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Differential Regulation of CCL22 Gene Expression in Murine Dendritic Cells and B Cells

Hormas Ghadially,2 Xiao-Lan Ross, Claudia Kerst, Jun Dong, Angelika B. Reske-Kunz, and Ralf Ross3

The activated T cell-attracting CC chemokine CCL22 is expressed by stimulated B cells and mature dendritic cells (DC). We have cloned and sequenced the complete mouse gene, including 4 kb of the 5'-flanking promoter region, and detected two distinct sites for initiation of transcription by 5'-RACE. Reporter gene assays indicate that the promoter reflects the specificity of the endogenous gene. Within the proximal promoter region, we identified potential binding sites for NF-κB, Ikaros, and a putative GC box. All three regions bind proteins. The NF-κB site was shown to specifically bind NF-κB subunits p50 and p65 from nuclear extracts of LPS-stimulated B cells, B cell line A20/2J, TNF-α-stimulated bone marrow-derived DC, and DC line XS106. Furthermore, promoter activity was affected by targeted mutagenesis of the NF-κB site and transactivation with p50 and p65. The region harboring the putative Ikaros site contributes to promoter activity, but the binding protein does not belong to the Ikaros family. The GC box was shown to specifically bind Sp1 using extracts from LPS-stimulated B cells and A20/2J but not from DC and DC line XS106. Additionally, Sp1 transactivated the promoter in A20/2J but not in XS106 cells, and mutation of the Sp1 site diminished transactivation. Furthermore, binding of the protein complex at the GC box is required for NF-κB activity, and the spatial alignment of the binding sites is of critical importance for promoter activity. Thus, identical and distinct proteins contribute to expression of CCL22 in DC and B cells.

C hemokines are a group of functionally and structurally related small proteins that are critically important in initiating and controlling the homing and migration of leukocytes (reviewed in Refs. 1 and 2). Other functions of individual chemokines or chemokine receptors include, e.g., the regulation of angiogenesis (3) and hematopoiesis (4). Based on the relative position of cysteine residues, four families of chemokines are distinguishable, namely CXC (α), CC (β), CX3C (δ), and C (γ). The genes of most of the CXC, as well as the CC chemokines, are clustered and show a similar exon/intron structure, hinting at duplication of common ancestor genes during evolution. For most chemokines, homologues have been identified in mice and humans, but there are exceptions such as the dendritic cell (DC)γ-chemokine 1 (no mouse homologue) (5) and IL-8 (no clear-cut murine homologue), suggesting relatively recent evolutionary rearrangements and differences in the equipment of the immune system of mice and humans.

The CCL22 is present in both mice and humans. In humans, CCL22 is also referred to as macrophage-derived chemokine or stimulated T cell chemotactic protein-1 and in mice as activated B cell-derived chemokine-1 or DC and B cell-derived chemokine (6–9). CCL22 has been shown to play an important role in various diseases and mouse disease models. For example, elevated levels of CCL22 have been found in affected tissue of patients suffering from atopic dermatitis (10), atopic asthma (10), and contact hypersensitivity (11). Furthermore, CCL22 has been shown to be critically involved in mouse models of allergen-induced lung inflammation (12), atopic dermatitis (13), and septic peritonitis (14).

The major function of CCL22 appears to be the induction of chemotactic migration of activated T cells (7–9, 15), predominantly of the Th2 type (16) and of CD4+CD25+ regulatory T cells (17), through its specific receptor CCR4. However, another as yet unidentified receptor has also been implicated (18, 19). The murine CCL22 is expressed by activated B lymphocytes and DC (8, 9). Murine epidermal Langerhans cells (LC), an exceptionally homogeneous population of immature DC, produce CCL22 mRNA only upon maturation (9), whereas other myeloid DC transcribe CCL22 constitutively (8). Epidermal LC and other immature DC represent sentinel cells of the immune system. Activation, e.g., by inflammatory stimuli, induces maturation into the most efficient APC known, which is outstanding in activating naive T cells (reviewed in Ref. 20). Applying a differential screening approach, we identified numerous genes differentially expressed during maturation of LC (21, 22). We used regulatory sequences of one of these genes, namely the actin-bundling protein fascin, to transcriptionally target DC (23, 24). Furthermore, the CCL22 gene was identified in this screening as the most abundantly expressed gene induced during maturation of murine epidermal LC (9). The high expression level and the tight regulation of expression, as indicated by the stage specificity, prompted us to isolate the gene and characterize the regulatory elements mediating expression in DC and B cells. Interestingly, DC and B cells use—at least in part—the same promoter elements but different protein complexes are formed.
Materials and Methods

Animals

BALB/c mice were bred in the Central Animal Core Facility of the University of Mainz under specified pathogen-free conditions. Mice were used at 2–5 mo of age.

Cells

Epidermal cell suspensions were prepared from pelts and used for LC enrichment either directly or following in vitro cultivation for 3 days as described previously (21). LC were enriched from epidermal cell suspensions by immunomagnetic separation with Dynal beads M-450 (Dyna-BioTech) loaded with anti-MHC class II mAb 269 (anti-mouse I-A\(^d\), I-E\(^e\), rat IgG2a as described previously; Ref. 21). MHC class II-positive, bead-coupled LC and cells without attached beads were enumerated using a Neubauer hemocytometer. A purity of ~92–95% was obtained as determined by the ratio of bead-rosetted to nonrosetted cells. Cell viability, determined by trypan blue exclusion, was >95%

Bone marrow-derived DC (BM-DC) were cultured as described previously (25) with modifications (26), and maturation was induced by adding either 50 ng/ml TNF-α (R&D Systems) or 1 μg/ml LPS (Sigma-Aldrich). MHC class II-positive cells were enriched 24 h later by immunomagnetic separation as described above.

