Characterization of the Promoter and the Transcriptional Regulation of the Lipoxin A4 Receptor (FPR2/ALX) Gene in Human Monocytes and Macrophages


J Immunol published online 13 January 2012
http://www.jimmunol.org/content/early/2012/01/13/jimmunol.1101788

Supplementary Material http://www.jimmunol.org/content/suppl/2012/01/13/jimmunol.1101788.8.DC1

Why The JI?
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Characterization of the Promoter and the Transcriptional Regulation of the Lipoxin A4 Receptor (FPR2/ALX) Gene in Human Monocytes and Macrophages

Vanessa Waechter,*† Mattia Schmid,*† Magdalena Herova,*† Angelika Weber,*† Viola Günther,‡ Jacqueline Marti-Jaun,*† Sophia Wüst,*† Marian Rössinger,*† Claudio Gemperle,*† and Martin Hersberger*†

The lipoxin A4 receptor FPR2/ALX plays an important part in host defense and inflammation. The receptor binds structurally diverse agonistic ligands, which mainly regulate chemotaxis and activation of leukocytes. However, little is known about the promoter region of the FPR2/ALX gene and its transcriptional regulation in leukocytes. We identified two TATA-less promoter regions, separated by 224 bp, that drive the expression of FPR2/ALX in macrophages. Both promoter regions increased transcription in a reporter assay, and the basal transcription factors OCT1 and SP1 were shown to bind to the first and the second promoter, respectively, and to transactivate transcription. Although monocytes expressed high levels of FPR2/ALX mRNA from the second promoter region, differentiation into macrophages abrogated FPR2/ALX expression. Stimulation of macrophages with a set of cytokines revealed that only IFN-γ and LPS increased FPR2/ALX expression from the first promoter to levels similar to those detected in monocytes. The upregulation by IFN-γ is in part mediated by the interaction of IFN regulatory factor 1 with an IFN-responsive sequence element transcription factor binding site located in the first promoter region of the FPR2/ALX gene. However, this upregulation on the mRNA level did not translate into FPR2/ALX protein expression in macrophages owing to reduced translation of the longer mRNA from the first promoter. In contrast, FPR2/ALX mRNA transcribed from the second promoter was translated into surface expression of FPR2/ALX in monocytes. These data support a model in which FPR2/ALX plays a role in chemotaxis and activation of monocytes; however, they also suggest that its function in resident tissue macrophages is limited. The Journal of Immunology, 2012, 188: 000–000.

The online version of this article contains supplemental material.

Abbreviations used in this article: ER-α, estrogen receptor α; GPR32, G protein-coupled receptor 32; IRF, IFN regulatory factor; IRSE, IFN-responsive sequence element; LXA₄, lipoxin A₄; P1, promoter region 1; P2, promoter region 2; PMN, polymorphonuclear neutrophil; RACE, rapid amplification of cDNA ends; SAA, serum amyloid A; dORF, upstream open reading frame; UTR, untranslated region.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12S16/03

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101788
mouse models lacking FPR2/ALX may derive from the deletion of an additional member of the FPR family, besides FPR2/ALX, in one of the mouse models (31). However, a general difficulty arises in attempting to make a direct correlation between function of the human and function of the murine FPR gene families, because humans have three FPR genes, whereas the murine FPR family comprises at least eight genes (2).

The opposing effects mediated by FPR2/ALX, upon binding of pro- and anti-inflammatory ligands, suggest that FPR2/ALX expression is restricted to certain cell types and that its expression is tightly regulated. Indeed, FPR2/ALX mRNA expression is in a limited number of primary cells involved in inflammation, such as monocytes (18, 32), neutrophils (33), NK cells (34), and possibly T cells (17, 35). However, regulation of FPR2/ALX expression is not well understood, with a limited number of regulators identified. In synovial fibroblasts, treatment with the proinflammatory TNF-α and IL-1β was shown to upregulate FPR2/ALX mRNA expression in a time-dependent manner, whereas IL-6 had little effect (36). In enterocytes, the proinflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-13 were shown to be the most potent inducers of FPR2/ALX expression, whereas IL-1β and LPS only slightly increased its expression (37). In mouse macrophages, an inverse correlation was found between FPR2/ALX expression and thromboxane A₂ signaling (38). In NK cells, IL-10 stimulation led to a moderate induction of FPR2/ALX mRNA (34), and the glucocorticoid analog dexamethasone increased the expression of FPR2/ALX mRNA in a time- and dose-dependent manner in PMNs (39, 40). Extensive studies in mouse macroglial cells identified TNF-α, CD40L, IFN-γ, and several ligands for TLRs as regulators of FPR2/ALX receptor expression and function (41–45). These findings indicate that FPR2/ALX mRNA expression may be regulated by pro- and anti-inflammatory stimuli and that the response to stimulation depends on the cell type investigated.

Because little is known about the promoter region of the FPR2/ALX gene and its transcriptional regulation in leukocytes, we set out to define the basal promoter of the FPR2/ALX gene and to investigate its transcriptional regulation in human macrophages. We located two TATA-less promoter regions in which the basal transcription factors OCT1 and SP1 were found to be necessary for FPR2/ALX transcription, respectively. We further show in primary human cells that monocytes express high levels of FPR2/ALX mRNA, whereas differentiation into macrophages abrogated this expression. IFN-γ stimulation of macrophages rescued FPR2/ALX mRNA expression in a dose-dependent manner, an effect at least partially mediated by IFN regulatory factor 1 (IRF1) via an IFN-responsive sequence element (IRSE) located in the first promoter of FPR2/ALX. However, this upregulation on the mRNA level did not translate into FPR2/ALX protein expression in IFN-γ-stimulated macrophages. These data support the model in which FPR2/ALX plays a role in chemotaxis and activation of monocytes, whereas its function in resident tissue macrophages seems restricted.

