This is a postprint of an article published in

De Buhr, M.F., Hedrich, H.-J., Westendorf, A.M., Obermeier, F., Hofmann, C., Zschemisch, N.-H., Buer, J., Bumann, D., Goyert, S.M., Bleich, A.
Analysis of Cd14 as a genetic modifier of experimental inflammatory bowel disease (IBD) in mice
(2009) Inflammatory Bowel Diseases, 15 (12), pp. 1824-1836.
.

Analysis of *Cd14* as a Genetic Modifier of Experimental Inflammatory Bowel Disease (IBD) in Mice

Short title: *Cd14* in experimental IBD

Maike F. de Buhr, Dr.,[1] Hans-J. Hedrich, DVM,[1] Astrid M. Westendorf, Dr.,[2] Florian Obermeier, MD,[3] Claudia Hofmann, Dr.,[3] Nils-H. Zschemisch, Dr.,[1] Jan Buer, MD[2] Dirk Bumann, Dr.,[4] Sanna M. Goyert, PhD,[5] Andre Bleich, PhD[1]

[1]Insitute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany; [2]Helmholtz Centre for Infection Research, Braunschweig, and Institute of Medical Microbiology, University Hospital Essen, Essen, Germany; [3]Department of Internal Medicine I, University of Regensburg, Regensburg, Germany; [4]Infection Biology, Biozentrum, University of Basel, Switzerland; [5]Department of Microbiology and Immunology, CUNY Medical School/Sophie Davis School of Biomedical Education, New York, USA

Corresponding Author: Andre Bleich, DVM, PhD, Institute for Laboratory Animal Science and Central Animal Facility, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany, Phone: +49 511 532 8714, Fax: +49 511 532 3710, E-mail: bleich.andre@mh-hannover.de

Sources of support: This work was supported by grants from the DFG (SFB621, HH*)* and Hannover Medical School (HILF, AB).

Abstract

Background and Aim: By combining QTL and gene expression analyses, we have previously identified *Cd14* as a potential candidate gene contributing to differential IBD susceptibility of C3H/HeJBir (C3/J)-*Il10*$^{-/-}$ (carrying IBD-resistance alleles at this QTL [*Cdcs6*]) and C57BL/6J (B6)-*Il10*$^{-/-}$ mice, corroborating studies that showed association of a *CD14*-promoter polymorphism with Crohn's disease and ulcerative colitis. Aim of the present study was to analyse the molecular mechanisms leading to differential intestinal expression of *Cd14* and its contribution to IBD development.

Methods: Intestinal CD14 expression was assessed by FACS, immunohistochemistry, and ELISA on supernatants of primary epithelial cell and tissue cultures. RAW264.7 cells were stimulated with LPS and PGN in presence or absence of CD14. *Cd14* alleles were sequenced and promoters cloned for luciferase assays in transfected RAW264.7 cells. Severity of typhlocolitis was compared between *Cd14*$^{-/-}$ and wild type mice in two distinct mouse models of IBD (acute DSS and *Il10*$^{-/-}$).

Results: In the gut, CD14 was detected mainly in its soluble form (sCD14), with higher expression in C3/J-*Il10*$^{-/-}$ mice. Polymorphisms in C3/J mice caused higher activity of the *Cd14*-promoter (luciferase assays). Intestinal sCD14 concentrations influenced LPS and PGN response of RAW264.7 cells. *In vivo*, genetic deletion of *Cd14* aggravated colitis in both mouse models of IBD.

Conclusions: Our study shows that *Cd14*-promoter polymorphisms affect CD14 expression and confirms the protective effect of CD14 against experimental IBD, potentially mediated by TLR2 and TLR4 dependent effects on intestinal barrier function. These findings support the concept that human *CD14*-promoter polymorphisms contribute to disease development.

Key Words: Animal models of IBD, Quantitative Trait Locus analysis, Inflammation in IBD, Toll-like receptors, Genetic susceptibility, Innate immune system, Pathology, Cytokine-deficiency induced colitis susceptibility

INTRODUCTION

A polymorphism in the promoter of the human *CD14* gene (-260T>C) has been associated with Crohn's disease (CD)[1-3] and ulcerative colitis (UC)[3-5], either alone or through interaction with polymorphisms in the *CARD15* gene.[2,6] However, other studies failed to detect association between *CD14* and inflammatory bowel diseases (IBD);[7,8] furthermore, the functional consequence of this polymorphism is not clearly defined.[9-11] Therefore, its role as a modifier of colitis has still to be elucidated. As we have previously identified *Cd14* as a potential modifier of colitis susceptibility in IL10-deficient (*Il10*$^{-/-}$) mice this model system might be of great value to clarify the human situation.[12-14]