B lymphocytes were purified from spleen cells using the Mouse B Cell Recovery kit (Cedarlane Laboratories). Purity as assessed by cytofluorometry was >95%. For stimulation, B cells were cultured for 2–3 days in the presence of 25 μg/ml LPS (Sigma-Aldrich) in IMDM (In Vitrogen Life Technologies) supplemented with 5% FCS (Sigma-Aldrich), 2 mM l-glutamine, 5 × 10\(^{-5}\) M 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin (complete medium). Naïve B cells were isolated by immunomagnetic separation using anti-IgM-coupled Dynabeads. The murine B cell lymphoma A2O/2J (27) was cultivated as described previously and stimulated with 25 μg/ml LPS for 24 h. The murine fibroblast line NIH 3T3 (28) was cultivated in complete medium. The murine immature and mature DC lines XS52 and XS106, respectively (29), kindly donated by Dr. A. Takashima (University of Texas Southwest Medical Center, Dallas, TX), were cultivated in complete medium supplemented with 10% supernatant of NS 47 cells (30) and 5 and 10 ng/ml, respectively, of mouse recombinant GM-CSF. GM-CSF was kindly provided by Drs. F. Seiler and D. Krumwieh (Behringwerke AG). Mouse rIL-4 was a kind donation of Dr. D. B. p52 and RelB were kind gifts by Dr. F. Weih (Forschungszentrum Karlsruhe, Karlsruhe, Germany). Expression plasmids for Sp1 and Sp3, respectively, luciferase activity was normalized to the amount of protein in the cell lysate.

Isolation of mRNA and RT-PCR

mRNA was isolated using the QuickPrep Micro mRNA Purification kit as recommended (Amersham Biosciences). Reverse transcription reaction was performed as described previously (31). RNA concentration and efficiency of reverse transcription reaction were assessed by RT-PCR using primers specific for the housekeeping gene hypoxanthine phosphoribosyltransferase as reported previously (21). Hypoxanthine phosphoribosyltransferase-standardized amounts of cDNA were subjected to PCR with CCL22-specific primers as described previously (9).

5’-RACE

5’-RACE was performed using the SMART cDNA Library Construction kit (Clontech) for first-strand synthesis. PCR was performed using the 5’-SMART PCR primer (5’-AAGCAGTGTGATCAACGCAGATG-3’; Clontech) and CCL22 gene-specific primer 1 (5’-ATGGATACCTGCCTCGGTGTCCCC-3’) or primer 2 (5’-GAGGAGCAAGGATGGTGG-3’), respectively. Products were separated on an agarose gel, blotted on a nylon membrane (Roche), and hybridized with a digoxigenin-labeled probe derived from CCL22 cDNA. Signals were detected using the STA-PROBE Synthesis kit (Roche). Alternatively, products from a PCR using primer 2 and the 5’-SMART PCR primer were sequenced using primer 1.

Isolation and characterization of a murine genomic CCL22 clone

A murine genomic DNA library derived from a female C57BL/6 mouse (library no. 703 (32)) was obtained from the Resource Center/Primary Database. The library was screened by hybridization with a probe containing the mouse CCL22 open reading frame (ORF) (9). Hybridization reactions and signal detection were performed as reported previously (33).

P1 clones selected following library screening were used for DNA preparation (NucleoBond Plasmid kit; Clontech). Hybridization reaction with mouse CCL22 cDNA probe was verified by Southern blot analysis. For additional characterization, restriction fragments of P1 clone DNA were sub cloned randomly into pZEO 2.1 (Invitrogen Life Technologies), and subclones hybridizing with mouse CCL22 cDNA probe were identified by colony filter hybridization.

DNA sequencing and sequence analysis

Nucleotide sequences were determined by cycle sequencing using the ABI PRISM Termination Cycle Sequencing Ready Reaction kit as recommended and analyzed on a PE 373A DNA sequencer (PerkinElmer). GC-rich DNA templates were linearized, heat-denatured (98°C for 5 min) and mixed with 5% (v/v) DMSO before sequencing.

Potential transcription factor binding sites were identified by MatInspector version 2.2 (http://transfac.gbf.de). Transcription factors mentioned in the text are named according to the first corresponding publication.

Construction of CCL22 promoter reporter gene plasmids

To assess activity of the CCL22 promoter, a fragment spanning a sequence −4 kb long containing part of the 5’-nontranscribed region and the complete 5’-untranslated region (UTR) was subcloned into promoterless firefly luciferase expression vector pGL3-Basic (Promega), resulting in reporter construct pGL-CCL22. Correct orientation was confirmed by sequencing with vector-specific primers (RVprimer3 and GLprimer2; Promega). 5’-Deletion constructs were obtained by restriction and religation or by nested deletion using the double-stranded Nested Deletion kit (Amersham Biosciences).

Transient cell transfection and luciferase assay

Cells were transfected using GenePORTER (Gene Therapy Systems) as recommended by the manufacturer. Briefly, 1 μg of reporter DNA and 0.1 μg of the coreporter (see next paragraph) were incubated with 4–8 μl of GenePORTER in 250 μl of serum-free culture medium for 1 h before 4 × 10\(^{6}\) cells were added. After 48 h 2 ml of complete medium were added.

Cells were analyzed 24 h posttransfection. To this end, cells were harvested, washed in PBS, and lysed in Passive Lysis Buffer (Promega). Cell lysates were analyzed sequentially for firefly and Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) in a Turner luminometer TD-20/20 (Turner Design) as recommended by the manufacturer.