Materials and Methods

**Materials**

The recombiant human cytokines IL-13, IL-1β, TNF-α, TGF-β, and IFN-γ; LPS; and the formylated peptide fMLF were purchased from Sigma-Aldrich (Buchs, Switzerland). IL-4 and IL-6 were purchased from R&D Systems Europe (Abingdon, U.K.). LXA₄ was purchased from Cayman Chemicals (Ann Arbor, MI). The TLR9 ligand, CpG, was synthesized by Microsynth (Balgach, Switzerland).

**Primary cell purification and cell culture**

Leukocytes from healthy volunteers were extracted from 60 ml heparinized blood or buffy coat (Blutspendezentrum, Zurich, Switzerland) with Histopaque-1077 gradient (Sigma-Aldrich). Peripheral blood monocytes were purified by capture with anti-CD14 Abs coupled to MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Monocytes were allowed to differentiate into macrophages for 7 d at 37°C in RPMI 1640 (Sigma-Aldrich) supplemented with 5% FCS (Bioconcept, Allschwil, Switzerland), 5% human AB serum (Sigma-Aldrich), 1% penicillin/streptomycin (Invitrogen, Basel, Switzerland), and 5% CO₂. Human THP-1 monocytes (46) were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (Bioconcept), 20 mM glutamine (Invitrogen), and 5% CO₂. For differentiation into macrophages, cells were stimulated for 2 d with PMA (Sigma-Aldrich) (47).

**Quantification of FPR2/ALX mRNA**

The reverse transcription was performed on 1 μg total RNA, using SuperScript III Reverse Transcriptase (Invitrogen) with random primers according to the manufacturer’s instructions. Each RT-PCR was performed in a total volume of 20 μl on a LightCycler System (Roche Diagnostics, Rotkreuz, Switzerland). FPR2/ALX mRNA quantification included 100 ng cDNA, 4 mM MgCl₂, 0.5 μM upper primer (all: 5’-GAACAGCAGGAAAGAGGAG-3’; or promoter region 1 [P1]: 5’-GGAACACCTGACACGTCCTTTTG-3’), 0.5 μM lower primer (5’-GAAAAGTGACCCCTACAAAGGA-3’), and 1× SYBR Green enzyme mix (Roche Diagnostics). GAPDH mRNA quantification included 100 ng cDNA, 3 mM MgCl₂, 0.5 μM upper primer (5’-CCAATGTTGCGCTGGTCTGGT-3’), and 0.5 μM lower primer (5’-TGGTCAGGTGTCTTTCCACAGTA-3’), and 1× SYBR Green enzyme mix (Roche Diagnostics). PCR reactions were performed under the following conditions. FPR2/ALX: preheating of the mixture for 10 min at 95°C, followed by 45 cycles of denaturation for 5 s at 95°C, annealing for 10 s at 68°C, and extension for 7 s at 72°C; GAPDH: preheating of the mixture for 10 min at 95°C, followed by 45 cycles of denaturation for 5 s at 95°C, annealing for 10 s at 59°C, and extension for 6 s at 72°C. The primers were designed over exon/intron boundaries, and amplified PCR products were checked for size on agarose gels and sequenced.

**Generation of promoter constructs**

Promoter constructs were amplified by PCR with Xhol upper primers and HindIII lower primers (Supplemental Table I). Constructs were subcloned into the pGL3 basic vector that contains the firefly luciferase gene (Promega, Roche, Switzerland), using the Xhol and HindIII restriction sites. Point mutations in transcription factor binding sites were introduced into P1-414 and P1-150, using the QuickChange II XL Site-Directed Mutagenesis Kit according to the manufacturer’s instructions (Stratagene, Basel, Switzerland). The primers (Microsynth) used to mutate P1-414 and promoter region 2 (P2)-150 were designed to result in the changes shown in Supplemental Table II. The ATG in the potential upstream open reading frame (uORF) was mutated in P2-639 and P2-1200, using the same method primers listed in Supplemental Table III.

The 5’-untranslated regions (UTRs) were amplified by RT-PCR with Xhol upper and HindIII lower primers (Supplemental Table III) from human monocyte mRNA. PCR products were TA subcloned into the pCRII vector (Invitrogen), and the 5’-UTRs were cut out by HindIII and XhoI, respectively. Constructs were subcloned into the pGL4.13 vector (Promega) between the SV40 promoter and the firefly luciferase gene, using the HindIII restriction site. All constructs were sequenced.

**Transcription assays**

For each experiment, 6 × 10⁶ THP-1 cells, 10 μg construct, and 0.25 μg internal control phRL-SV40 (Promega) were used. Electroporation was done under the following conditions: 200 V, 950 μl capacitance, and 900 μs. After electroporation, cells were seeded in RPMI 1640 with...
10% FCS and 20 mM glutamine. At 3 h later, cells were differentiated into macrophages with PMA for 48 h. For basal transcription assays, cells were washed with PBS and harvested. For the IFN-γ–induced transcription assay, cells were stimulated another 24 h with 50 ng/ml IFN-γ in RPMI 1640 with 0.5% FCS, washed with PBS, and harvested. Harvested cells were lysed in 250 μl 1× passive lysis buffer, and 50 μl was used for dual-luciferase reporter assays (Promega).

