Transcription Factor SP2 Enhanced the Expression of Cd14 in Colitis-Susceptible C3H/HeJ Bir

Nils-Holger Zschemisch, Inga Brüsch, Anne-Sophie Hambusch, André Bleich *

Institute for Laboratory Animal Science and Central Animal Facility, Hannover Medical School, Hannover, Germany

* bleich.andre@mh-hannover.de

Abstract

Genetic analysis in the IL10-deficient mouse model revealed a modifier locus of experimental inflammatory bowel disease (IBD) on chromosome 18, with the allele of the strain C3H/HeJ Bir (C3Bir) conferring resistance and the allele of C57BL/6J (B6) conferring susceptibility. Differential Cd14 expression was associated with this background specific susceptibility to intestinal inflammation. Polymorphisms of the Cd14 promoter were found to be likely causative for strain specific expression, and Cd14-knockout mice revealed a protective role of this gene-product in experimental IBD. In this study, luciferase reporter assays confirmed an increased activity of the C3Bir derived Cd14 promoter compared to the one of B6. Promoter truncation experiments and site-directed mutagenesis in both strains resulted in reduced Cd14 promoter activity and confirmed that a central AP1 and the proximal SP1 transcription factor binding sites mediated the basal activity of the Cd14 promoter in the mouse. Moreover, a T to C exchange at position -259 replaced putative STAT1 and CDX1 sites in the Cd14 promoter from B6 by a SP2 site in C3Bir. Ablation of the Sp2 site through truncation was associated with a decreased promoter activity. Site-directed mutagenesis also demonstrated that the inactivation of SP2 led to a substantial loss of promoter activity in C3Bir. Performing electrophoretic mobility shift and supershift assays demonstrated interaction of SP2 with its potential binding site. In addition, retroviral—mediated overexpression of the SP2 transcription factor in primary bone marrow macrophages derived from C3Bir mice caused a significant increase in Cd14 transcription. These data characterized SP2 as important factor responsible for higher Cd14 expression and reduced IBD susceptibility mediated by the C3Bir allele.

Introduction

CD14 was identified as a marker and modifier gene in human IBD [1–3] and found to be associated to experimental IBD in the IL10-deficient mouse model [4–7]. CD14 is synthesized in a soluble (sCD14) and a membrane bound (mCD14) form. mCD14 is expressed on mature...
macrophages and bound to the cell membrane by a glycosylphosphatidyl-inositol (GPI) anchor [8]. Together with lymphocyte antigen 96 and toll-like receptor 4 (TLR4) mCD14 forms a receptor for lipopolysaccharides (LPS); sCD14 circulates as a plasma protein due to a missing GPI anchor which is either not synthesized or it is post-translational detached by matrix metalloproteinase [9,10]. sCD14 competes with the membrane bound form for LPS and has an inhibiting effect on the activation of the complement system [11]. Less is known about the molecular mechanisms controlling expression and post-translational editing of soluble or membrane bound CD14. Landmann and co-workers demonstrated that Interferon-γ and IL4 down-regulate sCD14 expression in human monocytes and macrophages in vitro [12]. Synthesis of mCD14 and TLR4 is increased in patients with IBD and causes an enhanced reactivity to microbiota of the gut compared to healthy persons [13,14]. Regulatory mechanisms of CD14 expression were studied in mouse, rat and man. Methylation is increased in the CD14 promoter during childhood, inversely corresponding with reduced sCD14 levels [15]. Furthermore, promoter elements in the 5′UTR and flanking region determine molecular mechanisms controlling CD14 expression [4,16–18]. Several AP1 binding site were identified in the CD14 promoter in cow, mouse, rat and man mediating basal transcription activity [16–19]. SP1 regulatory elements found in the distal part of rat and human CD14 promoter also contribute to basal promoter activity, but SP1 sites closely adjacent to the transcription start are responsible for reduced CD14 transcription [17,18]. Furthermore, single nucleotide polymorphisms (SNP) modulated CD14 activity in cow [19–21]. Human CD14 promoter polymorphisms were involved in sensitization to allergens [22], atopic dermatitis [23], cardio-vascular diseases [24–27], tuberculosis and HIV infection [28–30], acute diarrhoea [31] and IBD [32–34]. Particularly the tantamount polymorphism C-260T/C-159T was found in patients with Crohn’s disease and ulcerative colitis [32,33,35–37].

We have identified Cd14 as a major candidate gene for experimental IBD in mice by performing genome wide linkage analysis and subsequent microarray studies [5]. For these analyses interleukin-10 deficient (Il10tmCgn, Il10-/−) mice were used that developed intestinal inflammation spontaneously in a microbiome dependent manner. In this model system, expression of disease depended on the mouse background strain. Quantitative trait locus analysis revealed at least ten loci that contributed to IBD susceptibility in Il10−/− mice on a C3Bir vs B6 background. A B6-derived susceptibility locus (Cdc56) determined low intestinal expression of CD14, while the C3Bir allele of Cd14 encoded high gene expression. In silico analysis of the Cd14 promoter in B6 as well as C3Bir demonstrated differences in transcription factor binding site equipment and co-location. Polymorphisms introduced STAT1 and SP2 binding sites in the Cd14 promoter of C3Bir mice while in B6 animals BCL6 and PPARγ elements were additionally present [4]. Therefore, the aim of this study was to identify regulatory factors that are associated with Cd14 promoter polymorphisms and thereby causing both strain specific Cd14 expression and disease susceptibility. Protective factors may serve as targets for prospective novel therapeutic approaches for IBD.