To normalize absolute firefly luciferase reporter expression values for differences in transfection efficiency and cell recovery, a Renilla luciferase expression construct was cotransfected. To avoid increased coreporter activity due to cytokine- or LPS-mediated stimulation of CMV promoter, pRL-EF1α was used as a coreporter construct (24). Renilla expression in pRL-EF1α is driven by the strong human elongation factor 1α (EF1α) gene promoter regulated mainly by GC box-binding transcription factors (34). Reporter activities are calculated as ratio of firefly luciferase test construct activity divided by activity of cotransfected Renilla luciferase reporter construct. For transactivation studies using expression plasmids for Sp1 and Sp3, respectively, luciferase activity was normalized to the amount of protein in the cell lysate.

Expression plasmids for NF-κB p50, p65, and c-Rel were kindly provided by Dr. S. Pettersson (Karolinska Institute, Stockholm, Sweden), and plasmids for the expression of NF-κB p52 and RelB were kind gifts by Dr. F. Weih (Forschungszentrum Karlsruhe, Karlsruhe, Germany). Expression plasmids for Sp1 and Sp3 (35) were kind gifts of Dr. G. Suske (Phillips-Universität, Marburg, Germany).

EMSA and site-directed mutagenesis

Nuclear proteins were isolated by the method of Schreiber et al. (36), and protein concentration was determined using the Roti-Quant Protein Assay kit (Roth). Complementary oligonucleotides were incubated for 10 min at 95°C and annealed by decreasing the temperature by 1°C/min to 4°C. Following agarose gel electrophoresis, double-stranded oligonucleotides were recovered from the gel by electroelution and end-labeled using T4 polynucleotide kinase (New England Biolabs). Radiolabeled DNA probe (25,000 cpm) was mixed with 1 μg of poly(dI: dC) (Amersham Biosciences), 10 μg of nuclear proteins, and binding buffer (25 mM HEPES (pH 7.5), 150 mM KCl, 5 mM DTT, and 10% glycerol). Complex formation was allowed to proceed for 30 min at room temperature. For competition experiments, 50-fold molar excess of unlabeled, double-stranded oligonucleotides were added to the binding reaction. For supershift experiments, nuclear proteins were incubated with 2 μg of specific Abs for 30 min on ice before the binding reaction. Complexes were separated on 5% nondenaturing polyacrylamide gels. Gels were dried afterward and exposed to Kodak MS films (Eastman Kodak) at −80°C.

Site-directed mutagenesis was performed as described previously (37). The sequence of oligonucleotides (top strand) for EMSA and site-directed mutagenesis were as follows: CCL22I 5’-ACCGCTAAGGG
RESULTS

Isolation of the murine CCL22 gene and identification of transcriptional initiation sites

The murine CCL22 gene was isolated from a genomic library with a cDNA probe encompassing the CCL22 ORF. As only the exon/intron boundaries of the gene had been reported previously (8), we subcloned and sequenced a continuous region of 11,728 bp, covering the murine CCL22 gene, including 4,096 bp of the 5'-flanking region (NCBI accession no. AJ315897). Analysis of the sequence confirmed that CCL22 is, as with other CC chemokines, encoded by three exons (Fig. 1A). The exons are separated by two introns of 1114 and 2758 bp, respectively. The sequences of the splice junctions are highly homologous to the corresponding human sequence and identical to the splice junctions in mouse reported by Schaniel et al. (8).

To identify the transcriptional initiation site(s), we performed 5'-RACE using mRNA of in vitro-cultured LC and LPS-activated B cells, previously shown to express CCL22 (9). Two gene-specific 3'-primers were used in combination with a common 5'-primer, and products were detected by hybridization with a probe containing the first exon of the CCL22 gene. Two lengths of 5'-UTR were identified in both cell types, indicating the presence of two distinct transcriptional start sites (Fig. 1B). Sequencing of the RACE products revealed that the majority of the transcripts in both cell types are initiated from a site 28 bp upstream of the translation initiation codon (position 4070 of sequence AJ315897), giving rise to a very short 5'-UTR. In both cell types, a fraction of the transcripts are initiated from a site 124 bp upstream of the start codon (position 3973 of sequence AJ315897), which corresponds to the start of the cDNA sequenced by Schaniel et al. (NCBI accession no. AF052505; Ref. 8). Interestingly, a nearly perfect TATA box sequence was found ∼32 bp upstream of the major transcription initiation site, indicating that the majority of the transcripts of both, cultured LC and activated B cells, arise from a promoter containing a TATA box.

The CCL22 promoter reflects the activity of the endogenous gene

Based on our previous observation that CCL22 is expressed by mature, but not immature, DC and by activated, but not resting, B cells, we searched for an adequate model system to study CCL22 expression. By RT-PCR, we found that the mature DC line X5106 expressed CCL22 but not the immature DC line XS52 and confirmed that LPS stimulation induced expression of CCL22 in the B lymphoma cell line A20/21 (data not shown). In addition, we used the CCL22-negative fibroblast cell line NIH 3T3. To analyze CCL22 promoter activity in these cell lines, a 4069-bp fragment directly upstream of the translational start site was inserted into the vector pGL3-Basic, driving expression of the adjacent firefly promoter activity in these cell lines, a 4069-bp fragment directly upstream of the translational start site was inserted into the vector pGL3-Basic, driving expression of the adjacent firefly

Statistical analysis of data

Statistical analysis of the experimental data was performed by the Student’s t test. A value of p < 0.05 was considered statistically significant.