Preparation of nuclear extracts

Nuclear extracts were prepared on ice from THP-1 macrophages as described in Ref. 48. Briefly, cells were resuspended in 500 μl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease inhibitors [Complete, Roche Diagnostics]) and were incubated on a rotating wheel for 30 min at 4°C, followed by the addition of 30 μl 10% Nonidet P-40. After vortexing, the nuclei were pelleted by centrifugation for 5 min at 13,000 rpm. Nuclei were then resuspended in 60 μl buffer C (20 mM HEPES, pH 7.9; 25% glycerol; 400 mM NaCl; 1 mM EDTA; 1 mM DTT; 1 mM PMSF; and protease inhibitors) and lysed by shaking for 15 min. After centrifugation for 5 min at 13,000 rpm, nuclear proteins in the supernatant were stored at −80°C until use. The protein concentration was measured with the M-TP Reagent (Beckman Coulter, Nyon, Switzerland).

EMSA

For EMSA, 2 pmol of dsDNA probes corresponding to a potential transcription factor binding site were end labeled with [γ-32P]-ATP (Hartmann Analytic, Braunschweig, Germany) by T4 polynucleotide kinase (Fermentas, Le-Mont-sur-Lausanne, Switzerland). Sequences of the EMSA probes are listed in Supplementary Table IV. A total of 10 μg THP-1 macrophage nuclear extracts and 2 μg freshly boiled polydeoxycytidylic-polydeoxythymidyl acid in binding buffer (24 μM HEPES, pH 7.9; 24% glycerol, 10 mM NaCl, 100 mM KCl, 10 mM MgCl2, and 1.2 mM DTT) was incubated in a 20-μl binding reaction. In the competition experiment, unlabeled competitor was added in 50× excess to the reaction. The labeled probes were added, and the reaction was incubated for 20 min at room temperature. Samples were analyzed on a 4% nondenaturing polyacrylamide gel (0.5% Tris/borate/EDTA, 4% acrylamide/bisacrylamide, 29:1, g[::-r]/g[::-r]) for 1 h and visualized by autoradiography. Gels were run at 150 V for 5 h, dried, and analyzed with the scanner FLA-7000 and the software Image Gauge (Fujifilm, Basel, Switzerland).

FACS analysis

FACS analysis was performed using the monoclonal APC-labeled anti-human FPR2/ALX Ab and the isotype control IgG2b (both from R&D Systems, Minneapolis, MN). As a positive control for permeabilization of the cells, the CD68 Ab was used (Clone EbioY1/82A; ebioscience, San Diego, CA). Briefly, cells were resuspended in PBS containing 2.5% FCS and incubated in the dark for 30 min at 4°C before analysis on a FACSCalibur analyzer (BD Biosciences, San Jose, CA). To permeabilize cells, PBS with Ca/Mg (Invitrogen) and containing 0.1% albumin and 0.1% Triton X-100 (Sigma-Aldrich) was added dropwise to the cells while vortexing. To avoid non-specific binding of Abs, permeabilized cells were blocked in 100% human serum (Sigma-Aldrich) for 15 min at room temperature. Differentiation of the resident macrophages into M1 and M2 macrophages was monitored using monoclonal anti-human CD80 and CD206 Abs (all from BD Biosciences) as markers for M1 and M2 macrophages, respectively.

Chemotaxis assay

A total of 105 monocytes or macrophages were placed on a 96-well membrane (5.7-mm diameter, 5-μm pore size; ChemiTx from Neuro Probe, Gaithersburg, MD) in RPMI 1640 containing 0.1% BSA (Sigma-Aldrich). The cells were allowed to migrate toward the chemotactic factors at the indicated concentrations for 90 min and 60 min, respectively. Migrated cells were fixed (2% paraformaldehyde) and stained with DAPI (Sigma-Aldrich), and migration was quantified as the total pixel count of DAPI-stained nuclei under the fluorescence microscope (two photos per membrane and three replicate wells per treatment). Migration indices were calculated over control values.

Statistical analysis

Statistical analysis was performed with StatView version 5.0.1 (SAS Institute, Cary, NC). The level of FPR2/ALX mRNA and the activities of the different FPR2/ALX promoters in the luciferase assays were compared using a two-sided t test. A two-sided p < 0.05 was considered significant.

Results

The FPR2/ALX gene has two promoters leading to two distinct transcription start sites

To define the transcription start site of the FPR2/ALX gene, 5’ RACE was used in primary macrophages. The amplification produced two distinct bands of 320 and 600 bp (Fig. 1A, lane 2), which represent two mRNAs containing both the coding exon and the first exon of the FPR2/ALX gene, but with one having a longer version of the first exon. These results suggest that FPR2/ALX has two transcription start sites (Fig. 1B) and that FPR2/ALX transcription may be regulated by two promoters. To characterize the transcriptional activity of these two potential promoter regions, different-sized fragments of the two regions were cloned upstream of a firefly luciferase reporter gene. As shown in Fig. 1C, all constructs containing promoter region P1 directed expression of the luciferase, and the shortest construct comprising 414 bp led to a 4-fold increase in transcription, compared with the empty vector. Similarly, all constructs containing sequences of promoter region P2 directed expression of the luciferase with a 4-fold increase (3.8 ± 0.3) increase in transcription of the shortest 150 bp promoter fragment. These results indicate that one core promoter each is located upstream of both transcription start sites. Both FPR2/ALX promoters are recognized by basal transcription factors

To characterize basal transcription by the two TATA-less promoters P1 and P2 of the FPR2/ALX gene, we analyzed these regions for transcription factor binding sites, using the bioinformatic tools MatInspector (http://www.genomatix.de) and Alibaba (http://www. gene-regulation.com). We identified eight putative transcription factor binding sites in promoter P1 implicated in the formation of the transcription preinitiation complex of TATA-less promoters, namely, one P1U1, P1, NFE2, GATA, and SP2 and three OCT1 binding sites. To identify the role of these transcription factor binding sites on basal transcription, we performed site-directed mutagenesis of their core binding sites (Supplemental Table II). These experiments revealed that an OCT1 binding site supports the basal transcription activity of promoter P1. A single A to T substitution in the OCT1 core binding site at position −103 resulted in an almost 50% reduction of luciferase expression (47 ± 29%) compared with the wild-type promoter P1-414 (Fig. 2A).

