether IRF-1, NF-κB and CREB-1 are also involved in the regulation of CCR5 transcription in various cell types.

Using established human monocytic and glioma cell lines, cultured monocyte-

derived DCs, and primary astrocytes and microglia, we determined that neither IRF-1 nor NF-κB are involved in the activation of CCR5 promoters, nor in the induction of endogenous CCR5 transcription. In contrast, we found that the CREB pathway regulates the activity of the CCR5 promoter. Considering the ubiquitous nature of CREB-1, these findings suggest that additional genetic or epigenetic mechanisms contribute to the cell-type specific transcriptional regulation of CCR5.

Materials & Methods

Cell culture

The cell lines THP-1, U251-MG and Tera-2 (ATCC, Manassas, Virginia) were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands), 100 IU/ml streptomycin and 100 IU/ml penicillin.

Transcription factor binding site search

Potential transcription factor binding sites were identified using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html), which searches the TRANSFAC database [17,18]. Cut of was set at 85% of the consensus TF binding site.

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared and electrophoretic mobility shift assays (EMSA) were performed as previously described [19]. Briefly, 2 μl of nuclear extracts were incubated with 2 ng of [^{33}P]-labeled dsDNA probe in binding buffer for 30 minutes on ice. Samples were run on a 6% polyacrylamide gel in 0.25x TBE buffer at 200 V for 150 minutes. The ds-oligonucleotides used as probe for transcription factor binding sites are depicted in Table 1.

For competition assays, nuclear extracts were incubated with an unlabeled consensus oligonucleotide (Table 1) for 30 minutes prior to incubation with the labeled oligonucleotides. For supershift assays, after oligonucleotide/nuclear extract incubation 1 μg of antibody was added and incubation was continued for an additional 60 minutes on ice. The antibodies used (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA) are depicted in Table 2.

Transient transfection

CCR5 promoter constructs were a kind gift from Prof. S. K. Ahuja (University of Texas Health Science Center at San Antonio, San Antonio, TX). Tera-2 cells were transfected with the CCR5 upstream promoter-luciferase reporter constructs pGL3-CCR5-pA1, pGL3-CCR5-pA2, pGL3-CCR5-pA3, pGL3-CCR5-pA4, the CCR5 downstream promoter constructs pGL3-CCR5-pB1, pGL3-CCR5-pB3, pGL3-CCR5-

Table 1. ds-oligonucleotide sequences used for EMSA

EMSA probe	Oligonucleotide sequence*	Location**
ISRE β2m[20]	5′-TAAGAAAAGGAAACTGAAAACG-3′	-
CCR5-ISRE-1	5′-CTCCGCATGGTGAAAGTAAGAACC-3′	-4026
CCR5-ISRE-2	5′-GCAATTAGCTTTACCTTTTCAGCTTCT-3′	-3367
CCR5-ISRE-3	5′-GGACTGCTGAAAGAGTAACTAAGAGTT-3′	-3255
κB β2m[20]	5′-ACGGGAAAGTCCCTC-3′	-
CCR5-κB-1	5′-GAACAGAGTGAAAATCCCCACTAAGA-3′	-2650
CCR5-κB-2	5′-CTTACTGTTGAAAAGCCCTGTGATCT-3′	-2340
CCR5-κB-3	5′-ATCCAGTGAGAAAAGCCCGTAAATAA-3′	-2155
CRE consensus[21]	5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′	-
CCR5-CRE-1	5′-AACACAAAAGTGGAGTAACGCACA-3′	-4369
CCR5-CRE-2	5′-CAGGTCTAGCACGTCATTTAACAG-3′	-4226
CCR5-CRE-3	5′-TATCTTGCCGAGGTCACAAAGCAA-3′	-4172
CCR5-CRE-B1	5′-GATTGGGGGCACGTAATTTTGCTG-3′	-3028
CCR5-CRE-B2	5′-AGCCAAGGTCACGGAAGCCCAGAG-3′	-2950
CCR5-CRE-B3	5′-AGATTTTCAGATGTCACCAACCGC-3′	-2866
CCR5-CRE-B4	5′-CCATATACTTATGTCATGTGGAAA-3′	-2788
CCR5-CRE-B5	5′-GGTTAATGTGAAGTCCAGGATCCC-3′	-2445
CCR5-CRE-B6	5′-TGGGCTTTTGACTAGATGAATGTA-3′	-2267
CCR5-CRE-B7	5′-TAGTGGGATGAGCAGAGAACAAAA-3′	-2187
CCR5-CRE-B8	5′-GCTTATTTTAAGCTCAACTTAAAA-3′	-2102
CCR5-CRE-B9	5′-TCTAGCTCTGATATCCTTTATTCT-3′	-2235
CCR5-CRE-B10	5′-CGTAAATAAACCTTCAGACCAGAG-3′	-2138
CCR5-CRE-B11	5′-ATTCTTTTCGCCTTCAATACACTT-3′	-2061
CCR5-CRE-B12	5′-ACTCCACCCTCCTTCAAAAGAAAC-3′	-2028
CCR5-CRE-B13	5′-TGATTTGCACAGCTCATCTGGCCA-3′	-1972

* Potential protein binding sites are underlined
** Location relative to start ORF (+1) according to accession no: AF031236 and AF031237[22]

pB4 [23] or the pGL3-Basic luciferase reporter plasmid (Promega, Madison, Wisconsin) in combination with the actin driven *Renilla* pGL3 reporter construct (pRL, Promega) as an internal control. pGL3-B250 and pGL3-β2m have been described previously [19,20].

Cells were transfected in triplicate with 1 μg of promoter construct and 0.1 μg of actin-pRL construct, using the calcium phosphate coprecipitation method [24]. For cytokine induction experiments, cells were treated with IFN-γ (500 U/ml; Boehringer-Ingelheim, Alkmaar, The Netherlands) or TNF-α (10 ng/ml; BioSource, Nivelles, Belgium) for 24 hours after transfection. For transcription factor induction experiments, cells were co-transfected with 0.5 μg of the previously described [25] pRc/RSV expression vectors of IRF-1, CREB-1, CBP, p300, P/CAF, ATF-1 or ICER. Cells were harvested 48 hours after

transfection and luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Primary cell culture and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from blood of normal healthy donors using a Ficoll gradient (Pharmacy Leiden University Medical Center, Leiden, The Netherlands). Isolated PBMC were stimulated with 10 μM Forskolin (Calbiochem, La Jolla, CA) for 4 to 6 h, after which RNA was isolated.

To obtain DCs, monocytes derived from freshly isolated PBMC were cultured in RPMI-1640 medium (Gibco BRL, Life Technologies, Breda, The Netherlands) with regular supplements and stimulated with a combination of 1000 U/ml recombinant human GM-CSF and 1000 U/ml IL-4 (both BioSource) for 7 days. Subsequently, the obtained cells were stimulated with LPS (100 ng/ml; Sigma-Aldrich, Steinheim, Germany) or IFN-γ (500 U/ml) for 24 hours each, after which RNA was isolated.

Human brain tissue was obtained by rapid autopsy according to standardized procedures under the management of the Netherlands Brain Bank, Amsterdam, The Netherlands. All patients or their next of kin had given written consent for autopsy and use of their brain tissue for research purposes. Brain samples were obtained from a patient without neurological complications and a patient with Multiple Sclerosis. Isolation of human adult astrocytes and microglia was performed as described previously [26]. Astrocytes were cultured in poly-L-lysine coated 75 cm^2-culture flasks (Greiner) in DMEM/HAMF10 (Gibco BRL Life Technologies) with regular supplements. For microglia isolation, cells were purified by a percoll/myelin-gradient buffer and plated in 25 cm^2-culture flasks (Greiner).