FIGURE 1. A, Schematic depiction of the murine CCL22 gene and CCL22 gene-derived reporter gene constructs. Exons are shown as boxes: ■ represents translated regions, and □ represents untranslated regions. The two transcriptional start sites are indicated by positions relative to the start of translation. Recognition sites of restriction enzymes are abbreviated as follows: B, BamHI; E, EcoRI; H, HindIII; and S, SpeI. A luciferase reporter gene construct of the 5'-flanking promoter region and a series of constructs obtained by successive 5'-truncation of the promoter region are represented below. B, Identification of transcriptional start sites of the CCL22 gene. 5'-RACE was performed on mRNA extracted from in vitro-cultured LC and LPS-activated B cells (BC), respectively. Products were separated by agarose gel electrophoresis and detected by Southern hybridization using a probe containing exon I of the CCL22 gene. C, Analysis of the promoter activity of the 5'-flanking region of the CCL22 gene. The indicated cell lines were transfected transiently with a reporter gene plasmid containing 4.1 kb of the 5'-flanking region of the CCL22 gene and cotransfected with a reporter plasmid coding for renilla luciferase driven by the EF1α promoter. Transcriptional activity was calculated as relative light unit relative to pGL3-Basic. Shown are the mean values (± SD) of two independent experiments performed in triplicate. D, Deletion analysis of the CCL22 promoter. Equimolar amounts of luciferase reporter plasmids with 5'-to 3'-deletions of the CCL22 promoter were transfected transiently into the indicated cell lines and cotransfected with a reporter plasmid coding for renilla luciferase driven by the EF1α promoter. A20/23 cells were activated with LPS (25 μg/ml) immediately after transfection or were left untreated. The numbers on the abscissae represent the length of the promoter fragment in kb. Transcriptional activity was calculated as relative light unit relative to pGL3-Basic. Shown are the mean values (± SD) of two independent experiments performed in triplicate.

The numbers on the abscissae represent the length of the promoter fragment in kb. Transcriptional activity was calculated as relative light unit relative to pGL3-Basic. Shown are the mean values (± SD) of two independent experiments performed in triplicate.
luciferase ORF (pGL-CCL22). This expression plasmid was transfected transiently into the cell lines, and firefly luciferase activity was measured 24 h posttransfection. A plasmid coding for renilla luciferase driven by the human EF1α promoter was cotransfected and used to standardize transfection efficiency. As shown in Fig. 1C, pGL-CCL22 mediated robust reporter activity in the mature DC line XS106, in LPS-stimulated A20/2J cells, and, to a markedly lesser extent (~4-fold less, p < 0.01), in unstimulated A20/2J cells. No activity was detected in XSS2 and NIH 3T3. The enhancement of CCL22 expression by LPS stimulation of A20/2J cells was concentration dependent with a maximal induction at 25 μg/ml LPS (data not shown). As the activity of the CCL22 promoter fragment reflected the activity of the endogenous gene, we concluded that the major positive or negative regulatory elements necessary for stage-specific CCL22 expression in these cell types were present in pGL-CCL22.

**Boundaries of the CCL22 promoter**

To define the extension of the CCL22 promoter and to detect regulatory regions within the promoter, we generated serial 5′-deletion constructs of pGL-CCL22. Constructs were transfected into XS106 and A20/2J cells, respectively, and analyzed for activity by luciferase assay. A20/2J cells were either stimulated with LPS directly after transfection or were left untreated. As shown in Fig. 1D, a reporter plasmid containing only the most proximal 250 bp of the CCL22 promoter was able to drive most if not all of the luciferase activity in A20/2J cells. Moreover, full inducibility of the promoter by LPS was retained. Additionally, when 120 bp were removed from the 5′-end of the promoter fragment, nearly all activity was abrogated in nonactivated and LPS-activated A20/2J cells, indicating that pivotal regulatory elements are located within this region.

In XS106 cells, deletion of the distal 2.5 kb of the promoter had little effect on reporter activity (Fig. 1D). However, additional deletion of the remaining 1.6-kb promoter fragment to 1.4 kb resulted in a ~2-fold increase (p < 0.001) in luciferase activity, hinting at negatively regulating elements within this 200-bp region. Deletion from 1.4 to 0.55 kb had no significant effect on reporter activity, whereas an additional 5′-terminal deletion to 0.25 kb decreased promoter activity back to the level of the full-length promoter. However, removal of additional 120 bp abrogated promoter activity nearly completely (p < 0.001).

Thus, a small proximal promoter region of 250 bp is sufficient to mediate at least a major part of promoter activity in both cell types. Pivotal regulatory elements are located between positions −250 and −130 bp. Therefore, we searched for putative transcription factor binding sites within the most proximal 250 bp of the promoter using MatInspector software. Three regions harboring conserved transcription factor binding motifs were found, containing a putative binding site for NF-κB and IκBα, as well as a G/C-rich element (Fig. 2A). EMSA indicate that all three regions bind nuclear proteins (Fig. 2B). Furthermore, differences in the complexes obtained with nuclear extracts derived from unstimulated vs LPS-stimulated A20/2J cells and XSS2 vs XS106 cells show that variable complexes are formed at box I and box III (Fig. 2B). To analyze the contribution of the three protein-binding regions to the activity of the CCL22 promoter, reporter plasmids were generated in which these three regions were deleted progressively (Fig. 2C). Deletion of box I, which contains the NF-κB binding site, resulted in loss of a major part of the inducibility of the promoter by LPS in A20/2J cells (p < 0.001). Additional deletion of box II had little effect on the residual promoter activity, whereas removal of box III reduced the activity of the reporter plasmid nearly to background level (p < 0.002). In XS106 cells, the deletion of box I resulted in a slight but statistically not significant decrease of promoter activity. However, the additional deletion of box II resulted in a significant decrease (p < 0.001). Most of the residual promoter activity was abrogated by the removal of all three boxes. These results indicate that all three regions contain cis-acting elements that contribute to the promoter activity in A20/2J and XS106 cells. However, the relative contribution is cell type dependent.
Three regulatory regions of the CCL22 promoter bind transcription factors, including NF-κB p50, p65, and Sp1

EMSA and supershift assays were conducted to evaluate the specificity of the protein complexes formed at box I–III and to identify the respective factors binding to these regions.