To verify that OCT1 binds to this sequence in promoter P1, we performed EMSA analysis with THP-1 nuclear extracts. Specific binding of the nuclear extracts to this OCT1 oligonucleotide was shown (Fig. 2B, lane 1) and could be competed in the presence of unlabeled wild-type, but not mutant, oligonucleotide (Fig. 2B, lane 2, OCT1, and lane 3, mOCT1). The presence of the A to T mutation at position −103 also dramatically reduced binding of nuclear proteins (Fig. 2B, lane 5). To show that OCT1 is part of the protein complex binding to the OCT1 oligonucleotide, nuclear extracts were coincubated with an OCT1 Ab. The observed supershift demonstrates that OCT1 binds to the OCT1 transcription factor binding site in promoter P1 (Fig. 2B, lane 4). This finding is further supported by the similar supershift observed with the designed OCT1 oligonucleotide used as a positive control (Fig. 2B, lane 6).
We also identified two putative basal transcription factor binding sites in promoter P2, namely, an NFY and SP1 binding site. Mutations in the core binding site of both NFY and SP1 (Supplemental Table II) resulted in an 83% (±3%) and a 91% (±3%) reduction of promoter activity, compared with the wild-type promoter P2-150, respectively (Fig. 2C). To confirm the binding of NFY and SP1 to promoter P2, we again performed EMSA analysis with THP-1 nuclear extracts. Direct binding of nuclear proteins to the SP1 and NFY oligonucleotides was confirmed (Fig. 2D, lanes 1 and 6), and a decrease in binding was observed for both tran-
Supplemental Table IV. Error bars display 95% confidence intervals. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over P1-414 and P2-150, respectively. B. EMSAs for the potential OCT1 binding site with nuclear extracts from THP-1 macrophages. The DNA-protein complex with OCT1 oligonucleotides was analyzed by gel electrophoresis and visualized by autoradiography (lane 1). For competition assays, the nuclear extracts were preincubated with excess of unlabeled OCT1 or mutant OCT1 oligonucleotides (OCT1: lane 2; mOCT1: lane 3). For supershift analysis, the nuclear extracts were preincubated with an OCT1 Ab (lane 4). The nuclear proteins were incubated with the mutant OCT1 oligonucleotide to test direct binding (lane 5). A control of supershift was done with OCT1 Ab and a designed OCT1 oligonucleotide (lane 6). D. EMSA for SP1 and NFY. DNA-protein complexes with SP1 and NFY oligonucleotides were visualized by autoradiography (lane 1 and lane 6, respectively). Competition assays were performed with three competitors for SP1: SP1 (lane 2), NFY (lane 3), and mutant SP1 (mSP1: lane 4). One competitor was used for NFY: SP1 (lane 7). SP1 Ab was used for supershift assays with the SP1 and NFY oligonucleotides (lane 5 and lane 8). The nuclear extracts were incubated with the mutant SP1 (mSP1: lane 9) and the mutant NFY oligonucleotides (mNFY: lane 10) to see abrogated direct binding. Sequences of the different oligonucleotides can be found in Supplemental Table IV. Error bars display ±SD. *p < 0.05, **p < 0.01. Ab, supershift with Ab.

**FIGURE 2.** Identification of basal transcription factor binding sites in the FPR2/ALX promoter region. Putative basal transcription factor binding sites for P1-414 and P2-150 were identified with the software Genomatix and Alibaba. A and C. Individual transcription factor binding sites were mutated in P1-414 (A) and P2-150 (C). For mutations introduced, see Supplemental Table II, which also delineates the order and location of the three potential OCT1 binding sites (P1-414mOCT1). Human THP-1 monocytes were transfected with the constructs and differentiated into macrophages, and luciferase activities were measured 48 h after transfection. Firefly luciferase activities were normalized to Renilla luciferase activities, and all experiments were done in triplicate. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over P1-414 and P2-150, respectively. B. EMSAs for the potential OCT1 binding site with nuclear extracts from THP-1 macrophages. The DNA-protein complex with OCT1 oligonucleotides was analyzed by gel electrophoresis and visualized by autoradiography (lane 1). For competition assays, the nuclear extracts were preincubated with excess of unlabeled OCT1 or mutant OCT1 oligonucleotides (OCT1: lane 2; mOCT1: lane 3). For supershift analysis, the nuclear extracts were preincubated with an OCT1 Ab (lane 4). The nuclear proteins were incubated with the mutant OCT1 oligonucleotide to test direct binding (lane 5). A control of supershift was done with OCT1 Ab and a designed OCT1 oligonucleotide (lane 6). D. EMSA for SP1 and NFY. DNA-protein complexes with SP1 and NFY oligonucleotides were visualized by autoradiography (lane 1 and lane 6, respectively). Competition assays were performed with three competitors for SP1: SP1 (lane 2), NFY (lane 3), and mutant SP1 (mSP1: lane 4). One competitor was used for NFY: SP1 (lane 7). SP1 Ab was used for supershift assays with the SP1 and NFY oligonucleotides (lane 5 and lane 8). The nuclear extracts were incubated with the mutant SP1 (mSP1: lane 9) and the mutant NFY oligonucleotides (mNFY: lane 10) to see abrogated direct binding. Sequences of the different oligonucleotides can be found in Supplemental Table IV. Error bars display ±SD. *p < 0.05, **p < 0.01. Ab, supershift with Ab.