Materials and Methods
Identification of transcription factor binding sites

The Cd14 promoter sequence from -1067 to +199 in B6 and -1067 to +203 in C3Bir as well as the human Cd14 promoter (GenBank: X74984 and U00699) were analyzed to identify putative transcription factor binding sites using the MatInspector Software (Genomatix Software GmbH, Munich, Germany).
Cell culture
RAW264.7 cells (ATCC: TIB-71, murine macrophages) were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Darmstadt, Germany), supplemented with 10% FCS (PAA, Pasching, Austria) and 5% Penicillin/Streptomycin (Biochrom, Berlin, Germany). When cells reached a confluence of 70% they were scraped and split 1:4. NIH3T3 cells (ATCC: CRL-1658, murine embryonic fibroblasts) were grown in DMEM with GlutaMax™ (Life Technologies), supplemented with 10% FCS (PAA) and 5% Penicillin/Streptomycin. Every third day or at reaching a confluence of 50% cells were split 1:10. L929 cells (murine fibroblasts derived from C3H/An mice) were seeded at 5 x 10⁵ on 75 cm² cell culture flasks and cultured in 55 ml DMEM (high glucose, GlutaMAX™ Supplement, pyruvate) supplemented with 10% FCS and 5% Penicillin/Streptomycin for 10 days [38]. Medium was harvested, filtered through a 45μm filter system and transferred to 50 ml tubes. L929 conditioned medium was stored at -20°C.

Isolation of primary bone marrow macrophages (BMM)
Bone marrow of tibia and femur from 6 C3Bir mice were flushed out using DMEM. The bone marrow suspension were filtered through a 70μm cell strainer and cells were centrifuged at 1000 rpm for 10 minutes at 4°C. The bone marrow cells were resuspended in BMM medium (DMEM, high glucose, GlutaMAX™ Supplement, pyruvate, Gibco 10569010) and seeded on a dozen 6 well plates. After four hours of culture BMM became adherent and medium was changed [38]. All cells were incubated with 5% CO₂ at 37°C.

Production of Moloney Murine Leukemia Virus (MMULV)-based retroviruses
6 x 10⁶ amphotrophic Phoenix-Ampho retrovirus producer cells were seeded on 10 cm cell culture dishes in DMEM (high glucose, GlutaMAX™ Supplement, pyruvate, Gibco 10569010) and grown overnight to a confluence of 80–90%. 32μg of the plasmid pBABE puro-Sp2 encoding for the murine SP2 transcription factor [39] or of the empty backbone vector pBABE puro were transiently transfected using 50 μl of Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. 24 and 48 hours after transfection the cell culture supernatant were harvested, centrifuged at 1200 rpm for 10 min at 4°C and the retroviral suspension was stored at -80°C.

Retroviral transduction
After 4 days of BMM culture the medium was changed to 3 ml fresh BMM medium. Cells were transduced with 200 μl retroviral suspension and 6μg/ml Polybrene (Merck Millipore, Billerica, MA) and centrifuged for 1 hour at 200xg at room temperature. After transduction infected BMMs and untreated control cells were cultured for additional 48 hours.

Quantitative Real-time gene expression analysis
After RNA isolation from BMM with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) 500 ng of total RNA were reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) following the supplier’s recommendations. 1 μl of the cDNA, the TaqMan Fast Advanced 2x Master Mix (Applied Biosystems, Fisher City, CA, USA) and a Cd14 TaqMan Gene Expression Assay (Mm00624086_cn, Applied Biosystems) were used to determine Cd14 expression with the StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt,
Germany). The expression of Actb as endogenous control was measured using a TaqMan Gene Expression Assay (Mm00607939_s1, Applied Biosystems). Results were statistically analysed performing unpaired t-test with the Prizm 5 software (GraphPad Software, LaJolla, CA). Significance levels were set to $p < 0.05$.

**Protein extraction and western blotting**

The two cell lines were tested for their basal Cd14 expression and expression after stimulation with LPS (0.1 ng/ml, Sigma-Aldrich, Munich, Germany) for 12 hours. Proteins were isolated from the cells by using NP40 buffer (1 mM PMSF, 1 mM DDT, 1 mM sodium ortho-vanadate, Complete Mini Protease inhibitor cocktail cocktail [Roche Applied Science, Mannheim, Germany]) and concentrations were defined by performing Bradford assays. 50 μg protein were used for SDS-PAGE followed by a semi-dry transfer to Optitran BA-S 85 membrane (Sigma-Aldrich, Munich, Germany) using Semi-Dry Blotter Maxi V20-SDB (Carl Roth, Karlsruhe, Germany) and transfer buffer (39 mM glycine, 0.037% SDS, 48 mM Tris, 20% methanol). Primary antibodies were applied to bind to CD14 (rat monoclonal [Sa14-2] to CD14 [FITC], Abcam, Cambridge, UK) or to beta actin (mouse monoclonal [mAbcam 8226] to beta Actin, Abcam). After labeling CD14 antibody with goat anti rat IgG [HRP] (AP136P; Millipore, Billerica, USA) and beta actin with F(ab')2 rabbit anti mouse IgG [HRP] (STAR21B; AbD serotec, Oxford, UK) as secondary antibodies, luminescence of the different bands was activated by using Roti-Lumin 1 and 2 solution (Carl Roth). Detection of the bands was performed by x-ray film (GE Healthcare, Munich, Germany)

**Cd14 promoter truncations**

Promoter truncations were prepared by PCR using Phusion Hot Start Polymerase and 5xHF-Buffer as preset by the manufacturer (NEB, Ipswich, UK), utilizing primers and annealing temperatures depicted in Supporting Information (S1 Table). Linearized pGL4.17 plasmid (Promega, Wisconsin, USA) containing Cd14 full-length promoters of C3Bir respectively B6 were served as templates [4]. For quality control the promoter fragments of C3Bir were flanked by KpnI and BglII,while B6 elements were flanked by Xhol and BglIII restriction sites. Cloning of the truncated promoter elements was performed using StrataClone PCR Cloning Kit (Agilent, San Diego, CA) according to the suppliers recommendations. The promoter sequences were subsequently excised from pSC-A amp/kan with Xhol/BglIII or KpnI/BglIII (NEB) and inserted into Xhol/BglIII or KpnI/BglIII digested luciferase reporter plasmid pGL4.17.

**Site-directed mutagenesis**

Mutagenesis of both full-length promoters in pGL4.17 was performed using Phusion Site-Directed Mutagenesis Kit (Biozym, Hessisch Oldendorf, Germany) according to the manufacturers recommendations with minor modifications. Therefore short base pair exchanges or insertion of Ascl or Pmel restriction endonuclease recognition site were introduced into the core sequence of the putative restriction factor binding sites. Primer sequences are depicted in Supporting Information (S2 and S3 Tables). After digestion with the methylation sensitive DpnI (NEB) to eliminate methylated templates, PCR products were ligated and transformed into XL10-Gold Kan multicompetent cells (Agilent).