Table 2. Antibodies used for EMSA supershift assays

Antibody name	Protein reactivity	Catalogue no.
IRF-1 (C-20)	IRF-1	sc-497
IRF-2 (C-19)	IRF-2	sc-498
IRF-4 (M-17)	IRF-4	sc-6059
ICSBP (C-19)	IRF-8	sc-6058
NF-κB p50 (NLS)	NF-κB p50	sc-114
NF-κB p65 (A)	NF-κB p65 (Rel A)	sc-109
RelB (C-19)	RelB p68	sc-226
cRel (C)	c-Rel p75	sc-71
CREB-1 (24H4B)	CREB-1 p43	sc-271
ATF-1 (C41-5.1)	ATF-1 p35	sc-243
ATF-1 (25C10G; ATF/CREB)	ATF-1, CREB-1, CREM-1	sc-270

For stimulation, astrocytes cultures grown in 75 cm^2 flasks were trypsinized and seeded into 25 cm^2 flasks at a density of approximately 1 x 10^5 astrocytes per flask. Cells were stimulated with LPS (100 ng/ml) or IFN-γ (500 U/ml) each for 8 hours prior to RNA-isolation. Microglia grown in 25 cm^2-culture flasks were stimulated with TNF-α (10 ng/ml or IFN-γ (500 U/ml) each for 8 h, followed by RNA-isolation.

RNA isolation and RT-PCR

Total RNA was isolated using the RNA-Bee extraction method (Tel-Test, Friendswood, Texas). cDNA was synthesized from 4 μg of each RNA sample using AMV reverse transcriptase (Promega). CCR5, NF-κB p50, HLA-DRA, CIITA-PIV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) products were amplified by PCR reaction. Primer sequences and condition used are depicted in Table 3. PCR cycles consisted of 30 seconds denaturation at 95°C, 30 seconds annealing and 30 seconds elongation at 72°C. For each PCR reaction 2,5 μl of 1:5 diluted cDNA was used in a total reaction volume of 25 μl, containing 1× PE PCR reaction buffer (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ), 0.5 mM of each dNTP, 10 pmol of each primer and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer). The PCR products were separated and visualized on an ethidium bromide stained agarose gel.

Table 3. PCR primer sequences and conditions

PCR product	Primer sequence (5′-3′)	T_{ann} *	Number of cycles	$MgCl_2$ concentration
CCR5	F-CTGAGACATCCGTTCCCCTA R-GCTCTTCAGCCTTTTGCAGT	60°C	32	3 mM
NF-κB p50	F-GAACTCCTCCATTGTGGAACC R-CCCGGAGATTTGCTGTCATG	62°C	32	1.5 mM
HLA-DRA	F-GGCCATAAGTGGAGTCCC R-CTATACTCCGATCACCAA	55°C	30	3 mM
CIITA-PIV	F-AGCTGGCGGGAGGGAGAGGCCACC R-CATACTGGTCCAGTTCCGCGATATTGG	60°C	35	1.75 mM
GAPDH	F-GGTCGGAGTCAACGGATTTG R-ATGAGCCCCAGCCTTCTCCAT	60°C	22	1.5 mM

* Tann: annealing temperature

Results

Location of potential binding sites in the CCR5 promoter region for transcription factors induced by inflammatory signaling pathways

The organization and promoter usage of the CCR5 gene has been elucidated previously by Mummidi *et al.* using luciferase-reporter constructs containing CCR5 regulatory regions (Figure 1) [23,27]. Two distinct functional promoter regions for the CCR5 gene were identified: a downstream promoter region, designated P1, and an upstream promoter region, designated P2. Using the TFSearch program we evaluated the CCR5 promoter regions for potential interferon-stimulated response elements (ISREs), which bind interferon regulatory factors (IRFs), κB elements, which bind nuclear factor κB (NF-κB), and cAMP-response elements (CRE elements) which can bind the common activator of transcription CREB-1 (cAMP-response element (CRE)-binding protein) and its family members.

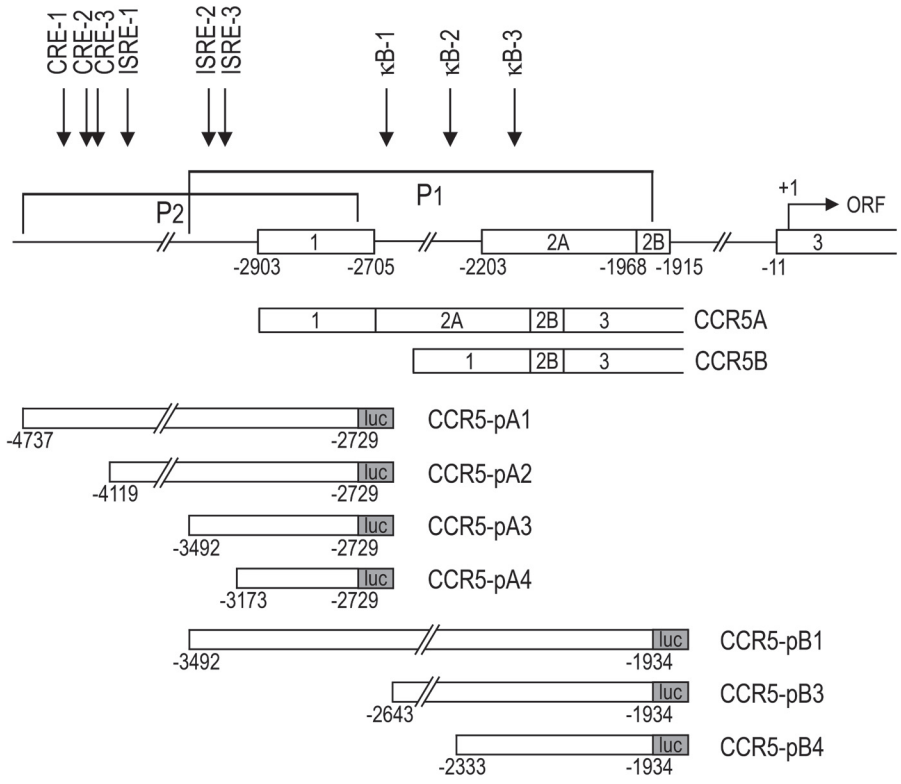


Figure 1. Gene and promoter organization of human CC chemokine receptor CCR5. Schematic representation of the CCR5 gene and its promoters. Exons (1 through 3) are depicted by boxes. Both promoter regions, the downstream promoter P1 and the upstream promoter P2, are indicated above the scheme. Locations of the identified putative transcription factor binding sites are depicted above. Underneath, promoter-luciferase-reporter constructs used in this study are shown. Two sets of constructs were used: the upstream promoter constructs CCR5-pA1 through -pA4 and the downstream promoter constructs CCR5-pB1, -pB3 and -pB4. Nucleotide positions for the exons and the promoter constructs are depicted relative to the ORF in exon 3.

Figure 1 shows the location of the TFSearch-identified potential binding sites for these transcription factors (see also Table 1). The potential NF-κB-binding sites (CCR5-κB-1 through κB-3) are situated only in the downstream promoter region, whereas the putative CRE sites (CCR5-CRE-1 through CRE-3) are all located in the upstream promoter region. One of the identified potential binding sites for proteins of the IRF family, CCR5-ISRE-1, is located in the upstream promoter region, whereas the other ISREs, CCR5-ISRE-2 and ISRE-3, are located in the region in which the upstream promoter and the downstream promoter overlap each other.