**FIGURE 2.** Identification of basal transcription factor binding sites in the FPR2/ALX promoter region. Putative basal transcription factor binding sites for P1-414 and P2-150 were identified with the software Genomatix and Alibaba. A and C. Individual transcription factor binding sites were mutated in P1-414 (A) and P2-150 (C). For mutations introduced, see Supplemental Table II, which also delineates the order and location of the three potential OCT1 binding sites (P1-414mOCT1). Human THP-1 monocytes were transfected with the constructs and differentiated into macrophages, and luciferase activities were measured 48 h after transfection. Firefly luciferase activities were normalized to Renilla luciferase activities, and all experiments were done in triplicate. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over P1-414 and P2-150, respectively. B. EMSAs for the potential OCT1 binding site with nuclear extracts from THP-1 macrophages. The DNA-protein complex with OCT1 oligonucleotides was analyzed by gel electrophoresis and visualized by autoradiography (lane 1). For competition assays, the nuclear extracts were preincubated with excess of unlabeled OCT1 or mutant OCT1 oligonucleotides (OCT1: lane 2; mOCT1: lane 3). For supershift analysis, the nuclear extracts were preincubated with an OCT1 Ab (lane 4). The nuclear proteins were incubated with the mutant OCT1 oligonucleotide to test direct binding (lane 5). A control of supershift was done with OCT1 Ab and a designed OCT1 oligonucleotide (lane 6). D. EMSA for SP1 and NFY. DNA-protein complexes with SP1 and NFY oligonucleotides were visualized by autoradiography (lane 1 and lane 6, respectively). Competition assays were performed with three competitors for SP1: SP1 (lane 2), NFY (lane 3), and mutant SP1 (mSP1: lane 4). One competitor was used for NFY: SP1 (lane 7). SP1 Ab was used for supershift assays with the SP1 and NFY oligonucleotides (lane 5 and lane 8). The nuclear extracts were incubated with the mutant SP1 (mSP1: lane 9) and the mutant NFY oligonucleotides (mNFY: lane 10) to see abrogated direct binding. Sequences of the different oligonucleotides can be found in Supplemental Table IV. Error bars display ±SD. *p < 0.05, **p < 0.01. Ab, supershift with Ab.

**FIGURE 2.** Identification of basal transcription factor binding sites in the FPR2/ALX promoter region. Putative basal transcription factor binding sites for P1-414 and P2-150 were identified with the software Genomatix and Alibaba. A and C. Individual transcription factor binding sites were mutated in P1-414 (A) and P2-150 (C). For mutations introduced, see Supplemental Table II, which also delineates the order and location of the three potential OCT1 binding sites (P1-414mOCT1). Human THP-1 monocytes were transfected with the constructs and differentiated into macrophages, and luciferase activities were measured 48 h after transfection. Firefly luciferase activities were normalized to Renilla luciferase activities, and all experiments were done in triplicate. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over P1-414 and P2-150, respectively. B. EMSAs for the potential OCT1 binding site with nuclear extracts from THP-1 macrophages. The DNA-protein complex with OCT1 oligonucleotides was analyzed by gel electrophoresis and visualized by autoradiography (lane 1). For competition assays, the nuclear extracts were preincubated with excess of unlabeled OCT1 or mutant OCT1 oligonucleotides (OCT1: lane 2; mOCT1: lane 3). For supershift analysis, the nuclear extracts were preincubated with an OCT1 Ab (lane 4). The nuclear proteins were incubated with the mutant OCT1 oligonucleotide to test direct binding (lane 5). A control of supershift was done with OCT1 Ab and a designed OCT1 oligonucleotide (lane 6). D. EMSA for SP1 and NFY. DNA-protein complexes with SP1 and NFY oligonucleotides were visualized by autoradiography (lane 1 and lane 6, respectively). Competition assays were performed with three competitors for SP1: SP1 (lane 2), NFY (lane 3), and mutant SP1 (mSP1: lane 4). One competitor was used for NFY: SP1 (lane 7). SP1 Ab was used for supershift assays with the SP1 and NFY oligonucleotides (lane 5 and lane 8). The nuclear extracts were incubated with the mutant SP1 (mSP1: lane 9) and the mutant NFY oligonucleotides (mNFY: lane 10) to see abrogated direct binding. Sequences of the different oligonucleotides can be found in Supplemental Table IV. Error bars display ±SD. *p < 0.05, **p < 0.01. Ab, supershift with Ab.

FPR2/ALX mRNA is downregulated during monocyte differentiation and upregulated in macrophages by IFN-γ