At the distal box I, harboring a NF-κB binding site, two DNA/protein complexes formed, using nuclear extracts from unstimulated A20/2J cells (Fig. 2B). Stimulation of the cells induced the formation of a third complex with a lower mobility. Similarly, three complexes were detected in the DC lines tested with the low mobility complex being prominent in the mature DC line XS106, as compared with the immature DC line XS52. Competition experiments showed that complex formation was sequence specific (Fig. 3A). An excess of unlabelled region I-oligonucleotide or an oligonucleotide with a NF-κB consensus-binding sequence was able to compete for complex formation, whereas an excess of an unrelated oligonucleotide, harboring an octamer-binding motif, did not affect complex formation and neither did an oligonucleotide containing two base substitutions in the NF-κB core sequence of region I.

To analyze whether indeed proteins of the NF-κB family bind to this region, we conducted supershift assays. An anti-p50 antisera supershifted a complex formed with nuclear extracts of LPS-activated A20/2J cells and XS106 cells, respectively (Fig. 3A). Addition of anti-p65 antisera resulted in decreased complex formation in XS106 cells and A20/2J cells. Antiserum or mAb directed against p52, RelB, or c-Rel had no substantial effect on complex formation (Fig. 3A).

At box II, harboring an Ikaros binding site, three DNA/protein complexes formed, regardless of whether the nuclear extracts were derived from unstimulated/LPS-stimulated A20/2J, XS52, or XS106 cells (Fig. 2B). Excess of unlabelled region II-oligonucleotide blocked complex formation (Fig. 3B). Notably, two base substitutions in the Ikaros core sequence did not affect competition, and an Ikaros consensus binding site did not compete for complex formation, both suggesting that proteins other than Ikaros bind to a distinct sequence of region II. This assumption was verified by supershift assays. Antiserum detecting all known differentially spliced Ikaros isoforms did not induce an additional shift (Fig. 3B). MatInspector detected solely the putative Ikaros site, which is obviously not involved. Thus, the binding site of region II or even the transcription factor binding to this site has not been identified previously.

Box III harbors a GC-rich sequence with putative binding sites for transcription factors of the Sp and AP-2 family. Nuclear proteins from unstimulated and LPS-activated A20/2J, as well as XS106, cells formed three distinct complexes, whereas proteins from XS52 cells formed only one complex (Fig. 2B). Competition experiments showed that formation of two of the complexes was specific, whereas the formation of the complex with the highest mobility was nonspecific. An unlabelled oligonucleotide with a two-base substitution in the G-rich sequence did not compete for complex formation, whereas an oligonucleotide with a known Sp1-binding sequence did compete for complex formation using nuclear extracts of A20/2J and XS106 cells (data not shown).

Binding of factors of the Sp and AP-2 family to box III was analyzed by supershift assays using polyclonal antisera against Sp1, Sp3, Sp4, AP-2α, AP-2β, and AP-2γ (Fig. 3C). The anti-Sp1 antisem supershifted complex 2 formed with nuclear proteins derived from LPS-stimulated A20/2J cells but not from XS106 cells. The other antisera did not have an effect on complex formation with nuclear proteins of LPS-stimulated A20/2J cells or XS106 cells. As region III also harbors a sequence with some similarity to the C/EBP consensus-binding sequence, we used mAb or antisera against the C/EBP isoforms α, β, γ, δ, ε, and ζ for supershift assays (Fig. 3C). No effect on complex formation with nuclear proteins of LPS-stimulated A20/2J cells or XS106 cells was observed (Fig. 3C). An antisem cross-reacting with the C/EBP isoforms α, β, δ, and ε did not have an effect either (data not shown). In conclusion, Sp1 binds to box III using extracts of LPS-stimulated A20/2J cells but not of XS106 cells. The corresponding factor derived from XS106 cells was not identifiable because antisera against all known factors with specificity for the putative binding sites of box III had no effect in supershift assays.

**FIGURE 3.** Identification of proteins binding to the binding sites in the CCL22 promoter. EMSA were performed using oligonucleotides of box I (A), box II (B), and box III (C), and nuclear extracts were isolated from the indicated cell lines in the presence or absence of a molar excess of a cold competitor or in the presence or absence of antisera. Antiserum were added to the nuclear proteins 30 min before adding the hot probe. A20/2J cells were activated with LPS (25 μg/ml) before the isolation. Competitors: A, wild-type box I (wt), consensus-binding sequence of Oct2a (Oct2a), consensus-binding sequence of NF-κB (NF-κB), and mutated box I (mut); and B, wild-type box II (wt), consensus-binding sequence of Ikaros (Ikaros), and mutated box II (mut). Data are representative of two independent experiments.

**Effect of site-directed mutagenesis of transcription factor binding sites on CCL22 promoter activity**

To further elucidate the importance of the identified binding sites of the CCL22 promoter and the relative contribution of the respective transcription factors, single nucleotides of the binding sites...
were substituted in luciferase reporter constructs (see Fig. 2A), and promoter activity was analyzed by luciferase assays.

Substitution of either two nucleotides of the NF-κB or of the Sp1 binding site drastically reduced promoter activity in LPS-stimulated A20/2J cells (\(p < 0.001\) and \(p < 0.002\), respectively), abrogating inducibility of the promoter (Fig. 4A). In XS106 cells, mutation of the Sp1 binding site had a strong effect on promoter activity as well (\(p < 0.001\)), indicating that exactly this site is
required, although it is not a known factor of the Sp1 family that
binds to this region, as shown. Mutation of the NF-κB site resulted
in a moderate but statistically not significant reduction of promoter
activity in XS106 cells as well (Fig. 4A).

In accordance with the finding that Ikaros and AP-2 do not bind
to the respective sites of the CCL22 promoter, base substitutions in
these sites had no effect on promoter activity in both cell lines.