Protein/DNA interactions at the identified putative transcription factor binding sites in the CCR5 promoter region

We tested the capacity of the identified regulatory sites in the promoter regions of the CCR5 gene to bind specific transcription factors *in vitro* by EMSA. Using extracts of THP-1 and U251 cells stimulated with TNF-α (which induces NF-κB activity), we studied *in vitro* complex formation at the κB sites present in the CCR5 downstream promoter. We could detect constitutive protein binding to the CCR5-κB-1 and CCR5-κB-2 probes but not to the CCR5-κB-3 probe using nuclear extracts of both cell types. Similar to binding to the κB element of the β2m promoter [20], the amount of protein binding to the CCR5-κB-1 was dramatically increased after TNF-α stimulation (Figure 2A). Binding of this TNF-α-induced factor to the CCR5-κB-1 was specific for the κB sequence in the oligonucleotide probe because it could be competed away with the β2m κB probe. In contrast, protein binding to CCR5-κB2 proved to be not specific.

To identify the proteins bound to these sites we performed a supershift analysis with antibodies directed against several members of the NF-κB family. We found that TNF-α stimulation of THP-1 and U251 cells induced binding of both subunits p50 and p65 of NF-κB to CCR5-κB-1 (Figure 2B). In addition, we could identify the constitutive binding to the CCR5-κB-1 site as binding of NF-κB p50 in THP-1 cells and binding of NF-κB p65 in U251 cells. In contrast, the complex found with CCR5-κB-2 could not be shifted with any of the antibodies, confirming that this binding is non-specific.

Next, *in vitro* binding to the putative ISREs was tested using nuclear extracts of U251 cells, either unstimulated or stimulated with IFN-γ. IFN-γ stimulation induced binding of one main and two minor protein complexes to CCR5-ISRE-1 and CCR5-ISRE-3, and to a lesser extent to CCR5-ISRE-2 (Figure 3A). Binding of these IFN-γ-induced complexes could be competed away with an ISRE consensus probe, indicating that the binding is specific for the ISRE sequence in the probes. In THP-1 cells we could detect only very weak binding to CCR5-ISRE-1 and CCR5-ISRE-3 (not shown).

Previously we have shown that IRF-1 is the main IRF that is induced by IFN-γ in U251 and THP-1 cells [20]. In addition, U251 cells constitutively express IRF-2, whereas THP-1 cells constitutively express both IRF-2 and the lymphoid/myeloid-specific factors IRF-4 and IRF-8 (data not shown, [20]). Supershift analysis revealed binding of IRF-1 to CCR5-ISRE-1 and CCR5-ISRE-3 using nuclear extracts from U251 cells, whereas IRF-2,

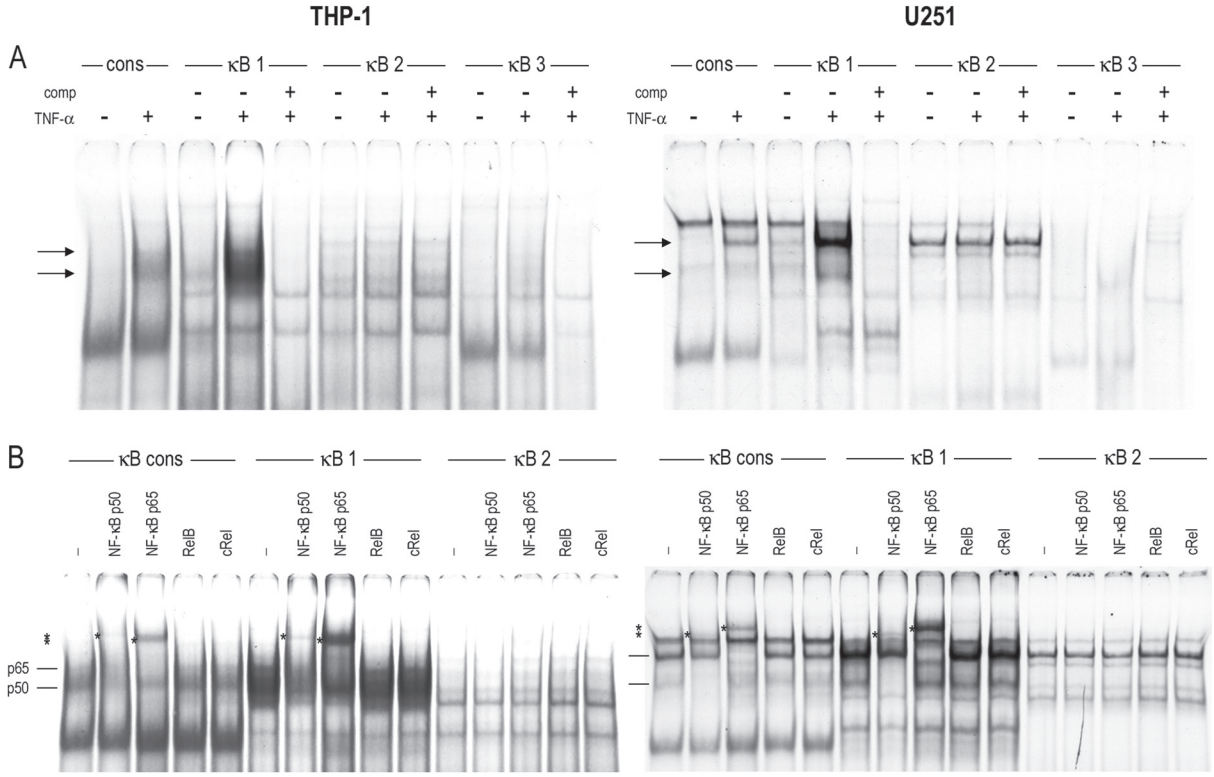


Figure 2. Transcription factor binding to the κB sites in the CCR5 downstream promoter. EMSA showing binding of complexes to the κB sites of CCR5 (κB-1 through –3) and the κB site of human β2m as a consensus probe (cons) using nuclear extracts of THP-1 cells (left panels) or U251 cells (right panels). **A** Cells were left untreated or stimulated with TNF-α (10 ng/ml) for 2 hours. EMSA analysis revealed formation of two complexes to CCR5-κB-1 upon TNF-α treatment (arrows). CCR-5-κB-2 showed non-specific complex binding constitutively, whereas CCR-5-κB-3 did not generate any significant binding, neither constitutive nor upon TNF-α treatment. **B** Using specific antibodies, the proteins binding to CCR5-κB-1 upon TNF-α treatment were identified as NF-κB p65 and p50. Binding of these factors to CCR5-κB-2 could not be detected. * supershifted complexes. Shown are representatives of 2 independent experiments

present in U251 cells, as determined by its binding to the β2m ISRE, did not bind to the CCR5 ISREs (Figure 3B). Furthermore, the CCR5-ISRE-1 and CCR5-ISRE-3 failed to bind IRF-4 and IRF-8 expressed in THP-1 nuclear extracts, while these factors did interact with the β2m-ISRE (results not shown, [20]). These findings indicate that only IRF-1 binds to CCR5-ISRE-1 and CCR5-ISRE-3 after IFN-γ stimulation.

Finally, we analyzed binding to the putative CRE sites in the CCR5 upstream promoter. We detected constitutive binding of three complexes in nuclear extracts from THP-1 and U251 cells to the CCR5-CRE-2 and -CRE-3, although binding to the latter was very weak (Figure 3C). Binding to both CCR5-CRE-sites was specific for the CRE-region in the probe, as determined by competition with a CRE consensus probe [21].