**Sequencing**

Amplification products were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Invitrogen, Darmstadt, Germany) with T7, T3 or Cd14 specific primers for 25 cycles (96°C
for 30 s, 60°C for 4 min). Reaction clean up was performed using innu-PREP-DYEpure-Kit (Analytic Jena, Jena, Germany) and sequencing was run at ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Alternatively samples were sent to MWG Eurofins Operon (Ebersberg, Germany).

Transfection efficiency test

XtremeGene HP DNA, XtremeGene 9 DNA (Roche Applied Science, Mannheim, Germany), Superfect and Polyfect Transfection Reagents (Qiagen, Venlo, Netherlands) were tested to achieve the highest efficiency by co-transfection of 2.5 μg pAdTrack-CMV and 2.5 μg plasmids CMV-β-Gal following the manufacturer’s instructions into RAW264.7 cells. 24 h and 48 h after transient transfection RAW264.7 cells were harvested using extraction buffer to determine transfection efficiency with a β-galactosidase assay [4].

Cd14 promoter activity

RAW264.7 cells were co-transfected with 4 μg promoter constructs containing pGL4.17 luciferase reporter plasmid and 1 μg CMV-β-Gal plasmid using Polyfect Transfection Reagent. Promoter activity and transfections efficiency were evaluated by performing luciferase and β-galactosidase assays on harvested cell extracts as described earlier [4]; luminescence was measured using 2030 Multilabel Reader Victor X3 (Perkin Elmer, Waltham, MA). Results were statistically analysed performing One-way Anova with Bonferroni’s Multiple Comparison Test with the Prizm 5 software (GraphPad Software, LaJolla, CA). Significance levels were set to p < 0.05.

Electrophoretic mobility shift assay (EMSA)

Physical interaction between a transcription factor and their putative binding site were confirmed performing EMSA and Supershift Assay using the Gelshift Chemiluminescent EMSA Kit (Active Motif, Rixensart, Belgium). Nuclear extracts (NE) were isolated from RAW264.7 cells using the Nuclear Extract Kit (Active Motif) following the suppliers’ recommendations. 50 μg of either the biotinylated Sp2 forward (5’ - GTTTTTCCGTCCCTCCCTAAACACT-3’) and reverse (5’ - AGTGTT TTTTAGGGAGGGACGAAATTG-3’) consensus oligonucleotides were dissolved in 10 x annealing buffer (200 mM Tris, pH 7.6; 100 mM MgCl2; 500 mM NaCl) with Tris-EDTA buffer, ph 7.6 and heated for 5 min at 70°C. The double stranded oligonucleotide then cooled down to room temperature over night inside the heating block together with the unlabeled Sp2 oligonucleotides. For the binding reaction of the transcription factor to the consensus oligonucleotides 10 μg NE was incubated with 20 fmol of the biotinylated consensus oligonucleotide and for competitive binding with additional 2–8 pmol unlabeled consensus oligonucleotide in the reaction buffer (1x binding buffer, 2.5% glycerol, 50 ng/μl Poly d(I-C), 0.05% NP-40) for 20 min at room temperature. The Supershift Assay was performed by a pre-incubation of the NE with 2 μg of antibodies (Santa Cruz Biotechnology) either against SP1 (H-225; sc-14027 X), SP2 (K-20; sc-643 X) or SP3 (D-20; sc-644 X) for 30 min on ice. Protein DNA complexes were separated electrophoretically in a 6% bisacrylamide TBE mini gel for 1 hour in 0.5% TBE buffer at 100 V followed by a semi-dry transfer (Semi-dry Blotter Model I; Phase, Lübeck, Germany) with 0.5% TBE buffer at 10 V for 15 min to the Hybond-N+ membrane (GE Healthcare, Little Chalfat, UK). After UV-crosslinking of the membrane at 312 nm for 15 min the detection of the mobility shifts were performed following the suppliers’ recommendations.
Results
Optimization of Cd14 promoter analysis system

To establish a reproducible and efficient transfection system, a suitable cell line as well as an appropriate transfection reagent had to be identified. Western blot analysis revealed a considerably higher basal CD14 expression in RAW264.7 than in NIH3T3 cells (Fig 1A). LPS stimulation did not show any effect on CD14 expression in NIH3T3 in contrast to the increased CD14 protein concentration in RAW264.7 cells. In addition, various chemical transfection methods were tested on RAW264.7 cells using the plasmids pAd-Track-CMV and pCMV-βGal to ensure reproducible high transient transfection efficiencies. GFP fluorescence and β-galactosidase assay revealed a significant lower expression level using XtremeGene 9 DNA (Fig 1C and 1F) after 24h and 48h after transfection compared to Superfect, Polyfect or XtremeGene HP transfection reagents achieving an high equal expression level (Fig 1B, 1D, 1E and 1F). Therefore, Superfect transfection reagent was chosen as tool for transient transfection of reporter plasmids into RAW264.7 cells.