Effect of overexpression of transcription factors on CCL22
promoter activity

Next, we examined the effect of overexpression of transcription
factors on the activity of the CCL22 promoter. CCL22 promoter/
luciferase reporter constructs were cotransfected with expression
vectors coding for the NF-κB proteins p50, p52, p65, RelB, and
c-Rel, either separately or as combination of two factors, to allow
for formation of functional units of homo- and heterodimers, re-
spectively. In A20/2J cells, overexpression of p65 alone increased
promoter activity ~2-fold (p < 0.001; Fig. 4C). Overexpression of c-Rel had a lesser effect (p < 0.05), and overexpression of p50,
p52, and RelB had no marked effect. Overexpression of both, p50
and p65, resulted in an ~10-fold increase of promoter activity
(p < 0.001), being by far the most effective combination of NF-
κB proteins in activation of the CCL22 promoter. In contrast to A20/2J
cells, XS106 cells require, as reported above, no stimulation to
show strong CCL22 promoter activity. Nevertheless, a combina-
tion of p50 and p65 further enhanced promoter activity (p < 0.001),
whereas all other combinations did not augment the already
high expression level (Fig. 4C). To investigate the effect of overexpression of NF-κB submits on the endogenous CCL22 mRNA production, A20/2J cells were transfected transiently with NF-κB expression plasmids and analyzed for CCL22 mRNA ex-
pression by RT-PCR. To assure comparable transfection effi-
ciency, cells were cotransfected with a renilla luciferase core-
porter, and samples displaying similar coreporter activity were
chosen for RNA isolation. Although overexpression of p50 alone
was not sufficient to induce CCL22 mRNA production, overex-
pression of p65 or of p50 together with p65 induced endogenous
CCL22 mRNA production (data not shown). Taken together, these
experiments show that p50 and p65 not only bind to region I of the
CCL22 promoter, as indicated by EMSA, but induce expression
acting probably as heterodimers.

The transcription factor Sp1, shown to bind to region III in
EMSA using nuclear extracts from LPS-stimulated A20/2J cells,
was analyzed likewise. Cotransfection of expression plasmids cod-
ing for Sp1 resulted in an ~6-fold increase in luciferase reporter
activity of CCL22 reporter constructs in A20/2J cells (p < 0.001; Fig. 4C). However, in XS106 cells, cotransfection of expression plasmids coding for Sp1 had no effect on promoter activity, again indicating that Sp1 is involved in induction of expression in A20/2J but not in XS106 cells. Overexpression of Sp3 had no significant effect on promoter activity of reporter constructs in both cell lines (Fig. 4C). Furthermore, overexpression of either Sp1 or Sp3 was not sufficient to induce endogenous CCL22 mRNA pro-
duction in A20/2J cells (data not shown).

Transactivation of the CCL22 promoter by overexpression of
Sp1 or NF-κB p50 + p65 in A20/2J cells or of NF-κB p50 + p65
in XS106 cells was abrogated by introducing a mutation in the
respective binding sites (all p < 0.001; Fig. 4B). Interestingly,
mutation of the Sp1 site affected inducibility of the promoter by
overexpression of p50 + p65 in A20/2J and XS106 cells (both p <
0.001; Fig. 4B), hinting at an interaction between the protein com-
plexes formed at region I and region III. Mutation of the AP-2 site
had, as expected, no effect on inducibility by overexpression of
Sp1 in A20/2J cells (data not shown).

Functional interaction of region I and III depends on the spatial
alignment within the CCL22 promoter

Interaction between members of the NF-κB family and Sp1 was
reported before, e.g., for the promoter of VCAM-1 or HIV-long-
terminal repeat (38, 39). Likewise, importance of interaction of the
protein complexes formed at box I and III of the CCL22 promoter
was suggested by the finding that mutation of the Sp1 binding site
markedly affected inducibility by overexpression of p50 + p65
(Fig. 4B). To demonstrate that the spatial alignment of box I and
III is important for the functional interaction of the protein com-
plexes, we inserted small DNA fragments of 18 and 24 bp, respec-
tively. To exclude that effects were caused by disruption of a so-far
undetected regulatory element or by disruption of a possible in-
teraction between box II and either boxes I or III, one fragment
was inserted between boxes I and II and the other fragment be-
tween box II and III. Promoter activity of both modified reporter
constructs was markedly reduced in LPS-stimulated A20/2J cells
and XS106 cells (all p < 0.001; Fig. 4D). Moreover, both inser-
tions reduced promoter activity to the same extent compared with
the wild-type construct. This reduction was more pronounced in
A20/2J cells than in XS106 cells.

Keeping in mind the importance of the spatial alignment of box
I and III, we replaced box II by an unrelated sequence of exactly
the same length to evaluate contribution of region II to promoter
activity. The sequence was selected to minimize sequence simi-
larity to avoid that it competes with the wild-type sequence for
transcription factor binding. A marked reduction (p < 0.001) of
promoter activity was observed in XS106 cells (Fig. 4D). In ac-
cordance with the data presented above (Fig. 2C), the relative loss
of activity by replacement of box II was less pronounced in LPS-
stimulated A20/2J cells (p < 0.02; Fig. 4D).

Distinct proteins from primary DC and B cells bind to region I
and region III of the CCL22 promoter

To investigate whether the same transcription factors are involved
in primary cells as in the cell lines tested, EMSA and supershift
assays were performed with nuclear extracts from primary B cells
and BM-DC.

Stimulation of primary B cells with LPS enhanced complex for-
mation (data not shown), an antiserum against NF-κB p50 induced
a supershift, and an antiserum against p65 resulted in decreased
complex formation, using nuclear extracts from LPS-activated B
cells (Fig. 5).