The CREB family consists of three members: CREB-1, activating transcription factor 1 (ATF-1) and cAMP responsive element modulator (CREM), which all bind to CRE sequences as homo- or heterodimers [28]. With antibodies directed against either CREB-1 or ATF-1 and an antibody that recognizes CREB-1, ATF-1 and CREM-1, we determined

that all three antibodies induced a supershift of one or more of the complexes bound to CCR5-CRE-2 and CCR5-CRE-3 (Figure 3D). The supershift patterns revealed that both CREB-1 and ATF-1 bind to CCR5-CRE-2 as well as CCR5-CRE-3. In addition, the middle complex formed at CCR5-CRE-2 was identified as CREM-1 binding, because it was only shifted by the antibody directed against multiple members of the CREB family (ATF/CREB), whereas the upper and lower complexes were shifted by both this antibody and the antibodies against CREB-1 and ATF-1, respectively (Figure 3D).

Taken together, these analyses reveal that the CCR5 promoter contains at least two ISREs, one κB site and one CRE site that can be bound *in vitro* by their respective transcription factors that are present in THP-1 or U251 cells, either constitutively or induced by IFN-γ or TNF-α.

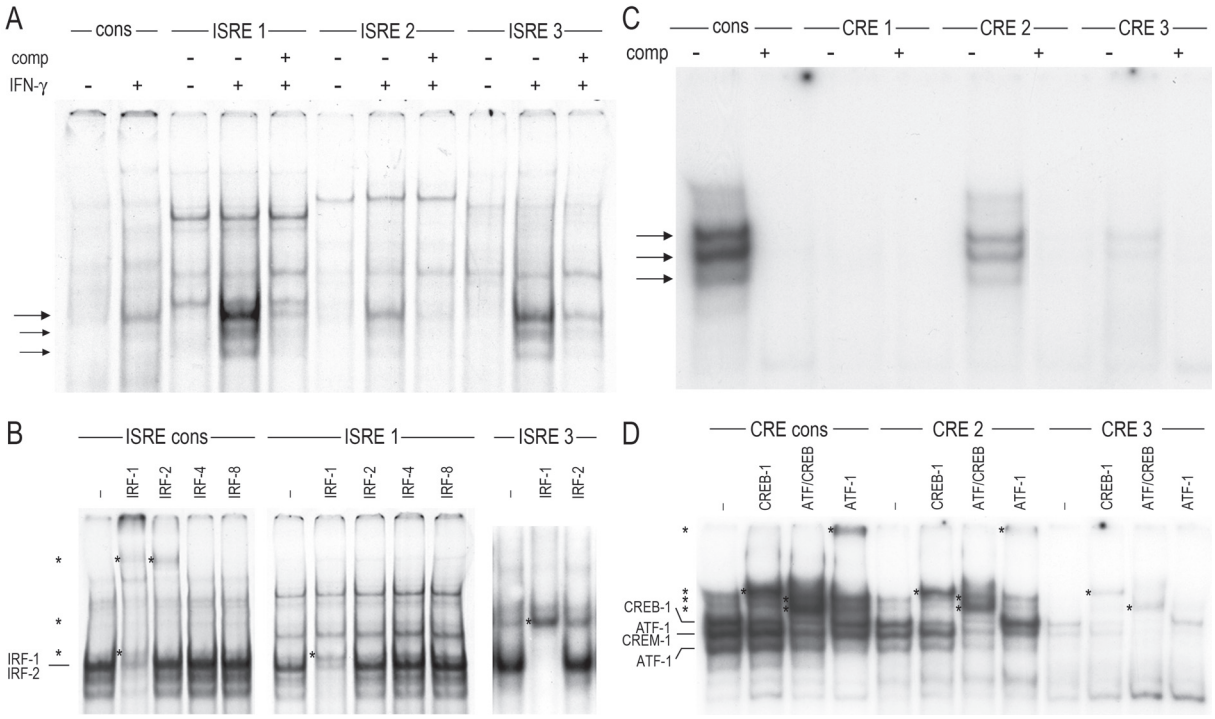


Figure 3. Transcription factor binding to the ISREs and CRE sites in the CCR5 promoter. **A** EMSA showing binding of complexes to the ISREs of CCR5 (ISRE-1 through ISRE-3) and the ISRE of human β2m as a consensus probe (cons) using nuclear extracts of U251 cells either unstimulated or stimulated with 500 U/ml IFN-γ for 2 hours. IFN-γ stimulation induced formation of one major (large arrow) and two minor (small arrows) complexes to CCR5-ISRE-1 and CCR5-ISRE-3 and weak binding of these complexes to CCR5-ISRE-2, specific for the ISRE in the probes. **B** Using specific antibodies, the proteins binding to the β2m-ISRE upon IFN-γ treatment were identified as IRF-1 and IRF-2, whereas the complexes binding to ISRE-1 and ISRE-3 contained only IRF-1. **C** EMSA showing binding of complexes to the CRE sites located in the CCR5 upstream promoter (CRE-1 through CRE-3) and a CRE consensus probe (cons) using nuclear extracts of U251 cells. Binding of three complexes (arrows) to CCR5-CRE-2 and to a lesser extent CCR5-CRE-3, specific for the CRE in the probes, was detected. **D** Using specific antibodies, the proteins binding to CCR5-CRE-2 and CCR5-CRE-3 were identified as CREB-1 and ATF-1. In addition, the CCR5-CRE-2 binding complex was revealed to contain also CREM-1. * supershifted complexes. Shown are representatives of 2 independent experiments.

Transactivation capacity of the putative regulatory sites

To test whether the binding sites that displayed *in vitro* binding of transcription factors in EMSA are actually functional, we performed luciferase-reporter assays in the cytokine-responsive teratocarcinoma cell line Tera-2 using various promoter constructs generated by Mummidi *et al.* [23] (Figure 1).