SNPs changed the patterns of putative transcription factor binding sites

In promoter sequences of C3Bir and B6, identical recognitions sites for AP1 (-417 to -407, -367 to -356, +3 to +14) and SP1 (+141 to +156) were identified by bioinformational analysis of putative transcription factor binding sites (Fig 2). However, polymorphisms in the Cd14 promoter sequences induced strain specific recognition sequences in C3Bir and B6 (Fig 2). We identified a cluster of alternative sites for ATF2 (-1055 to -1038), BRN4 (-1044 to -1026), GSH1 (-1041 to -1025), CUX1 (-1040 to -1022) LMX1B (-1040 to -1026) and BARX2 (-1039 to -1022) caused by the exchanges from T to C at position -1041 and from G to A at position -1034 in the C3Bir allele, while in the B6 allele a binding site for OCT1 was found covering the sequence -1044 to -1030 (Fig 2). The C to T substitution at position -876 introduced a PPARγ site (-878 to -856) in the C3Bir allele instead of binding motifs for NMP4 (-885 to -875) and PAX6 (-885 to -867) found in the Cd14 promoter of B6. Additionally, a LHX3 site (-824 to -810) in the B6 Cd14 promoter was replaced in the C3Bir allele by an OCT6 (-835 to -823) site and STAT1 sites (-833 to -815; -832 to -830) caused by an A to G exchange. The loss of E2F (-601 to -585) and NRF2 (-601-581) found in the B6 allele caused by a C-596T polymorphism at position was compensated by the introduction of OCT1 (-606 to -592) and EVI-1 (-604 to -588) sites in the C3Bir allele of Cd14. The C-360T polymorphism introduced binding sites for PPARγ homodimers (-376 to -854), PPARγ-RXR heterodimers (-366 to -344), and NRC2c family members (-366 to -345) while the G-328T polymorphism generated an additional BRN5 site (-329 to -311) in the B6 allele. A cluster of alternative transcription factor binding motifs was found in the C3Bir allele containing PAX3 (-268 to -250), PAX1 (-267 to -249), SP2 (-244 to -229), and ELF2 (-255 to -235) caused by T to C and G to C substitutions at positions -260 and -244, respectively. However, in the complementary region of the Cd14 promoter from B6 unique binding site for STAT1 (-266 to -248), CDX1 (-264 to -246), and BCL6 (-263 to -247) (Fig 2) were identified. Upstream of the modifications the C-75T polymorphism introduced a PAX2 binding motif (-75 to -53) in the B6 allele, while the C-49T exchange led to a MOK2 binding site (-50 to -30) in B6 eliminating the E2F site (-51 to -35) found in the C3Bir allele (Fig 2) [4].

Truncations of the Cd14 promoter

To figure out which binding sites were associated with the strain specific Cd14 expression, the full-length promoter constructs of both strains were truncated to map regions essential for transcription regulation.
Fig 1. Transfection system establishment. A Western blot analysis of CD14 expression in untreated and LPS stimulated murine RAW264.7 macrophages and NIH/3T3 fibroblasts, β-actin as endogenous control. Transfection efficiency test by determination of GFP-positive RAW264.7 cells by cyto fluorescence 24 hours after transfection with B Xtreme Gene HP, C Xtreme Gene 9 DNA, D Superfect, E Polyfect, and F by β-galactosidase assay, ***: p < 0.05.

doi:10.1371/journal.pone.0155821.g001
Luciferase reporter assay showed that 5′ truncation of the B6 -1067/+199 full-length promoter of Cd14 to -722/+199 increased promoter activity significantly sixfold to more than 3 x $10^5$ associated with the loss of OCT1 binding sites. Therefore, the distal part of the promoter contained a stimulating element. Further truncation to -844/+199 did not change the reporter expression (Fig 3A). Cutting off the region up to -474/+199 in the next steps reduced the luciferase transcription to the level of the full-length promoter. These results suggested that the sequence between -977 and -474 containing an NRF2 binding site has a stimulatory impact (Fig 3A). Continuing truncation to -385/+199 led to a reconstitution of the high reporter expression as detected in the -977/+199 and -844/+199 promoter constructs. This result was associated with the loss of the distal AP1 binding site while the removal of the PPARγ and proximal AP1 sites by reduction of the promoter to -298/+199 obtained no significant change in the reporter activity. After ablation of the -298 to -181 part of the promoter containing STAT1 and CDX1 the luciferase expression dropped again to 60177 counts (SD ± 11309) demonstrating the loss of an activating transcription factor binding site between -298 and -181. Further truncation to -52/+199 and +85/+199 induced only weak expression decreases compared to the -181/+199 promoter fragment (Fig 3A).

-1067/+203 full-length Cd14 promoter showed a higher activity in the luciferase assay as reported previously [4]. In C3Bir the truncations to -722/+203 bp deprived of CUX1, PPARγ and STAT1 binding sites and to -474/+203 depleting an OCT1 binding motif resulted in a stepwise but not significant reduction of reporter activity (Fig 3B). Like in the B6 Cd14 promoter allele the loss of the distal AP1 binding site by reduction to -385/+203 led to a strong increase of the reporter expression. The ablation of the proximal AP1 by cutting off the region between -385 and -298 did not change the luciferase activity significantly as seen in the B6 allele (Fig 3B). Further truncation to -181/+203 associated with the loss of the SP2 recognition motif led to strong decrease in reporter activity compared the the -385/+203 and -298/+203 constructs. Reduction of the C3Bir allele of the Cd14 promoter to -52/+203 resulted again in a strong increase of the luciferase activity suggesting an activation element between -181 and -52 while the deletion of the AP1 site at the 3′ end shutted down the luciferase expression (Fig 3B).

These results implicated inhibiting functions of the distal OCT1 binding site in Cd14 promoter of B6 and strong activating properties of the STAT1/CDX1 binding motif located between -298 and -181. The depletion of the region between -474 and -385 containing the
distal AP1 site led in both promoter alleles to a significant increase in reporter activity. The drop of reporter activity induced by cutting off the region containing the SP2 binding site of the \textit{Cd14} promoter of C3Bir demonstrated a stimulating influence of the -298 to -181 sequence. These experiments also confirmed that the proximal SP1 site regulated the basal activity of the both alleles [17, 18].

**Fig 3. Truncations of the \textit{Cd14} promoter alleles.** Constructs of the 5' truncated \textit{Cd14} promoter were cloned in the pGL4.17 reporter plasmid and transfected in RAW264.7 cells. \textbf{A} The strong luciferase activity increase after removal of the distal 100 bp and of the sequence between -474 and -385 in the B6 allele indicated inhibitory elements in these regions. Activating binding motifs were identified through the drop of luciferase activity after cutting of the -844 to -722 and -298 to -181 regions. \textbf{B} Stepwise truncation of the C3Bir allele revealed significant expression peaks after removal of the -474 to -385 region and the -181 to -52 sequence while the -298 to -181 and the -52 to +85 regions seemed to contain stimulating binding motifs. ***: P < 0.05.

doi:10.1371/journal.pone.0155821.g003
Modulation of *Cd14* promoter activity through site-directed mutagenesis