To investigate complex formation in DC, we used nuclear ex-
tracts of BM-DC instead of LC because in BM-DC analysis is not
hampered by the rapid changes of CCL22 expression observed in
LC (Refs. 8 and 9 and our unpublished results). We obtained dis-

tinct stages of maturation in BM-DC by stimulation with TNF-α
and LPS, respectively; the latter representing a stronger maturation
stimulus than TNF-α. Three distinct complexes were formed using
nuclear extracts from TNF-α-stimulated BM-DC. An antiserum
against NF-κB p50 induced a supershift, and an antiserum against
p65 resulted in decreased formation of the preexisting complexes,
wheras a new low molecular mass complex appears (Fig. 5). In addi-
tion, an antiserum against RelB reduced complex formation.

However, when extracts from LPS-stimulated BM-DC were used,
addition of an antiserum against p50 supershifted both complexes,
whereas the addition of an antiserum against p65 had little effect
on complex formation. Instead, addition of an antiserum against
RelB induced a marked decrease in formation of the lower mobili-
ity complex, indicating that p50/RelB rather than p50/p65 het-
erodimers from nuclear extracts of LPS-stimulated BM-DC bind to
region I.
sive in suggesting that the NF-

tors involved. The data obtained from EMSA, supershift analysis,

250-bp region. Interestingly, the same regulatory elements within

lements active in DC and B cells being located in a small proximal

sion are included in a 4.1-kb promoter region, with the major el-

130 kb (19). The separate location and the extensive differences

kines are clustered. Likewise, in mice these three chemokines are

mokine locus on chromosome 17q11.2, where most CC chemo-

human chromosome 16q13 (40), apart from the common CC che-

expression profiles of CCL22 and CCL17 are similar, this may suggest a

to other CC chemokines on amino acid levels (9) indicate a diver-

130 kb (19). The separate location and the extensive differences

ings region III as a probe. As had been observed for the B cell line

We next conducted EMSA with proteins from primary cells us-

ing region III as a probe. As had been observed for the B cell line

A20/2J, extracts from LPS-activated splenic B cells formed three

complexes, of which complex 2 was supershifted using an anti-Sp1

antisera. When proteins isolated from LPS-matured BM-DC were

used, three complexes were detected, of which none was

affected by the addition of antisera against Sp1 or Sp3 (Fig. 5).

Discussion

The mouse CC chemokine CCL22 is involved critically in regu-

lation of immune responses (8–12, 14, 15). Of interest are its tight

regulation of expression and high expression levels in mature DC

and activated B cells. Thus, we characterized the CCL22 gene and

its regulation. CCL22 is clustered with CCL17 and CX3CL1 on

human chromosome 16q13 (40), apart from the common CC che-
mokine locus on chromosome 17q11.2, where most CC chemoki-

nes are clustered. Likewise, in mice these three chemokines are

clustered separately on a short chromosomal fragment spanning

<130 kb (19). The separate location and the extensive differences
to other CC chemokines on amino acid levels (9) indicate a diver-

gent evolution of this locus. Taking into account that the expres-

sion profiles of CCL22 and CCL17 are similar, this may suggest a

common regulation of gene expression of these two chemokines.

Our data indicate that in case of the CCL22 gene, the regulatory

elements required to mediate a strong and highly specific expres-
sion are included in a 4.1-kb promoter region, with the major el-

elements active in DC and B cells being located in a small proximal

250-bp region. Interestingly, the same regulatory elements within

this proximal region (box I–III) are required in all cells analyzed,

and there are similarities but there are also differences in the fac-
tors involved. The data obtained from EMSA, supershift analysis,
site-directed mutagenesis, and transactivation studies are conclus-
ive in suggesting that the NF-κB proteins p50 and p65 act to-

tgether, probably as heterodimers, to activate the CCL22 gene by

binding to box I of the promoter in nearly all cells tested, namely

the B cell line A20/2J, the DC line XSI06, primary LPS-stimu-

lated B cells, and TNF-α-stimulated DC. Moreover, overexpres-
sion of p50 and p65 enhanced the endogenous CCL22 expression

in A20/2J cells synergistically, as determined by RT-PCR (data not

shown). However, the fact that the mutation as well as the deletion

of the NF-κB site had only a small and statistically not significant

effect indicates that in this cell line, which expresses CCL22 con-

stitutively, this NF-κB site is of minor importance.

In LPS-stimulated DC, p65 is replaced by RelB. A slightly de-

creased complex formation was already observed with antisera

against RelB in TNF-α-stimulated DC (Fig. 5). Because LPS rep-

resents the stronger maturation stimulus compared with TNF-α,

this may suggest that RelB replaces p65 during maturation of DC.

However, in contrast to p65 overexpression of RelB does not en-
hance CCL22 promoter activity in A20/2J and XSI06 cells, either

alone or in combination with other proteins of the NF-κB family.

Thus, replacement of p65 by RelB may rather reduce than enhance

CCL22 expression. Accordingly, RelB had been shown to be the

most actively translocated NF-κB protein in maturing DC (41).

Saccani et al. (42) recently demonstrated that p65-containing
dimers are replaced completely by RelB-containing dimers at the

human CCL22 promoter during maturation of DC. A similar re-

placement of p65 by RelB at the IL-12p40 promoter correlated

with a transcriptional shutdown of the promoter and a drop of

polymerase II recruitment. However, they observed that RNA

polymerase II levels at the CCL22 promoter peaked when RelB

recruitment was maximal and that expression of the IκBα super-

repressor, which affects translocation of p65- and c-Rel- but not

RelB-containing dimers, rather enhanced than suppressed CCL22

transcription (42). This may suggest that the human and mouse

CCL22 promoters differ with respect to RelB activation.