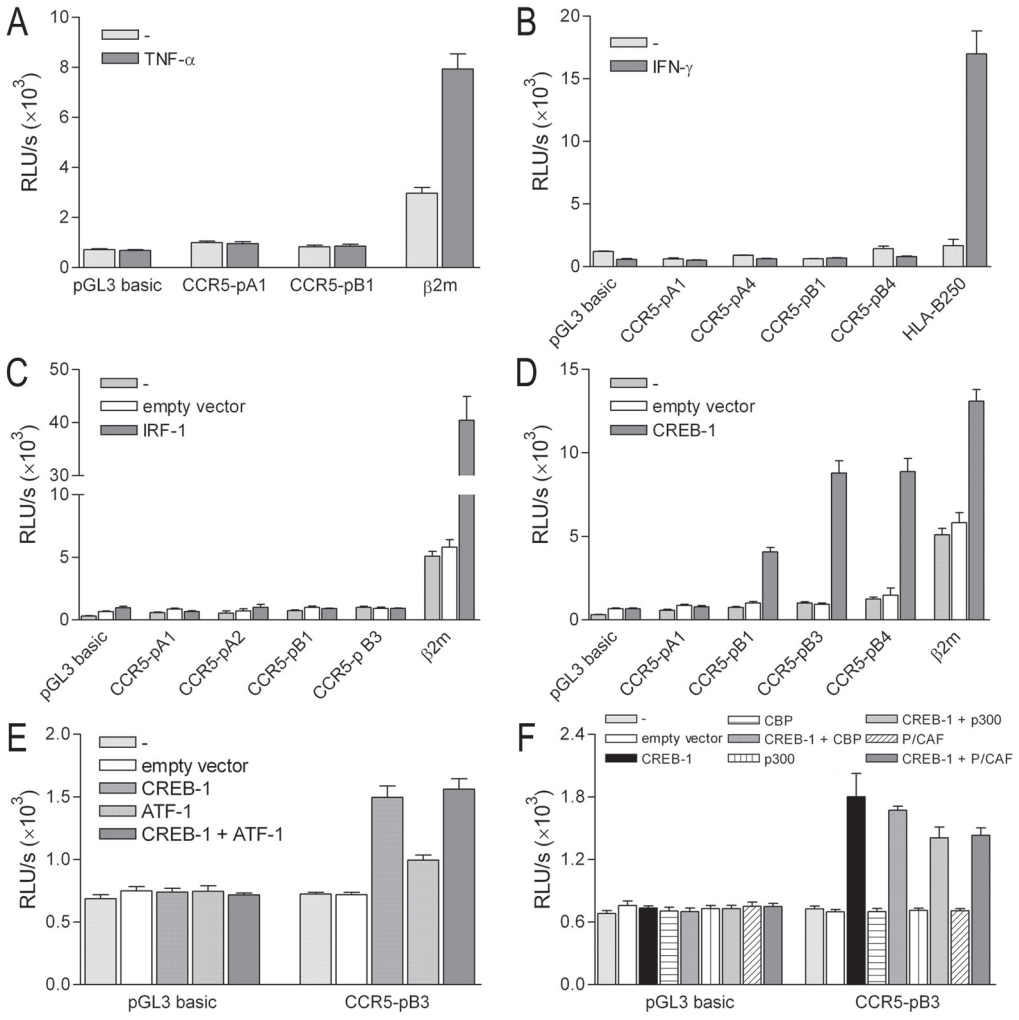
Transient transfection of the CCR5 promoter constructs revealed that none displayed any constitutive activity (Figure 4). This notion is in line with the absence of CCR5 transcription in Tera-2 cells (unpublished observations). We then first investigated whether we could activate the CCR5 downstream promoter, which contains the *in vitro* NF-κB binding CCR5-κB-1 element, by TNF-α treatment. Exposure of Tera-2 cells to TNF-α revealed that the full-length CCR5 downstream promoter construct (CCR5-pB1) could not be activated by TNF-α, whereas a promoter construct containing the β2m promoter, responsive to TNF-α [20], did display enhanced activity upon TNF-α treatment (Figure 4A). These results indicate that the putative CCR5-κB-1 site found in the CCR5 promoter is not functional, despite *in vitro* NF-κB protein binding. In addition, we were not able to induce activity of the CCR5-pA1 upstream promoter construct by TNF-α treatment (Figure 4A), thereby excluding the presence of functional non-consensus NF-κB binding sites in the CCR5 promoter region, which remained undetected by the TFSearch analysis.

Next we determined whether the CCR5 promoter constructs were responsive to IFN-γ and the IFN-γ-induced transcription factor IRF-1. IFN-γ treatment did not result in the activation of the CCR5-pA1 upstream promoter construct that contains both CCR5-ISRE-1 and CCR5-ISRE-3, or the CCR5-pB1 downstream promoter construct that contains CCR5-ISRE-3. The activity of these constructs was comparable to the activity of the truncated constructs (CCR5-pA4 and CCR5-pB4) lacking these sites, and the control plasmid. In contrast, the IFN-γ-responsive HLA-B promoter [19] did show activity upon IFN-γ treatment (Figure 4B). In addition, cotransfection of an expression vector of IRF-1 did not lead to activation of the two CCR5 upstream promoter constructs containing both CCR5-ISREs (CCR5-pA1 and CCR5-pA2), or the CCR5-ISRE-3-containing downstream construct (CCR5-pB1) (Figure 4C). Again, activity did not rise above activity of a construct lacking CCR5-ISREs (CCR5-pB3) or the control plasmid, while another promoter known to be activated by IFN-γ, the β2m promoter [20], was activated by IRF-1 (Figure 4C). This demonstrates that, like the CCR5-κB-1 site, the putative CCR5-ISREs in the CCR5 promoter are not functional, although they do bind IRF-1 *in vitro*. Furthermore, these analyses exclude the presence of non-consensus ISREs in the full-length promoter-reporter constructs, which could potentially bind IRFs leading to IFNγ-mediated induction of CCR5.

Finally, we tested whether CREB-1, which can bind to the CRE-2 and CRE-3 sites *in vitro*, is able to transactivate the CCR5 upstream promoter. Cotransfection of the full-length upstream promoter construct, which contains both CCR5-CRE sites, with

an expression vector for CREB-1 did not lead to activation of this promoter construct (Figure 4D, CCR5-pA1). Furthermore, CREB-1 cotransfection was not able to activate any of the truncated upstream promoter constructs, CCR5-pA2 through –pA3, either (not shown). Surprisingly however, CREB-1 did activate the full-length downstream promoter CCR5-pB1 and to an even higher extent the two truncated downstream promoter constructs. The activity of the latter two reached a level comparable to CREB-1 induced activity of a β2m promoter construct (Figure 4D). These findings implicate that, whereas the CRE sites found in the upstream promoter are not functional, the downstream promoter region must contain at least one functional CREB-binding site.

In addition to this, we tested the capacity of ATF-1 to activate the CCR5 promoter and the cooperation of CREB-1 with several coactivators using the construct which displayed maximal CREB-1 transactivation, CCR5-pB3. We found that similar to CREB-1, ATF-1 induced CCR5 promoter activity (Figure 4E). However, its transactivation capacity was considerably lower than that of CREB-1, and ATF-1 could not further enhance CREB-1-induced transactivation. Subsequently, we also investigated whether



the coactivators CBP (CREB-binding protein), p300 and P/CAF (p300/CREB-associated factor) [28,29] would modulate CREB-1 mediated activation of CCR5. Cotransfection of the CREB-responsive CCR5-pB3 construct with CREB-1 and these coactivators revealed that CBP, p300 and P/CAF did not enhance CREB-1 induction of CCR5 promoter activity (Figure 4F), indicating that exogenous expression of CREB-1 is sufficient to activate the CCR5 promoter.

Taken together, these data suggest that the CREB pathway is involved in the transactivation of the CCR5 promoter region, whereas the IRF and the NF-κB pathways are not.

Protein binding to CRE-sites in the downstream promoter

Because we found that CREB-1 is capable of activating CCR5 downstream promoter constructs, we analyzed the CCR5 downstream promoter region more closely for potential CRE-sites. Figure 5A depicts the position of putative CRE sites identified in the downstream promoter region (designated CCR5-CRE-B1 through -CRE-B13). Using EMSA, we observed protein/DNA complex formation with several of the CCR5-CRE-B sites (Figure 5B), which we further analyzed by supershift analysis. CCR5-CRE-B1 through –CRE-B4 are located in the region that is included in the downstream CCR5 promoter construct CCR5-pB1. However, this region is also included in the upstream promoter constructs (CCR5-pA1 through -4), which are not responsive to CREB-1 (Figure 1 and 5A). Of these sites, CCR5-CRE-B1 and -CRE-B3 displayed a clear binding of CREB-1 and a weaker binding of ATF-1 (Figure 5C). In addition, CCR5-CRE-B1 bound another lower-weight complex, which could not be shifted away with the used antibodies directed against several members of the CREB-1 family. We detected weak binding of CREB-1 to CCR5-CRE-B2 and CREB-1 and ATF-1 to -CRE-B4, which also bound other complexes that were not shifted with these antibodies.