In an effort to understand the role of FPR2/ALX in resident tissue macrophages during inflammation, we investigated the level of FPR2/ALX mRNA during the differentiation of primary monocytes to macrophages. Our results show that monocytes, but not 7-d-old macrophages, express FPR2/ALX mRNA (Fig. 3A). These results indicate that macrophages need external stimuli to produce FPR2/ALX. Such an upregulation of FPR2/ALX mRNA has been shown for different types of cytokines; however, whether this is true in macrophages has not been investigated. To answer this question, primary macrophages were stimulated with several pro- and anti-inflammatory cytokines known to be secreted during inflammation and with some TLR ligands (Fig. 3B). No stimulation of FPR2/ALX mRNA transcription was observed with IL-1β, IL-4, IL-13, TGF-β, and a ligand for TLR9 (CpG). However, stimulation of macrophages with IFN-γ and the TLR4 ligand LPS increased FPR2/ALX mRNA to levels similar to those in monocytes. Significant upregulation of FPR2/ALX mRNA by TNF-α and IL-6 was also observed; however, the observed mRNA levels remained low. Thus IFN-γ and LPS-stimulation, which led to classically activated M1 macrophages (50), also led to the rescue of FPR2/ALX. Such an upregulation of FPR2/ALX mRNA has been shown for different types of cytokines; however, whether this is true in macrophages has not been investigated. To answer this question, primary macrophages were stimulated with several pro- and anti-inflammatory cytokines known to be secreted during inflammation and with some TLR ligands (Fig. 3B). No stimulation of FPR2/ALX mRNA transcription was observed with IL-1β, IL-4, IL-13, TGF-β, and a ligand for TLR9 (CpG). However, stimulation of macrophages with IFN-γ and the TLR4 ligand LPS increased FPR2/ALX mRNA to levels similar to those in monocytes. Significant upregulation of FPR2/ALX mRNA by TNF-α and IL-6 was also observed; however, the observed mRNA levels remained low. Thus IFN-γ and LPS-stimulation, which led to classically activated M1 macrophages (50), also led to the rescue of FPR2/ALX. Such an upregulation of FPR2/ALX mRNA has been shown for different types of cytokines; however, whether this is true in macrophages has not been investigated.
FIGURE 3. Regulation of FPR2/ALX mRNA expression. FPR2/ALX mRNA was determined by real-time RT-PCR in primary monocytes and macrophages. A, Monocytes extracted from buffy coat (day 0) were allowed to differentiate into macrophages for 1, 3, 6, and 7 d. FPR2/ALX mRNA values were normalized to GAPDH mRNA levels, and results represent fold induction compared with monocyte expression levels at day 0. B, Total RNA was extracted from 7-d-old macrophages stimulated for 24 h with the mentioned cytokines and TLR ligands (TNF-α: 1 ng/ml; IL-1β: 1 ng/ml; IFN-γ: 50 ng/ml; IL-6: 10 ng/ml; IL-4: 10 ng/ml; IL-13: 10 ng/ml; TGF-β: 1 ng/ml; LPS: 100 ng/ml; CpG: 100 ng/ml). Values represent fold induction compared with nonstimulated cells. C, Ratio of FPR2/ALX over GAPDH mRNA expression levels of cells stimulated with different concentrations of IFN-γ (-, no stimulation; 10 ng/ml; 25 ng/ml; 50 ng/ml). Error bars display ±SD; n = 3. *p < 0.05, **p < 0.01.

INF-γ increases transcriptional activity of the first promoter via an IRSE

To locate the IFN-γ response element in the FPR2/ALX promoter, THP-1 macrophages were subjected to 24-h stimulation with IFN-γ after electroporation of the different constructs of promoters P1 and P2. As seen in Fig. 4A, IFN-γ stimulation resulted in a 1.5-fold (±0.6) increase in luciferase expression for the first promoter P1, compared with nonstimulated cells, whereas IFN-γ had no stimulatory effect on the second promoter P2. These results suggest that P1 contains transcriptional elements, which promote the IFN-γ-dependent stimulation of the FPR2/ALX gene. MatInspector located two putative binding sites for IRF4 and IRF7 within this region. These transcription factors have already been shown to induce transcription upon stimulation with IFNs (51, 52). To investigate whether one or both of these transcription factor binding sites are necessary for the IFN-γ response of the FPR2/ALX gene, their core binding sites were mutated on the shortest P1-414 luciferase construct (Supplemental Table II). Site-directed mutagenesis of the core binding site for IRF4 resulted in a 48% decrease in basal transcriptional activity, compared with that in the wild-type construct, whereas site-directed mutagenesis of IRF7 did not alter the promoter activity (Fig. 4B). In addition, when cells were stimulated with IFN-γ, the construct containing the mutant IRF4 binding site showed decreased transcription even compared with nonstimulated cells (p = 0.038) (Fig. 4C). Taken together, these results indicate that the transcription factor binding site for IRF4 is important for basal and IFN-γ–induced transcription of FPR2/ALX. EMSA confirmed the binding of proteins from nuclear extracts from nonstimulated (Fig. 4D, lane 1) and, even more, from IFN-γ–stimulated macrophages (Fig. 4D, lane 2) to the IRF4 oligonucleotide. Of interest, stimulation of macrophages with IFN-γ changed the size of DNA-protein complexes, indicating differential regulation for basal and induced transcription. This binding could be competed with unlabeled IRF4 oligonucleotide (Fig. 4D, lane 3, IRF4), but less so with a mutant version (Fig. 4D, lane 4, mIRF4), supporting a role for this transcription factor binding site in IFN-γ stimulation. However, incubation of the IRF4 oligonucleotide with nuclear extracts in the presence of an IRF4 Ab did not result in a supershift of the protein-DNA complex (Fig 4D, lane 7), arguing against an involvement of IRF4 in IFN-γ stimulation of the FPR2/ALX gene. Because members of the IRF family of transcription factors bind to IRSE (53) and the IRF4 site also matches this IRSE consensus sequence, we probed the protein-DNA complexes with several Abs against transcription factors of the IRF family. As can be seen in Fig. 4D, only incubation with the IRF1 Ab resulted in a supershift of the protein-DNA complexes (lane 10). In summary, these results indicate that IFN-γ stimulates transcription of the FPR2/ALX gene via IRF1 binding to the ISRE located in the first promoter P1.

FPR2/ALX protein is expressed in monocytes, but not in macrophages, after IFN-γ stimulation

To investigate whether mRNA expression during monocyte differentiation correlates with FPR2/ALX protein expression and function, we studied FPR2/ALX cell surface expression by FACS analysis and the receptor function by chemotaxis assays. As expected from the mRNA expression pattern, monocytes expressed the FPR2/ALX protein on their cell surface (Fig. 5A), whereas 9-d-old macrophages did not (Fig. 5B). To determine whether this loss of FPR2/ALX expression has functional consequences, we investigated the transmigration of monocytes and macrophages toward the FPR2/ALX ligand LXA₄ (18) (Fig. 6). Although both monocytes and macrophages migrated toward the FPR1 ligand fMLF, only monocytes migrated toward the FPR2/ALX ligand LXA₄. This inability of LXA₄ to stimulate macrophage chemotaxis and the lack of FPR2/ALX protein expression on macrophages argue for a loss of functional FPR2/ALX receptor in differentiated macrophages.