To proof the predicted influence of the transcription factor binding sites on *Cd14* expression site-directed mutagenesis followed by luciferase reporter assays were performed. Mutagenesis of the distal Oct1 binding site in the full length promoter of the B6 allele induced a significant reduction of the reporter activity while destruction of the NRF2 binding motif led to a almost complete shut down of the *Cd14* activity (Fig 4A). As expected, disruption of the distal AP1 binding site caused a strong decrease of reporter activity, while the simultaneous mutagenesis of the Stat1/CDX1 binding motifs and the ablation of the proximal SP1 site resulted in silencing of the B6 allele of the *Cd14* promoter (Fig 4A). As in the B6 allele, the mutagenesis of the distal AP1 site induced a significant drop of the promoter activity confirming the activating influence of AP1 (Fig 4B). The disruption of the SP2 binding site diminished reporter activity significantly to 10049 (SD± 2584) compared to the activity of wild type C3Bir allele with 89503 (SD± 13839). Like in the B6 allele of the *Cd14* promoter the experimental mutation of the 3' SP1 site caused a reduction of the reporter activity (Fig 4B). These data suggested that the distal AP1 site and the proximal SP1 were responsible for the basal activity of the *Cd14* promoter in both mouse strains. The results demonstrated that the OCT1 binding site in the -1067 to -977 region of the B6 allele did not mediate the proposed inhibitory effect while the NRF2 binding

![Fig 4](fig4.png)

*Fig 4. *Cd14* promoter activity after site-directed mutagenesis. A* After inactivation of OCT1, Nrf2 and proximal AP1 binding sites in the B6 allele reduced activities were detected while in promoter constructs lacking Stat1/Cdx2 and SP1 led to complete transcriptional silencing. **B** In the C3Bir allele deletion of AP1 and SP2 binding sites resulted in significant transcriptional decrease, while after the mutation of SP1 only a slight reduction of Luciferase expression was seen. ***: P < 0.05.

doi:10.1371/journal.pone.0155821.g004
motif had an unexpected activating effect. The predicted stimulating effect caused by the STAT1/CDX1 binding site in the B6 allele was confirmed by the mutagenesis experiment. However, the disruption of the SP2 recognition motif in the C3Bir allele induced to the expected reduction of promoter activity and led to the hypothesis that SP2 as stimulating factor was in charge for the higher expression level of Cd14 in C3Bir compared to B6.

Physical interaction of SP2 with the C3Bir allele of the Cd14 promoter

EMSA using a consensus oligonucleotide representing the transcription factor binding sites for SP2 derived from the C3Bir allele formed two complexes with the protein extracts isolated from RAW264.7 cells. In lane 2 of the EMSA (Fig 5A) the complex I remained in the upper part of the gel demonstrating the formation of a large protein-DNA aggregation. Also the faster migrating complex II was identified. Moreover, competitive application of unlabelled oligonucleotide abolished dose-dependent changes in migration shifts (Fig 5A, lanes 3–5). Incubation of oligonucleotide-protein complexes with an SP2 specific antibody induced formation of specific complexes III-IV (Fig 5, lane 6) which were absent using SP1 or SP3 specific antibodies (Fig 5, lanes 7 and 8). Moreover, while the infection of proliferating primary BMMs in vitro (Fig 5B) with an MMULV-based control retrovirus reduced the expression of Cd14 compared to untreated BMMs (Fig 5C) the retroviral-mediated overexpression of the mouse SP2 protein in primary BMMs caused a significant increase in Cd14 expression in two independent experiments (Fig 5C). These results clearly revealed physical interaction between SP2 with the murine Cd14 promoter.

Discussion

The Cd14 promoter of the mouse strains C3H/HeJ Bir and C57BL6/J display common transcription factor binding sites. Three AP1 and a single SP1 recognition site in close proximity to the transcription start site were identified. These results confirmed the work of Matsuura and colleagues who localized an AP1 binding elements in the Cd14 promoter derived from BALB/c mice identical to AP1 site at -367 to -356 in the B6 and C3Bir alleles [16,19]. AP1 binding sites were also found in rat, cattle and human CD14 promoter controlling the basic promoter activity like in the mouse [17,18]. Similarly, SP1 sites are well known regulatory elements in the Cd14 promoter of different species [17–19]. The SP1 site in the mouse Cd14 promoter in spatial proximity to the translation start site mediates basal promoter activity in both alleles revealed a reduced transcription rate after truncation to the shortest +85 fragment. In the human CD14 promoter the truncation to the -128 fragment containing the proximal SP1 site also resulted in a sparse reduction of reporter activity [17], while the shortening of the rat Cd14 promoter to the -142 construct including the 3’ end SP1 binding motif caused a drop of reporter expression to 20% compared to the full length promoter [18]. Moreover, the activating influence of the proximal SP1 site in the rat was confirmed by the significant lower reporter expression found after mutagenesis of the SP1 site in the full length promoter [18]. These data demonstrate that the 3’ SP1 site in rodents are associated with the maintenance of basal Cd14 promoter activity in contrast to the inhibiting function in the human CD14 promoter. Besides these corresponding features of the B6 and C3Bir allele we try to identify inhibiting transcription factor binding sites in the B6 allele that are lost or replaced by a stimulating binding motif in the C3Bir allele explaining the higher transcriptional level of Cd14 in the C3Bir strain.

Truncation experiment revealed an inhibitory element in the distal -1067 to -977 region of the B6 allele containing an OCT1 binding site that was replaced by a CUX1 binding motif in the C3Bir allele through a T to C exchange at position -1041. Mutagenesis of this OCT1 consensus sequence demonstrated clearly that the OCT1 site has activating functions. The
Fig 5. Physical interaction of SP2 with the Cd14 promoter derived from C3Bir. A, SP2 electrophoretic mobility shift was performed running a biotin-labelled Sp2 consensus oligonucleotide alone (lane 1) or incubated with a nuclear extracts isolated from RAW264.7 cells (lane 2). In the latter two complex formations (I and II) from the Sp2 consensus oligo with nuclear extract components were detected. Competition of increasing amounts of unlabelled SP2 oligo for binding to the nuclear extract proteins led to a stepwise disappearance of biotin-Sp2-oligo protein complexes (lane 3–5). After pre-incubation of the nuclear extracts with an SP2 specific antibody new complexes (III-V) were established in this supershift assay demonstrating physical interaction of the SP2 protein with its consensus oligo (lane 6). Specific SP2 DNA complex formation was also demonstrated by the failure of SP1 and SP3 antibodies to bind to the protein DNA complexes (lane 7 and 8).