Remarkably, the factor Sp1 binds to box III in B cells and the B

cell line A20/2J but not in DC and the DC line XSI06. Moreover,

Sp1 is able to transactivate the promoter in A20/2J but not in

XSI06 cells, indicating that either the concentration of the factor

replacing Sp1 in XSI06 is not limiting the expression or Sp1 can-

not substitute for this factor in XSI06 cells. Nevertheless, exactly

the same site is required for activation of CCL22 expression in

XSI06 cells because substitution of two nucleotides of the Sp1

binding site disrupts activity of the promoter in XSI06 cells. Fur-

thermore, the complexes formed at box I and box III interact in

both cell lines because a change of the spatial alignment of the

boxes affects activity of the promoter in both cases (Fig. 4D).

Given these close similarities and the fact that neither Sp1 nor

another known member of the Sp1 family binds to the promoter in

XSI06 cells and DC, a so-far unknown member of the Sp1 family

may be active in DC. The fact that LPS did not induce an addi-
tional complex to be formed at region III by proteins isolated from

A20/2J indicates that Sp1 is already present in the nucleus of these
cells before LPS activation.

The functional interaction of Sp1 and NF-κB at the CCL22 pro-
moter, indicated (1) by the importance of the spatial alignment of

the binding sites within the promoter and (2) by the fact that a

functional Sp1 site is required for NF-κB transactivation, was

demonstrated for other promoters previously (38, 39). The fact that

the distance between the NF-κB and the Sp1 binding sites is con-

siderably greater in the CCL22 promoter may suggest involvement

of additional proteins. Such proteins may bind to the promoter

themselves, e.g., at box II or even adjacent to the binding sites for

Sp1 or NF-κB.

 Likely candidates are C/EBP proteins because region III harbors

a sequence with similarity to the C/EBP consensus-binding se-

quence, and interaction of NF-κB and C/EBP was demonstrated

previously (43). The fact that we did not detect binding of C/EBP

isoforms α, β, γ, δ, ε, and ξ in supershift assays (Fig. 3C) does

not completely rule out an involvement because unstable higher

order complexes may remain unnoticed. C/EBP isoforms α, β, and δ

were detected in nuclear extracts of A20/2J, and XSI06 cells and
cotransfection of C/EBP α + δ increased promoter activity in

A20/2J cells ~5-fold, indicating that heterodimers of C/EBP α and

FIGURE 5. Identification of proteins isolated from LPS-activated B
cells and TNF-α- or LPS-matured BM-DC binding to box I and III, re-
spectively. EMSA were performed on oligonucleotides of box I and box

III, respectively, using nuclear extracts isolated from the indicated cells in

the presence or absence of a molar excess of a cold competitor or in the

presence or absence of antisera. Antisera were added to the nuclear proteins

30 min before adding hot probe. Data are representative of two independent

experiments.

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δ may activate the promoter (data not shown). However, in XS106 cells, overexpression of C/EBP isoforms α, β, and δ, either separately or as combination of two factors, had no effect on reporter activity (data not shown).

An additional difference in regulation of the CCL22 promoter in A20/2J and XS106 cells was indicated by luciferase assays following 5′-terminal deletion of the promoter region, hinting at an enhancing promoter element located between 550 and 250 bp upstream of the translational start site, which is active in XS106 cells but not in A20/2J cells. Because we detected a putative binding site for GATA proteins in this region, we overexpressed GATA-1, GATA-2, and GATA-3 but did not detect an effect on reporter gene expression in A20/2J and XS106 cells (data not shown).

Interestingly, a very recent study by Nakayama et al. (44) investigated the roles of two adjacent NF-κB sites and a single AP-1 site of the human CCL22 promoter in induction by EBV-encoded LMP-1 in human B cells. Although we identified only one NF-κB site in the murine CCL22 promoter, a putative AP-1 site is also present in the murine promoter (position 97–100 relative to the translation start site). However, because this site is situated between the two transcriptional start sites, it is unlikely that AP-1 is involved in the regulation of the murine CCL22 promoter. However, it cannot be ruled out that AP-1 is involved in the initiation of transcription of only a fraction of the products.

Remarkably, no site with substantial homology to an Ikappa binding site is present in the human CCL22 promoter region, but a very G-rich element, similar to the Sp1 site identified here, is located 175 bp upstream of the translation start codon of the human CCL22 promoter.

Although the CCL22 promoter reflects the specificity of the endogenous gene, there may be additional levels of regulation of CCL22 expression not analyzed in detail yet. Saccani and Nataoli (45) detected, for example, acetylation of histones H3 and H4 at the human CCL22 locus during activation, a common mechanism of gene activation on chromatin level (46). Enhancer elements are often located far upstream, downstream, or in introns, especially in the first intron. The latter was tested by incorporating a 1.2-kb downstream region, including the first intron of the CCL22 gene into a luciferase reporter construct, but no regulatory sequences were detected (data not shown).

The usage of two transcriptional initiation sites 28 and 124 bp upstream of the translational start codon, respectively, offers additional possibilities for regulation of CCL22 expression. Quantitative differences in the usage of these start sites were detected in cultured LC and LPS-activated B cells. The longer 5′-UTR contains two small ORF (mini ORF) (position −120 to −96 and −116 to −80, respectively). Such mini ORF in the 5′-UTR have been reported to interfere with translation of the corresponding proteins (47), raising the possibility that CCL22 production is controlled not only on the transcriptional but also on the translational level. However, the significance of these mini ORF in the 5′-UTR of a proportion of the CCL22 transcripts is not clear.

Considering the pivotal role of DC in initiation of novel immune responses and the promising strategies to transcriptionally target DC for therapeutic use (23, 24, 48), thorough knowledge of gene regulation in DC will help to manipulate DC on this level.

Disclosures

The authors have no financial conflict of interest.

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