To determine whether the substantial increase in FPR2/ALX mRNA upon IFN-γ stimulation of 7-d-old macrophages translates into de novo FPR2/ALX membrane expression, we analyzed FPR2/ALX cell surface expression on 9-d-old M1 macrophages following IFN-γ stimulation. No membrane expression of FPR2/ALX was observed between 2 and 72 h following IFN-γ or LPS stimulation, although the mRNA was increased >80-fold after 24
IFN-γ redistribution of FPR2/ALX from the cell surface.

To investigate whether differential promoter usage could explain the translational repression of FPR2/ALX mRNA following IFN-γ stimulation, we quantified the different-length mRNAs from the two promoters in primary monocytes, macrophages, and IFN-γ–stimulated M1 macrophages further increased FPR2/ALX gene transcription from promoter P1 (100 ± 19%), corroborating our localization of the IFN-γ–responsive IRF4 binding site in this first promoter P1. Hence, a difference in promoter use can be noted between neutrophils and monocytes expressing FPR2/ALX protein on their cell surface and the IFN-γ–stimulated M1 macrophages not expressing the protein.

The longer 5′-UTR in the mRNAs from promoter P1 reduces translation of the FPR2/ALX protein

The mRNAs derived from the two promoters of the FPR2/ALX gene differ in the longer 5′-UTR region present in the mRNA from promoter P1 and in a differential splicing of exon 2 observed in the mRNA from promoter P2 (Fig. 7B). To investigate whether the longer 5′-UTR region or whether exon 2 of FPR2/ALX mRNA influences translation of the mRNA, we subcloned all 5′-UTRs of the observed FPR2/ALX mRNAs into the 5′-UTR of the luciferase gene and measured the translation of the luciferase protein in THP-1 macrophages (Fig. 7C). Luciferase activity was similar in the control plasmid (SV40) and in the construct transcribing the shorter 5′-UTR deriving from promoter P2 (SV40-P2). However, the presence of the longer 5′-UTR deriving from promoter P1

h (Fig. 5C) and no chemotaxis was observed for IFN-γ–stimulated macrophages to LXA4 (data not shown). To dissect whether this posttranscriptional regulation of FPR2/ALX expression resulted from an inhibition of translation or from a redistribution of the FPR2/ALX receptor from the membrane to an intracellular compartment, we investigated intracellular localization of the FPR2/ALX receptor from the membrane to an intracellular complex to ISRE of the IRF4 oligonucleotide; OCT1, binding of protein complexes to OCT1 of the IRF4 oligonucleotide.

FIGURE 4. Location of the IFN-γ response element. A, THP-1 monocytes were transfected with the different constructs of promoter P1 and P2 and differentiated into macrophages for 2 d. Cells were further stimulated with IFN-γ for 24 h, and luciferase activities were measured and normalized to the activity of the Renilla luciferase. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction of the respective construct compared with nonstimulated cells. B and C, Site-directed mutagenesis of the putative IFN response elements IRF4 and IRF7 in construct P1-414 (for mutations, see Supplemental Table II). Luciferase activity was measured 48 h after transfection (B) and following 24 h stimulation with IFN-γ (C). Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over the P1-414 construct (B) or over the respective construct in nonstimulated cells (C). D, EMSA for IRF4 with the nuclear extracts from THP-1 macrophages (lane 1) and THP-1 macrophages stimulated for 24 h with IFN-γ (lane 2). For competition assays, the stimulated nuclear extracts were preincubated with excess of unlabeled IRF4 (lane 3) or mutant IRF4 oligonucleotides (mIRF4: lane 4). As the binding site overlaps with a putative OCT1 transcription factor binding site, nuclear extracts from nonstimulated cells were preincubated with an excess of unlabeled designed OCT1 oligonucleotide (lane 5). The nuclear extracts from stimulated macrophages were incubated with the mutant IRF4 oligonucleotide to test direct binding (lane 6). For supershift assays, IRF4, ISBCP, OCT1, and IRF1 Abs were preincubated with the IRF4 oligonucleotide (lanes 7–10). Sequences of oligonucleotides can be found in Supplemental Table IV. Error bars display ±SD. *p < 0.05, **p < 0.01. Ab, supershift of the ISRE oligonucleotide-protein complexes with Ab; ISRE, binding of protein complexes to ISRE of the IRF4 oligonucleotide; OCT1, binding of protein complexes to OCT1 of the IRF4 oligonucleotide.

IFN-γ stimulation of macrophages increases transcription from promoter P1 in primary macrophages

To investigate whether differential promoter usage could explain the translational silencing of FPR2/ALX expression and against the mRNA from promoter P2 (79 ± 4%) and, to a lesser extent, from promoter P1 (21 ± 4%). In contrast, macrophages transcribed the FPR2/ALX gene from promoter P1 (100 ± 20%). IFN-γ stimulation of macrophages further increased FPR2/ALX gene transcription from promoter P1 (100 ± 19%), corroborating our localization of the IFN-γ–responsive IRF4 binding site in this first promoter P1. Hence, a difference in promoter use can be noted between neutrophils and monocytes expressing FPR2/ALX protein on their cell surface and the IFN-γ–stimulated M1 macrophages not expressing the protein.