B MMs after four days of culture. The scale bar represents 10 μm at a 10x magnification. C The MMULV-mediated overexpression of mouse SP2 in primary BMMs derived from C3Bir mice correlated with a higher Cd14 transcription rate compared to untreated and control virus infected macrophages. ***: p<0.01; ** p<0.05.

doi:10.1371/journal.pone.0155821.g005
stimulating effects of OCT1 were already shown on B cells and in the expression of Lipoxin A4 in human monocytes [40,41]. Therefore, the activation by OCT1 must be superimposed by yet unknown repressive elements in the -1067 to -977 region. Also the NRF2 binding site between -722 and -474 of the B6 allele of Cd14 mediates transcriptional activation revealed by the transcriptional down-regulation after mutagenesis. On the other hand the elimination of the sequence containing the NRF2 site by truncation did not change reporter activity suggesting that also in the -722 to -474 region of the B6 allele of the cd14 a unknown inhibitory element seems to neutralize NRF2 stimulation. Overexpression of NRF2 (Nuclear factor (erythroid-derived 2)-like 2) in rat alveolar macrophages after transient tranfection in vitro increased the Cd14 protein level while the expression of an anti-NRF2 siRNA accomplished the opposite effect [42]. NRF2 supported the maintenance of the intestinal barrier and reduced the colonic inflammation in chronic kidney disease [43]. Moreover, NRF2-deficient mice have been shown to be more susceptible to dextran sulfate sodium (DSS)-induced colitis than wild type animals [44] suggesting that expression of the protective the Cd14 protein and therewith the colitis manifestation in B6 mice was modulated by the NRF2 transcription factor.

Following elimination of the Cd14 promoter region from the B6 allele containing the STAT1/CDX1 transcription factor binding site between -298 and -181 as well as after the mutagenesis of these binding motifs the reporter expression was diminished emphasizing the stimulatory function of STAT1 or CDX1. The transcription CDX1 was characterized as an intestine-specific homeoprotein but in contrast to the hypersensitivity to DSS-induced intestinal inflammation after the deletion of the closely related factor CDX2 the ablation of CDX1 has no effect of colitis susceptibility [45, 46]. On the other hand Rahimi and colleagues were able to show that the IL10-dependent induction of Cd14 expression was mediated by STAT1 in human moncytic cells [47] indicating an innovative activating function of CDX1 and STAT1 in the transcriptional regulation of Cd14 in B6 mice.

In the Cd14 allele derived from C3Bir the STAT1/CDX1 consensus sequences were disrupted creating a new SP2 binding motif introduced through a T to C substitution at position -244. We showed that the depletion of the SP2 element from the promoter by truncation and mutation led to a significant transcriptional down-regulation. Moreover, EMSA and supershift experiments proofed the protein-DNA interaction between SP2 and its C3Bir specific binding site [48]. Moreover, LeVan and colleagues reported that the ratio of Sp3 to Sp1 and Sp2 transcription factors influenced the expression of human CD14 in monocytes and hepatocytes [49]. The retroviral transduction of primary BMMs with an empty control virus led to a reduced Cd14 expression compared to untreated cells. Also Bösinger and colleagues found that the retroviral infection with HIV correlated with a lower expression of Cd14 and TLR4 [46]. Compensating these inhibitory effects of the retrovirus by itself the retroviral-mediated overexpression of SP2 induced almost a doubling of Cd14 transcription compared to wild type BMMs. Concerning the low binding affinity of SP2 to DNA [48] these results demonstrate the binding and activating capacities of SP2 to the Cd14 promoter allele of C3Bir. While the SP1 and SP3 bind the same target gene sequences mediated by conserved carboxy-terminal zinc finger domains, SP2 binds in a complex with the transcription factor NF-κ to a trimeric histone-fold CCAAT box motif to SP2 specific target genes [50]. Genome wide binding studies of SP2 using murine embryonic fibroblasts of unclear origin did not identify Cd14 as a major target of SP2 [39, 50,51], which might be due to the fact that most other mouse strain except C3H lack the SP2 binding site [21]. Therefrom, Cd14 was characterized as positively regulated target gene of SP2 in C3Bir mice. SP2 may contribute to the differential expression level between B6 and C3Bir mice and was identified as innovative target for the therapy of inflammatory bowel diseases supporting the upregulation of the protective CD14 protein.
Supporting Information

S1 Table.
(DOCX)

S2 Table.
(DOCX)

S3 Table.
(DOCX)

Acknowledgments
We thank Prof. Guntram Suske, Philipps-Universität Marburg, who kindly provided the plasmids pBABE puro and pBABE puro Sp2.

Author Contributions
Conceived and designed the experiments: NHZ ASH AB. Performed the experiments: NHZ ASH IB. Analyzed the data: NHZ ASH IB. Contributed reagents/materials/analysis tools: NHZ ASH IB. Wrote the paper: NHZ ASH IB AB.