In addition to these sites, two other sites displayed protein binding in EMSA analysis. These sites, CCR5-CRE-B7 and -CRE-B13, are located in the region that is included

Figure 4. Transactivation capacity of the regulatory sites in the CCR5 promoter. Transient transfection of promoter constructs in Tera-2 cells. **A** Transient transfection of the full-length CCR5 upstream (CCR5-pA1) and downstream (CCR5-pB1) promoter constructs. Cells were left untreated or treated with TNF-α (10 ng/ml) for 24 h, leading to activation of only the β2m promoter. **B** Transient transfection of full-length and truncated upstream (CCR5-pA1 and CCR5-pA4) and downstream (CCR5-pB1 and CCR5-pB4) promoter constructs and the HLA-B250 promoter construct, as a positive control. Cells were left untreated or treated with IFN-γ (500 U/ml) for 24 h, leading to HLA-B250 promoter activity only. **C** Transient transfection of CCR5 promoter constructs and a β2m promoter construct as a positive control with an IRF-1 expression vector or empty control vector, showing lack of CCR5 promoter transactivation by IRF-1. **D** Transient transfection of CCR5 promoter constructs with a CREB-1 expression vector, showing transactivation of the downstream promoter constructs by CREB-1. **E** Cotransfection of the CCR5 downstream promoter construct CCR5-pB3 with CREB-1 and ATF-1 expression vectors, revealing low transactivation activity of ATF-1 and absent enhancement of CREB-1 induced promoter activity. **F** Cotransfection of the CCR5 downstream promoter construct CCR5-pB3 with CREB-1 and CBP, p300 and P/CAF expression vectors, revealing lack of co-induction of the CCR5 promoter by CBP, p300 and P/CAF. Depicted are relative light units (RLU) per second obtained for luciferase activity and normalized with Renilla luciferase activity. Shown are mean + SEM of 3 independent experiments (A and C-F), or a representative of 3 independent experiments (B), performed in triplicate.

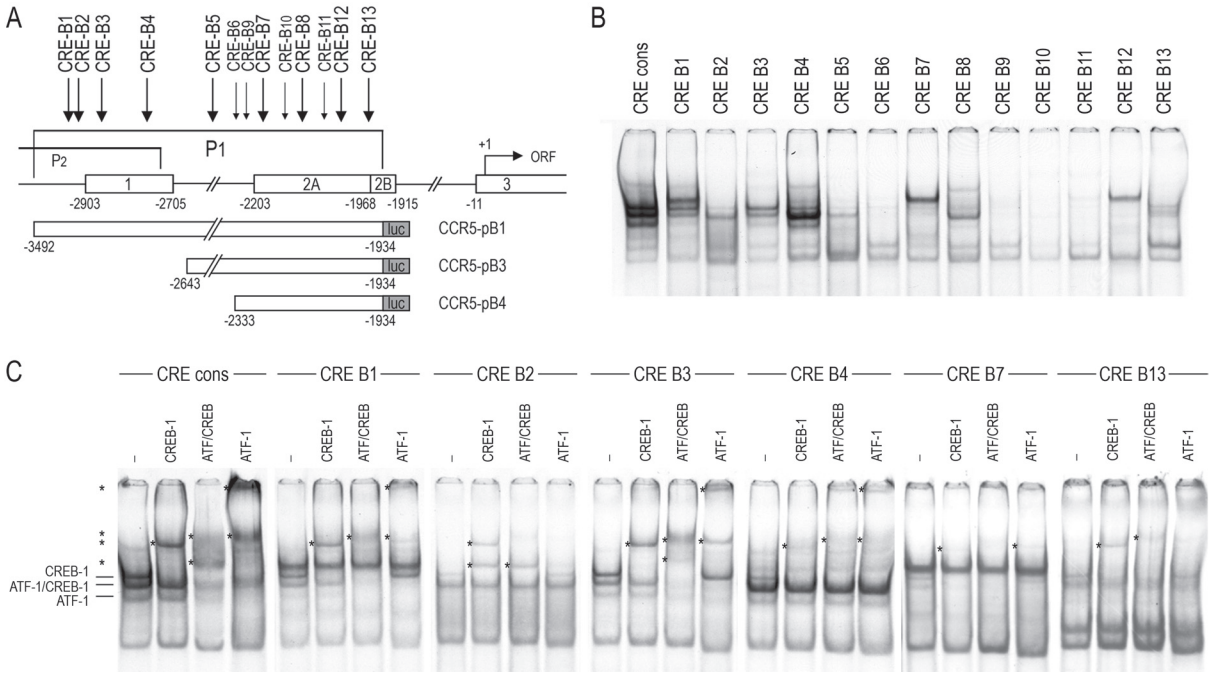


Figure 5. Transcription factor binding to the CRE sites in the CCR5 downstream promoter. **A** Scheme depicting the CCR5 gene and the downstream promoter region (P1). Locations of the identified putative CRE sites in the downstream promoter regions are depicted above. Sites at which significant protein binding was detected are indicated by large arrows, whereas sites that did not generate protein binding are indicated by small arrows. Underneath, the downstream promoter-luciferase-reporter constructs are shown. **B** EMSA showing binding of complexes to the CRE sites in the CCR5 downstream promoter region (CRE-B1 through –B13) and a CRE consensus probe (cons) using nuclear extracts of U251 cells. Protein/DNA complexes are formed with probe CRE-B1 through –B5, -B7, -B8, -B12 and –B13. **C** Using specific antibodies, the complexes binding to CCR5-CRE-B1, -B3, -B4 and -B7 were shown to contain both CREB-1 and ATF-1, whereas the complexes formed with CCR5-CRE-B2, and CRE-B13 contained only CREB-1. * supershifted complexes. Shown are representatives of 2 independent experiments.

in all CREB-responsive downstream promoter constructs (Figure 5A). CCR5-CRE-B7 displayed rather weak binding of both CREB-1 and ATF-1, whereas we only found weak binding of CREB-1 to CCR5-CRE-B13 (Figure 5C). In addition, we found very weak binding of CREB-1 and ATF-1 to CCR5-CRE-B5, located in promoter constructs CCR5-pB1 and CCR5-pB3, and hardly detectable binding of CREB-1 to CCR5-CRE-B8 and CCR5-CRE-B12, located in all downstream promoter constructs (not shown).

Modulation of endogenous CCR5 expression

Finally, we evaluated whether stimulation of the investigated signaling pathways could lead to transcription activation or modulation of CCR5 expression in monocyte-derived DCs and in primary cells originating from the central nervous system. First, we analysed primary human monocyte-derived DCs for constitutive and LPS- or IFN-γ-induced CCR5 expression. Neither LPS (a potent inducer of NF-κB activation) nor IFN-γ did enhance the constitutive transcription of CCR5 in monocyte-derived DCs significantly, as determined by RT-PCR (Figure 6A,C). Likewise, LPS and IFN-γ did not modulate the constitutive transcription of HLA-DRA, whereas both stimuli did

enhance expression of the p50 subunit of NF-κB in these monocyte-derived DCs.