The longer 5′-UTR in the mRNAs from promoter P1 reduces translation of the FPR2/ALX protein

The mRNAs derived from the two promoters of the FPR2/ALX gene differ in the longer 5′-UTR region present in the mRNA from promoter P1 and in a differential splicing of exon 2 observed in the mRNA from promoter P2 (Fig. 7B). To investigate whether the longer 5′-UTR region or whether exon 2 of FPR2/ALX mRNA influences translation of the mRNA, we subcloned all 5′-UTRs of the observed FPR2/ALX mRNAs into the 5′-UTR of the luciferase gene and measured the translation of the luciferase protein in THP-1 macrophages (Fig. 7C). Luciferase activity was similar in the control plasmid (SV40) and in the construct transcribing the shorter 5′-UTR deriving from promoter P2 (SV40-P2). However, the presence of the longer 5′-UTR deriving from promoter P1
(SV40-P1) led to an almost 50% reduction in translation of the luciferase protein (53 ± 22%). Similarly, the presence of exon 2 (SV40-P2+Ex2), which was observed in only a minor splice variant of FPR2/ALX mRNA from promoter P2, led to a drastically reduced translation of the luciferase protein. These results indicate that the preference of macrophages for promoter P1 could at least partially explain the lack of expression of FPR2/ALX in IFN-γ-stimulated M1 macrophages.

Discussion

We identified and characterized two promoter regions in the FPR2/ALX gene separated by 224 bp, which drive FPR2/ALX mRNA expression in monocytes and macrophages. We also showed that transcription of the FPR2/ALX gene from these promoters decreases during differentiation of primary human monocytes to macrophages and that the low transcription in macrophages can be rescued by IFN-γ and LPS stimulation, leading to classically activated M1 macrophages. On the molecular level, the transcription factors OCT1 and SP1 are involved in the initiation of transcription in the TATA-less promoters 1 and 2 of the FPR2/ALX gene, respectively. We have further located the IFN-γ response element to promoter 1 and have shown that the transcription factor IRF1 binds an IRES binding site in promoter 1 for IFN-γ stimulation of FPR2/ALX mRNA. Intriguingly, the increased FPR2/ALX mRNA transcription following IFN-γ stimulation does not translate into functional receptor expression on the cell surface of M1 macrophages. This lack of translation is caused by the preferential usage of promoter P1 in macrophages, resulting in an mRNA with a longer 5′-UTR having reduced translation efficiency. In contrast, monocytes transcribe FPR2/ALX mRNA from promoter P2 and express the functional receptor on their cell surface.

Two distinct promoters are located in the FPR2/ALX gene, which result in alternative 5′-UTRs of the FPR2/ALX mRNA in monocytes and macrophages. Both promoters lack the TATA box at position −25 but have basal transcription factor binding sites present, which were previously shown to initiate transcription of TATA-less promoters. The promoter P1 contains an OCT1 binding site, which plays a critical role in the preinitiation complex recruitment of promoters that lack TATA boxes. For instance, OCT1 was able to replace TBP via its interaction with the general transcription factor.
TFIIB for transcription of the lipoprotein lipase gene (54). Similarly, the promoter P2 contains a basal transcription factor binding site for SP1, which is known to bind CpG islands and also to drive gene transcription of TATA-less promoters (55). This promoter P2 is preferentially used for basal transcription in neutrophils and monocytes expressing the FPR2/ALX receptor on their cell surface, whereas macrophages use only the first promoter P1.

Promoter P1 is responsible for low basal transcription in macrophages and is responsive to IFN-γ stimulation. The signaling pathway of IFN-γ stimulation involves either the activation of the JAK/STAT pathway or an increase in transcription of IRFs (56), both of which lead to enhanced transcription of downstream genes. In this article, we show that IRF1 supports the stimulated FPR2/ALX expression through binding to an IRSE binding site (57) in the first promoter of the FPR2/ALX gene. IRF1 is one of the highest induced members of the IRF family and was shown to be highly upregulated by IFN-γ in THP-1 monocytes (58). This upregulation resulted in a strong increase in IRF1 binding to the IRSE element in the β2-microglobulin promoter (58), similar to the binding shown in our EMSA experiment with the IRSE element in the first promoter P1 of the FPR2/ALX gene. In line with these results, we mainly observed FPR2/ALX mRNA from promoter P1 in IFN-γ–stimulated primary macrophages. Although monocytes preferentially transcribe the FPR2/ALX gene from promoter P2, stimulation of macrophages with IFN-γ and LPS (data not shown) results in transcription from promoter P1. This alternate mRNA has a longer 5′-UTR.
LXA₄ was shown to recruit monocytes to the site of inflammation (68). In addition, LXA₄ has recently been demonstrated to modulate a direct effect of LXA₄ not only on monocytes but macrophages, which support tissue homeostasis (66). These findings show that macrophages do not express the FPR2/ALX receptor. In contrast, treatment of monocytes with the FPR2/ALX ligand shows that macrophages do not express the FPR2/ALX receptor. Alternatively, LXA₄-mediated chemotaxis could prime monocytes through the FPR2/ALX receptor to polarize into M2 macrophages instead of polarizing into resident macrophages without stimulation. Indeed, it appears from the macrophage cell line U937 that LXA₄ signaling leads to M2 polarization of these macrophages through FPR2/ALX (72); however, direct experimental evidence for the priming of primary monocytes by LXA₄, leading to polarized M2 macrophages, is currently lacking.

In summary, we have characterized the regulatory sequences and some components of the transcription machinery that drive expression of the FPR2/ALX gene in monocytes and macrophages. Our data also suggest that FPR2/ALX mRNA is expressed in monocytes, but not in macrophages. This finding supports the model in which FPR2/ALX is a chemotactic receptor in monocytes but has a limited role in resident tissue macrophages.

Disclosures
The authors have no financial conflicts of interest.

References


microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. *Genome Res.* 19: 1175–1183.