References
1. Frolova L, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulation expression of TLR2 in terminal ileum of patients with ulcerative colitis. 2008; J Histochem Cytochem. 56: 267–274. PMID:18040078
2. Yun J, Xu CT, Pan BR Epidemiology and gene markers of ulcerative colitis in the Chinese. World J Gastroenterol. 2009; 15: 788–803. PMID:19230040
3. Lakatos PL, Kiss LS, Palatka K, Altorjay I, Antal-Szalmas P, Palyu E, et al. Serum lipopolysaccharide-binding protein and soluble CD14 are markers of disease activity in patients with Crohn’s disease. 2011; Inflamm Bowel Dis. 15: 767–777. doi:10.1002/ibd.21402 PMID: 20865702
4. de Buhr MF, Hedrich HJ, Westendorf AM, Obermeier F, Hofmann C, Zschemisch, et al. Analysis of Cd14 as a genetic modifier in experimental inflammatory bowel diseases (IBD) in mice. 2009; Inflamm Bowel Dis. 15: 1824–1836. doi:10.1002/ibd.21030 PMID: 19637339
5. de Buhr M, Mähler M, Geffers R, Hansen W, Westendorf AM, Lauber J, et al. Cd14, Gbp1, and Pla2g2a: three major candidate genes for experimental IBD identified by combining QTL and microarray analysis. 2006; Physiol Genomics. 25: 426–434. PMID:16705022
6. Bleich A, Büchler G, Bechwith J, Peteil LM, Affourtit JP, King BL, et al. Cdc51 a major colitis susceptibility locus in mice; subcongenic analysis reveals genetic complexity. 2010; Inflamm Bowel Dis. 16: 765–775. doi:10.1002/ibd.21146 PMID: 19856416
7. Büchler G, Wos-Oxley ML, Smoczek A, Z schemisch NH, Neumann D, Pieper DH et al. Strain-specific colitis susceptibility in IL-10-deficient mice depends on complex gut microbiota-host reactions. 2012; Inflamm Bowel Dis. 18: 943–954. doi: 10.1002/ibd.21895 PMID:22238116
8. Simmons DL, Tan S, Tenen DG, Nicholson-Weller A, Seed B. Monocyte antigen CD14 is a phospholipids anchored membrane protein. 1989; Blood. 73: 284–289. PMID:2462937
9. Dziarski R, Viriyakosol S, Kirkland TN, Gupta D, Soluble CD14 enhances membrane CD14-mediated responses to peptidoglycan: structural requirements differ for those responses to lipopolysaccharide. 2000; Infect Immun. 68: 5254–5260. PMID:10948152
10. Bufler P, Stiegler G, Schuchmann M, Hess S, Krüger C, Stelter F, et al. Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. 1995; Eur J Immunol. 25: 604–610. PMID:7533093
11. Haziot A, Rong GW, Bazil V, Silver J, Goyert SM Recombinant soluble CD14 inhibits LPS-induced tumor necrosis factor-alpha production by cells in whole blood. J Immunol. 1994; 141: 547–552.
12. Landmann R, Fisscher AE, Olbrecht JP. (1992) Interferon-gamma and interleukin-4 down regulate soluble CD14 release in human monocytes and macrophages. 1992; J Leukoc Biol. 52: 323–330. PMID:1381744
13. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of Toll-like receptor 4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. 2001; J Immunol. 167: 1609–1616. PMID: 11466383

14. Suzuki M, Hisamatsu T, Podolsky DK. Gamma interferon augments the intracellular pathways for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intercellular Toll-like receptor 4- MD-2 complex. 2003; Infect Immun. 71: 3503–3511. PMID: 12761135

15. Munthe-Kaas MC, Torjussen TM, Gervin K, Lødrup Carlsen KC, Carlsen KH, Granum B, et al. CD14 polymorphisms and serum CD14 levels through childhood: A role for gene methylation? 2010; J Allergy Clin Immunol. 125: 1361–1368. doi: 10.1016/j.jaci.2010.02.010 PMID: 20398919

16. Matsuura K, Ishida T, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S. Identification of a tissue-specific regulatory element within the murine CD14 gene. 1992; J Biol Chem. 267: 21787–21794. PMID: 1383228

17. Zhang DE, Hetherington CJ, Tan S, Dziennis SE, Gonzalez DA, Chen HM, et al. Sp1 is a critical factor for the monocytic specific expression of human CD14. 1994; J Biol Chem. 269: 11425–11434. PMID: 7512665

18. Liu S, Shapiro RA, Nie S, Zhu D, Vodovotz Y, Billiar TR. Characterization of the rat CD14 promoter and its regulation by transcription factors AP1 and Sp family proteins in hepatocytes. 2000; Gene. 250: 37–147.

19. Ibeagha-Awenu EM, Lee JW, Ibeagha AE, Zhao X. Bovine cd14 gene characterization and relationship between polymorphisms and surface expression on monocytes and polymorphonuclear neutrophils. 2008; BMC Genetics. 9: 50. doi:10.1186/1471-2156-9-50 PMID: 18691417

20. Vychodilova-Krenkova L, Matiasovic J, Horin P. Single nucleotide polymorphisms in four functionally related immune response genes in the horse: CD14, TLR4, Cε and FcεRI alpha. 2005; Int J Immunogenet. 32: 277–283. PMID: 16166949

21. Liu HH, Hu Y, Zheng M, Suhoski MM, Engleman EG, Dill DL, et al. Cd14 SNPs regulate the innate immune response. 2012; Mol Immunol. 51: 112–127. doi: 10.1016/j.molimm.2012.02.112 PMID: 22445606

22. Tan CY, Chen YL, Wu LS, Liu CF, Chang WT, Wang JY. (2006) Association of Cd14 promoter polymorphisms and soluble levels in mite allergen sensitization of children in Taiwan. 2006; J Hum Genet. 1: 59–67.

23. Lange J, Heinzmann A, Zehle C, Kopp M. CT genotype of promoter polymorphism C159T in the CD14 gene associated with lower prevalence of atopic dermatitis and lower IL-13 production. 2005; Pediatr Allergy Immunol. 16: 456–457. PMID: 16101942

24. Lichy C, Meiser H, Grond-Ginsbach C, Buggle F, Dörfer C, Grau A. Lipopolysaccharide receptor CD14 polymorphism and risk of stroke in a south-german population. 2002; J Neurol. 249: 821–823. PMID: 12140663

25. Lima-Neto LG, Hirata RD, Luchessi AD, Silbiger VN, Stephano MA, Sampaio M, et al. Cd14 and IL6 polymorphisms are associated with a pro-atherogenic profile in young adults with acute myocardial infarction. J Thromb Thrombolysis. 2013 Oct; 36(3): 332–40.