In *Il10*$^{-/-}$ mice, severity of IBD depends on the inbred strain carrying the IL10-mutation, indicating a major contribution from genetic background modifier genes. Previous linkage studies using an F2 and a first backcross population of highly susceptible C3H/HeJBir (C3/J)-*Il10*$^{-/-}$ and partially resistant C57BL/6J (B6)-*Il10*$^{-/-}$ mice revealed 10 quantitative trait loci (QTL) that were associated with colitis susceptibility in these mice (*Cdcs1-10*, Cytokine-deficiency induced colitis susceptibility 1-10).[12,13,15] In the segregating populations used for QTL-analyses, i.e. under hybrid combinations, the susceptibel C3/J as well as the nominally resistant B6 parental genome contributed susceptibility alleles to the colitis subphenotypes. This is considered as particularly relevant in understanding why colitis heritability in outbred human populations is so complex;[12,13] however, similar findings are often observed in QTL-studies. By combining mapping results with gene-expression analyses, we were able to identify eight candidate genes which may contribute to differential colitis susceptibility. Among these genes, *Cd14* was considered to be a major candidate because of very distinct expression differences in the C3 and B6 genetic backgrounds,[14] its function, and the association of the promoter-polymorphism with CD and UC. The mouse *Cd14* gene is located within the interval of *Cdcs6*, a QTL at which the B6- and not the C3/J-allele contributed to colitis susceptibility.

CD14 is constitutively expressed as a membrane bound form (mCD14) on the surface of human monocytes, macrophages, and neutrophils. It is essential for LPS-signal transduction via TLR4 and also interacts with TLR2 for the detection of lipoproteins leading to NFκB activation.[16] A soluble form of CD14 (sCD14) is present in serum and shows ambiguous functions, as it promotes LPS-response in cells that do not express mCD14[17] but also reduces pro-inflammatory responses of mCD14 positive cells to LPS at high concentrations,[18,19] probably by competitive binding. Data indicate that extra vascular sCD14 increases resistance against bacterial infections.[20]

A role of CD14 in IBD is not defined yet. So far, it was found to be expressed only at low levels in not inflamed intestine.[21,22] However, we have identified considerable higher expression in the gut on mRNA and protein levels in the C3 than in the B6 genetic

background, even under germ-free conditions. We have furthermore excluded the possibility that the *Tlr4*$^{Lps\text{-}d}$ allele of C3/J mice coding for a defective LPS-receptor causes expression differences. Besides expression differences in the intestine, we have observed that CD14 is differentially expressed on peritoneal macrophages of both strains after antigen stimulation.[14]

As the role of CD14 is controversially discussed in human IBD, aim of this study was to elucidate the nature of differential intestinal *Cd14* expression and whether it might contribute to IBD-susceptibility in mice. Because our genetic analyses showed that C3 mice carry IBD-resistance alleles at this QTL we hypothesized that CD14 protects against colitis.

MATERIAL AND METHODS

Mice

C3H/HeN.129S1-*Cd14*^{tm1Smg} (C3/N-*Cd14*$^{-/-}$) and C57BL/6J.129S1^{tm1Smg} (B6-*Cd14*$^{-/-}$) mice as well as a colony of C57BL/6J.129P2-*Il10*^{tm1Cgn} (B6-*Il10*$^{-/-}$) mice for the breeding of B6-*Il10*$^{-/-}$*Cd14*$^{-/-}$ double deficient mice were produced and maintained under conventional SPF conditions as described previously.[14] Routine microbiological monitoring according to FELASA recommendations[23] did not reveal any evidence of infection with common murine pathogens except for *H. hepaticus* and *H. typhlonius*. All other mice (C3H/HeJBir.129P2-*Il10*^{tm1Cgn} [C3/J-*Il10*$^{-/-}$] and wild-type C3H/HeJZtm [C3/J], B6-*Il10*$^{-/-}$ and C57BL/6JZtm [B6], C3H/HeNZtm [C3/N]) mice were maintained under strict SPF conditions.[14] Routine microbiological monitoring did not reveal any evidence of infection with common murine pathogens. All congenic mice were backcrossed for at least 10 generations.

This study was conducted in accordance with the German Animal Welfare Law and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes. All experiments were approved by the Local Institutional Animal Care and Research Advisory committee and authorised by the local government (AZ # 0407/1237).

Promoter analyses

Coding and promoter region (1067 bp upstream of the start codon) of the *Cd14* gene were sequenced in B6 and C3/J mice after amplification using primer pairs 5'-CCA-CCG-CTG-TAA-AGG-AAA-GAA-AC-3' and 5'-ACC-CAC-TGA-ACC-ATC-TTG-ACT-GC-3' (annealing temperature: 56°C), 5'-ATT-TGA-ACG-GTG-GGA-AAT-TG-3' and 5'-CAG-AAG-CAA-CAG-CAA-CAA-GC-3', 5'-AAT-GAT-GAC-GAT-GAC-GAC-GA-3' and 5'-CAC-CGT-TCA-AAT-CCA-GCT-CT-3' (annealing temperature: 57°C). Subsequently, *Cd14*-promoters of both strains were amplified by PCR using oligonucleotides 5'-ATC-TCG-AGA-ATG-ATG-ACG-ATG-ACG-AC-3' and 5'-CCA-GAT-CTA-TCT-GAG-AAG-TTG-CAG-GAA-CAA-C-3' flanked by XhoI and BglII restriction sites and Easy-A High Fidelity PCR Cloning Enzyme