Subsequently, we investigated CCR5 transcription in cultured astrocytes and microglia stimulated with LPS, TNF-α or IFN-γ by RT-PCR. While cultured monocyte-derived DCs constitutively express CCR5, we could not detect constitutive CCR5 expression in cultured astrocytes (Figure 6A,C). In addition, LPS stimulation did not induce CCR5 expression in these cells, whereas NF-κB p50 expression was induced by LPS treatment. Furthermore, we were unable to detect CCR5 expression in astrocytes upon IFN-γ treatment, while HLA-DRA expression was induced (Figure 6A,C). In contrast, primary cultured microglia did express CCR5 constitutively (Figure 6B,C). Consistent with the results obtained with primary monocyte-derived DCs, neither IFN-γ nor TNF-α treatment resulted in enhanced CCR5 expression, whereas these treatments did lead to enhanced expression of the inducible Class II Transactivator (CIITA) PIV isoform or the induction of NF-κB p50 expression (Figure 6B,C). Likewise, we were not able to induce CCR5 mRNA expression by either IFN-γ or TNF- α treatment in U251 or THP-1 cells (data not shown).

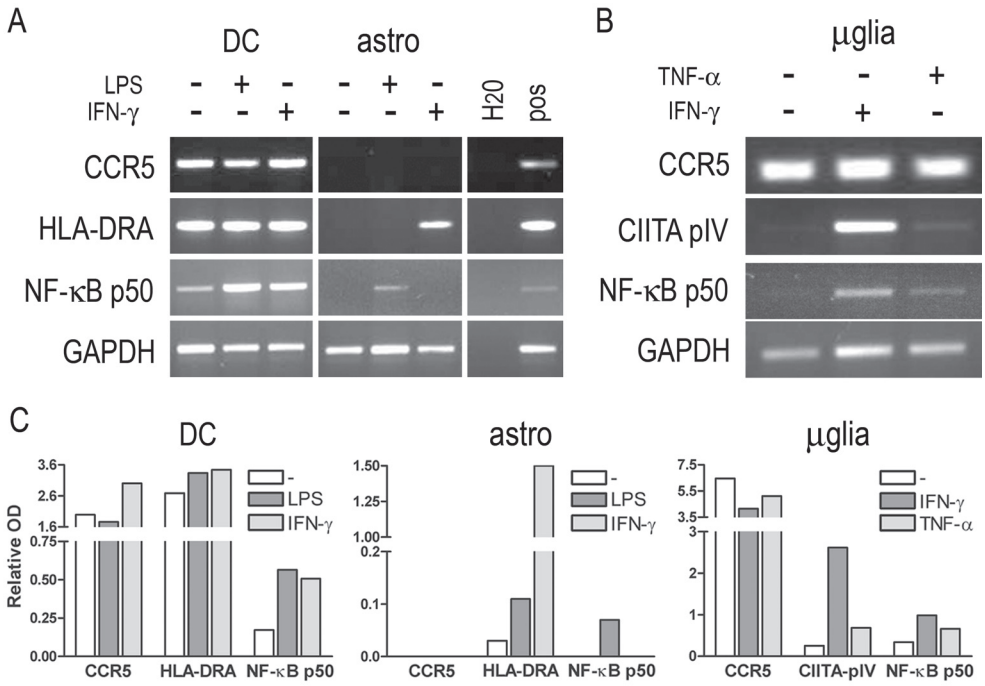


Figure 6. Induction of CCR5 transcription by inflammatory stimuli. **A** mRNA expression levels of CCR5, HLA-DRA and NF-κB p50 in human monocyte-derived DCs (DC) and human primary astrocytes (astro) after stimulation with LPS (100 ng/ml) or IFN-γ (500 U/ml) for 24 (DC) or 8 (astro) hours, as determined by RT-PCR. CCR5 expression is not enhanced in DCs nor induced in astrocytes by either LPS or IFN-γ treatment, whereas NF-κB p50 expression is enhanced in DCs by either treatment, and HLA-DR and NF-κB p50 expression is induced in astrocytes by IFN-γ and TNF-α, respectively. **B** mRNA expression levels of CCR5, CIITA-PIV and NF-κB p50 in human primary microglia (μglia) after stimulation with TNF-α (10 ng/ml) or IFN-γ (500 U/ml) for 8 hours, as determined by RT-PCR. IFN-γ or TNF-α do not enhance CCR5 expression in microglia, whereas expression of CIITA-PIV and NF-κB p50 is enhanced by either treatment. **C** Quantification of the PCR products. Relative optical densities (OD) corrected for GAPDH expression are shown.

Finally, we investigated whether the CREB pathway is involved in endogenous expression of CCR5 as found in T-cells. Therefore, we treated human PBMC with forskolin, an agent that induces the CREB family member inducible cAMP early repressor (ICER) in various cell types, including PBMC [30,31]. ICER competes with CREB for DNA binding and acts as a repressor of CREB-mediated transcription [30]. We found that CCR5 expression was markedly reduced in PBMC after forskolin treatment (Figure 7A,B). Therefore, we examined whether ICER affects CCR5 promoter activity, by cotransfecting the CREB-responsive CCR5 promoter construct CCR5-pB3 with CREB-1 and ICER expression constructs. Indeed, we found that ICER strongly inhibited CREB-induced transactivation of the CCR5 promoter (Figure 7C).

These findings confirm that of the signaling pathways investigated, only the cAMP/CREB pathway contributes to CCR5 transcriptional activation.

Discussion

In the current study we have evaluated the contribution of IRF-1, NF-κB and CREB-1 to the transcriptional regulation of the chemokine receptor CCR5. Our results indicate that only CREB-1 is involved in the transcriptional regulation of CCR5 and that CCR5 expression is not induced nor modulated by IRF-1 and NF-κB.

Although it has been previously suggested that NF-κB could upregulate CCR5 in T-cells [32], our data indicate that the identified NF-κB binding sites do not seem to play a role in the transcriptional regulation of CCR5 expression in the cell types investigated in this study. This is illustrated by the lack of CCR5-promoter activation by TNF-α and the failure of LPS and TNF-α to induce or enhance endogenous CCR5 transcription. At the same time, these agents did induce activity of the NF-κB responsive β2m promoter and transcription of the NF-κB subunit p50.

By monitoring endogenous CCR5 expression we showed a lack of inducibility by IFN-γ of CCR5 in several cell types, while at the same time IFN-γ-induced expression of HLA-DRA or activation of IFN-γ-responsive CIITA-PIV isotype was noted. The lack of IFN-γ-mediated activation of endogenous CCR5 transcription is corroborated by the failure of IFN-γ and IRF-1 to activate the promoter construct containing CCR5-ISRE-1 and -3, that displayed *in vitro* IRF-1 binding. The lack of CCR5 promoter activation by IFN-γ and IRF-1 does not seem to result from the presence of a region upstream of the ISREs [32,33] that inhibits IFN-γ-mediated transactivation, because the truncated constructs, which did include CCR5-ISRE-3, were not responsive to IFN-γ or IRF-1 either. In addition, it also seems unlikely that binding of an inhibitory IRF causes the unresponsiveness of the ISRE-containing promoter constructs. Previously we have shown that IRF-2 inhibits the transactivation of the β2m promoter by IRF-1 [20]. However, whereas we did find binding of IRF-2 to the ISRE of β2m in U251 cells, we could detect no binding of IRF family members other than IRF-1 to CCR5-ISRE-1 and CCR5-ISRE-3 (Figure 3B).