26. Hubacek JA, Rothe G, Pit'ha J, Skodov Z, Stanék V, Poledne R, et al. C(-260)>T polymorphism in the promoter of the CD14 monocyte receptor gene as a risk factor for myocardial infarction. 1999; Circulation. 99: 3218–3220. PMID: 10385492

27. Pu H, Yin J, Zhang D, Wang Y, Zhou R, Jiang L, et al. The association between CD14 gene C-260T polymorphism and coronary heart disease risk: a meta-analysis. Mol Biol Rep. 2013 Jun; 40(6):4001–8. doi: 10.1007/s11033-012-2478-y PMID: 23277403

28. Alavi-Naini S, Salimi S, Sharifi-Mood B, Dovoodikia AA, Moody B, Naghavi A. Association between CD14 C-159T polymorphism and serum soluble CD14 with bacterial SP2 Transcription Factor in Colitis.
32. Griga T, Klein W, Epplen JT, Hebler U, Stachon A, May B. CD14 expression on monocytes and soluble CD14 plasma levels in correlation to the promoter polymorphisms of the endotoxin receptor CD14 gene in patients with inactive Crohn’s disease. 2005; Hepatogastroenterology. 52: 808–811. PMID: 15966209

33. Obana N, Takahashi S, Kinouchi Y, Negoro K, Takagi S, Hiwatashi N, et al. Ulcerative colitis is associated with a promoter polymorphism of lipopolysaccharide receptor gene, CD14. 2002; Scand J Gastroenterol. 37: 699–704. PMID: 12162249

34. Huebner C, Petermann I, Lam WJ, Shelling AN, Ferguson LR. Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. 2010; Inflamm Bowel Dis. 16: 282–295. doi: 10.1002/ibd.20143 PMID: 19653290

35. Kim EJ, Chung WC, Lee KM, Paik CN, Jung SH, Lee BI, et al. Association between toll-like receptors/CD14 gene polymorphisms and inflammatory bowel disease in Korean population. 2012 1; J Korean Med Sci. 27: 72–77. doi: 10.3346/jkms.2012.27.1.72 PMID: 22219617

36. Wang Z, Hu J, Zhou J, Zhong J. Association between CD14 gene C-260T polymorphism and inflammatory bowel diseases: a meta-analysis. PlosOne. 2012; 7(9):e45144. doi: 10.1371/journal.pone.0045144 PMID: 22978913

37. Baumgart DC, Büning C, Geerdts L, Schmidt HH, Genschel J, Fiedler T, et al. The c.1-260C>G (p.D299G) variant of the toll-like receptor 4 (TLR4) genes is associated with inflammatory bowel disease. 2007; Digestion. 76: 196–202. doi: 10.1159/000112646 PMID: 18174680

38. Weischenfeldt J, Porse B. Bone marrow-derived macrophages (BMM): Isolation and Application. 2008; CSH Protoc. 2008 Dec 1; 2008:pdb.prot5080. doi: 10.1101/pdb.prot5080

39. Baur F, Nau K, Sadic D, Allweis L, Elsässer HP, Gillemans N, et al. Specificity Protein 2 (Sp2) is essential for mouse development and autonomous proliferation of mouse embryonic fibroblasts. PLoS One. 2010 Mar 8; 5(3):e9587. doi: 10.1371/journal.pone.0009587 PMID: 20221402

40. Waechter V, Schmid M, Herova M, Weber A, Günther V, Marti-Jaun J. Characterization of the promoter and the transcriptional regulation of the lipoxin A4 receptor (FPR2/ALX) gene in human monocytes and macrophages. 2012; J Immunol. 188: 1856–1867. doi: 10.4049/jimmunol.1101788 PMID: 22246625

41. van Heel DA, Udalova IA, McGovern DP, Kinouchi Y, Hull J, et al. Inflammatory bowel disease is associated with a TNF polymorphism that affects an interaction between the OCT1 and NF-κB transcription factors. 2002; Hum Mol Genet. 11: 1281–1289. PMID: 12019209

42. Staitieh BS, Fan X, Neveu W, Guidot DM. Nrf2 regulates PU.1 expression and activity in the alveolar macrophage. 2015; Am J Physiol Lung Cell Mol Physiol. 308: L1086–L1093. doi: 10.1152/ajplung.00355.2014 PMID: 25840997

43. Lau WL, Liu SH, Pahlevan S, Yuan J, Khazaal M, Ni Z, et al. Role of Nrf2 dysfunction in uremia-associated intestinal inflammation and epithelial barrier disruption. 2015; Dig Dis Sci. 60: 1215–1222. doi: 10.1007/s10620-014-3428-4 PMID: 25399330

44. Khor TO, Huang MT, Kwon H, Chan JY, Reddy BS, Kong AN. Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. 2006; Cancer Res. 66: 11580–11584. PMID: 17178849

45. Crissey MAS, Guo RJ, Fogg F, Li H, Katz JP, Silberg DG, et al. The homeodomain transcription factor Cdx1 does not behave as an oncogene in mouse intestine. 2008; Neoplasia. 10: 8–19. PMID: 18231635

46. Calon A, Gross I, Lhermitte B, Martin E, Beck F, Duclos B, et al. Different effects of Cdx1 and Cdx2 homeobox genes in a murine model of intestinal inflammation. 2007; Gut. 56: 1688–1695. PMID: 17595234

47. Rahimi AA, Gee K, Mishra S, Lim W, Kumar A. STAT-1 mediates the stimulatory effect of IL-10 on CD14 expression in human mononuclear cells. 2005; J Immunol. 174: 7823–7832. PMID: 15944287

48. Moorfield KS, Fry SJ, Horowitz JM. Sp2 DNA binding activity and trans-activation are negatively regulated in mammalian cells. 2004; J Biol Chem. 279: 13911–13924. PMID: 14726517

49. LeVan TD, Bloom JW, Bailey TJ, Karp CL, Halonen M, Martinez FD, A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. 2001; J Immunol. 167: 5838–5844. PMID: 11698458

50. Völkel S, Stelow B, Finkernagel F, Stiewe T, Nist A, Suske G. Zinc finger independent genome-wide binding of Sp2 potentiates recruitment of histone-Fold protein nfd-1 distinguishing It from Sp1 and Sp3. PLoS Genet. 2015 Mar 20; 11(3):e1005102. doi: 10.1371/journal.pgen.1005102 PMID: 25793500

51. Terrados G, Finkernagel F, Stelow B, Sadic D, Neubert J, Herdt O, et al. Genome-wide localization and expression profiling establish Sp2 as a sequence-specific transcription factor regulating vitally important genes. 2012; Nucleic Acids Res. 40: 7844–7857. doi: 10.1093/nar/gks544 PMID: 22884502