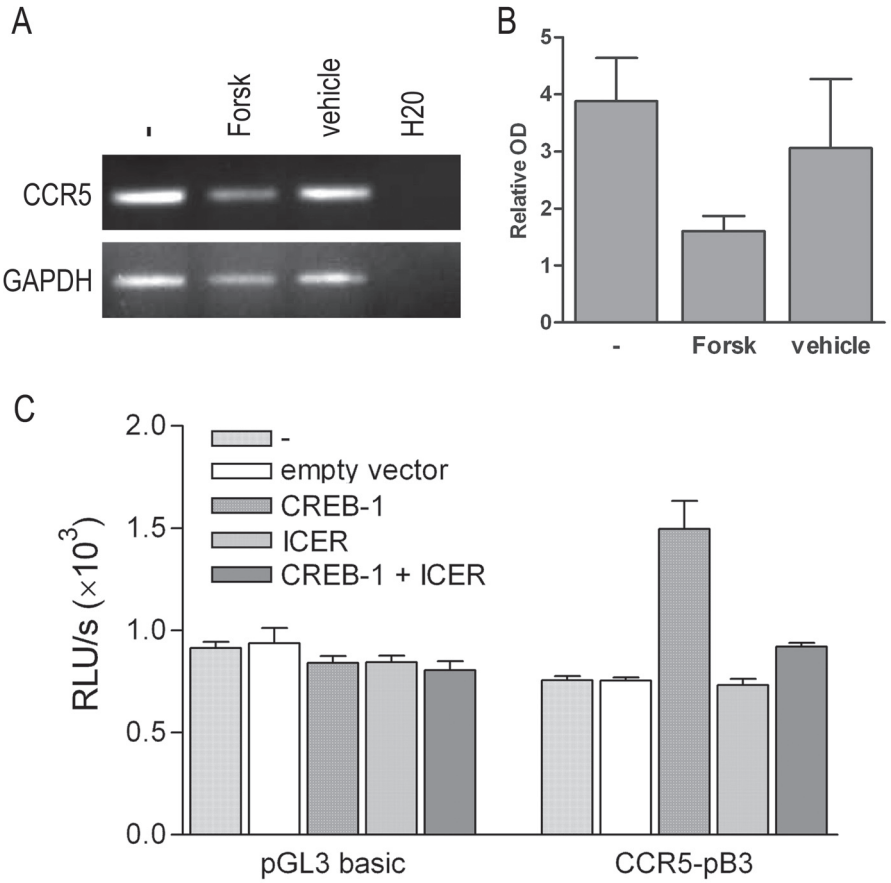


Figure 7. Inhibition of CCR5 transcription and CCR5 promoter activation by forskolin and ICER, respectively. **A** mRNA expression levels of CCR5 in human PBMC treated with forskolin (Forsk; 10 μM) for 4 hours, as determined by RT-PCR. Forskolin treatment reduces amounts of CCR5 transcript in PBMC. Shown is a representative of 2 independent experiments. **B** Quantification of the PCR products. Mean relative optical densities + SEM, corrected for GAPDH expression, of two independent experiments are shown. **C** Transient transfection of the CREB-responsive CCR5 downstream promoter construct CCR5-pB3 with CREB-1 and ICER expression vectors, showing inhibition of CREB-1 induced CCR5 promoter activity by ICER. Depicted are relative light units (RLU) per second obtained for luciferase activity and normalized with Renilla luciferase activity expressed as mean + SEM. Shown is a representative or 3 independent experiments performed in triplicate.

In contrast to the lack of CCR5 transcriptional regulation through IFN-γ and TNF-α-induced activating pathways, our findings indicate that CREB-1 is involved in the transcriptional regulation of CCR5. Of the identified CRE sites in the downstream promoter, CCR5-CRE-B1 and CCR5-CRE-B3 showed the most prominent binding of CREB-1/ATF-1. However, these sites are located in the region that is also included in the upstream promoter constructs, which are not responsive to CREB-1 cotransfection. Therefore, it is reasonable to assume that these sites most likely do not contribute to the transactivation of the downstream promoter by CREB-1. In contrast, CCR5-CRE-B7 and CCR5-CRE-B13, which displayed weaker binding of CREB-1/ATF-1 and CREB-1, respectively, are located in the region which all downstream promoter constructs encompass and therefore most likely do contribute to the transactivation of the CCR5

promoter by CREB-1. The CRE site in the upstream promoter region, CCR5-CRE-2, proved also not to contribute to CCR5 transcription despite binding of CREB-1/ATF-1 (Figure 3D). However, this site also seems to bind CREM-1. It is known that, depending on the isoform obtained from alternative splicing, CREM-1 can act as either an activator or repressor of transcription [28,34]. For example, ICER is the collective name of a family of inducible CREM isoforms that lack a transactivation domain and therefore are natural antagonists of CREB-1, repressing its transactivation function [30]. Therefore it could be argued that the CREM-isoform binding to CCR5-CRE-2 might represent a repressive CREM isoform or ICER, inhibiting activation of the CCR5 upstream promoter by CREB-1. However, EMSA analysis revealed considerable binding of both CREB-1 and ATF-1, which suggests that the CREM-binding to CCR5-CRE2 is not able to compete with the binding of CREB-1 and ATF-1.

The fact that the upstream promoter constructs are not responsive to CREB-1, whereas the downstream promoter constructs are, might suggest a repressive function of the upstream promoter region. This hypothesis is underscored by the fact that the longest downstream promoter construct (CCR5-pB1), including part of the upstream region (Figure 1), is less responsive to CREB-1 than the truncated downstream promoter constructs CCR5-pB3 and -pB4 (Figure 4D). Therefore it seems that this upstream region indeed suppresses CREB-1-mediated activation of the downstream promoter. These findings corroborate those of others, that mapped a repressive element, corresponding to the region upstream of -2750, affecting the CCR5 promoter [32,33]. This region is included in CCR5-pB1 and not in CCR5-pB3 and –pB4 (Figure 1).

Many genes contain consensus sites for CREB binding and as such the transcription factor CREB-1 has been implicated in a wide variety of cellular processes [35]. In the central nervous system CREB-1 plays an important role in various neuronal processes, such as neuronal development, neuroprotection and disease [36]. In addition, various signaling pathways, including neurotrophin-mediated signaling, result in enhanced CREB-1 expression [36]. In effect, CREB-1 plays an important role in the transcriptional control of anti-apoptotic factors such as Bcl-2 following neurotrophin signaling [37]. Furthermore, CREB-1 has also been implicated in axonal regeneration in the injured CNS [38]. Previously, we have detected enhanced expression of CREB-1 in MS affected microglia, which could reflect a stress signaling-induced upregulation of *CREB-1* transcription [12]. In addition, a concomitant increase in CCR5 expression on MS affected microglia has also been documented [10,11]. The fact that we now have shown that CREB-1 is a bona fide inducer of CCR5 promoter activation is in line with these observations.

In conclusion, we show that CREB-1 is an important inducer of CCR5 expression. Interestingly, whereas CREB-1 is ubiquitously expressed and its expression can be induced by various stimuli, expression of CCR5 in healthy subjects is confined to T-lymphocytes, DCs, macrophages, monocytes and microglia. This specificity of CCR5 expression could be the result of differences in signal transduction pathways that lead to differential phosphorylation and additional modifications of CREB-1, altering its

transcriptional activation state [28,29]. In addition, differential expression of CCR5 could be due to cell-specific splicing of the *CREM* gene, giving rise to either activating or repressive CREM isoforms that could affect CREB-1 transactivation of the CCR5 promoter [34]. However, these mechanisms are also quite common and therefore are not likely to account for the cell-specific expression of CCR5. Thus, these data suggest the existence of additional regulatory constraints imposed on *CCR5*, which control tissue-specific expression. Alterations in these regulatory mechanisms might also explain the aberrant expression of CCR5 in MS and in a number of other inflammatory diseases.

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