(Stratagene, La Jolla, CA, USA). PCR products comprising the region -1067 to +199 (B6)/+203 (C3/J) in exon 2 were directly cloned into StrataClone PCR Cloning Vector pSC-A and transformed in SoloPack Competent Cells (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. *Cd14* promoters were confirmed by sequencing and inserted into XhoI and BglII sites of the pGL4.10 Luciferase Reporter Vector (Promega, Madison, WI, USA). Plasmids were prepared using EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA (5 µg) was transfected into RAW264.7 macrophages for 3 h using Superfect Transfection Reagent (Qiagen, Hilden, Germany). Each transfection reaction contained 1 µg β–Galactosidase reporter plasmid pCMV-Gal as internal standard and 4 µg pGL4.10 or *Cd14*-Luciferase reporter constructs. Macrophages were stimulated with 0.1 ng/ml LPS (Sigma-Aldrich, Munich, Germany) and harvested after 48 h of incubation together with unstimulated macrophages as controls. Luciferase reporter assays were performed as described elsewhere.[24]

DSS-induced colitis

Age-matched (8-10 weeks) mice were treated with 3.5% (wt/vol) DSS (mol wt 36,000-50,000 [MP Biomedicals, Eschwege, Germany]) in their drinking water over five days, control mice received autoclaved distilled water. In order to assure similar states of bacterial flora, mice were transferred onto bedding of their counterparts frequently and at least four weeks before the experiments started. Clinical assessment including determination of body weight, stool consistency, and the presence of blood in the faeces was performed daily. After treatment, mice were euthanized by CO_2 asphyxiation and exsanguination; blood was collected by cardiac puncture for determination of IL1β levels in serum. Spleens were weighed.

Scoring of intestinal lesions in DSS-treated and *Il10*$^{-/-}$ mice

Caeca and colons (including the rectum and anus) were removed, and processed for histology as described previously;[15] colons were prepared as a modified "Swiss role", caeca were cut into halves after fixation. Histopathologic lesions in DSS-treated mice were graded as follows: epithelial changes (crypts unchanged: 0, loss of basal 1/3: 1, basal 2/3: 2, complete: 3, loss of crypts plus ulceration: 4), cellular infiltration (none: 0, infiltrates in lamina propria: 1, in lamina propria plus oedema formation: 2, in lamina propria and submucosa: 3), area involved (none: 0, 10-30%: 1, 40-60%: 2, >60%: 3; for each parameter individually applied). This scheme was independently applied to caecum, proximal, medial and distal colon, yielding scores from 0-13 for each segment and 0-39 for total colon and 0-52 for colon plus caecum.

In *Il10*$^{-/-}$ and *Il10*$^{-/-}$*Cd14*$^{-/-}$ mice, colitis activity was monitored by histological grading of intestinal lesions according to the "TJL-score" described previously.[15] In short, four general criteria were evaluated in the above mentioned intestinal sections: 1) severity, 2) degree of hyperplasia, 3) degree of ulceration, if present, and 4) percentage of area involved. A range of 1-3 was used for each category. All 4 criteria were added and provided a total score for the section of large intestine evaluated, each ranging from 0 to 12. A total colon score (maximum possible = 36) was determined by adding the total score of all three sections of the colon. Furthermore, colitis activity was characterized by determination of red blood cell numbers and the hematocrit, and determination of cytokine secretion of isolated MLN cells after stimulation over 24 hours in anti-CD3 coated wells (2.5 µg/well; BD, Pharmingen, Hamburg, Germany) together with anti-CD28 (1 µg/ml; BD Pharmingen (Heidelberg, Germany) as described elsewhere.[25]

Immunofluorescence microscopy

Cryosections of colons were stained with biotin conjugated rat anti-mouse CD14-antibody (1:50 [eBioscience, San Diego, CA, USA]) or with a biotin-conjugated rat IgG2a isotype control (1:50 [eBioscience, San Diego, CA, USA]), followed by incubation with streptavidine-horse radish peroxidase (1:100 [Invitrogen, Karlsruhe, Germany]) and with DAPI (1:1,000 [Applichem, Darmstadt, Germany]). Tyramide signal amplification was performed according to the manufacturer's manual with a TSA-kit containing Alexa Fluor 647-conjugated tyramide (Molecular Probes/Invitrogen, Karlsruhe, Germany) for detection of the horse-radish peroxidase.

FACS-analyses

For isolation of intestinal cells colons were opened longitudinally and incubated in 10 ml PBS with 1 mM Di-thiotreitol (DTT) (Fluka Riedel-de Haën, Seelze, Germany), rinsed in 20 ml HBSS without Mg^{2+}, Ca^{2+} and with 0.25 mM EDTA (Biochrom, Berlin, Germany) and incubated for 15 min on a shaker at 37°C. Cells of remaining tissue were detached by vortexing in PBS. All cell containing media were centrifuged at 500 g for 10 min and washed in FACS buffer (PBS, 2% FCS, 2 mM EDTA [Biochrom, Berlin, Germany]). Cells were stained with FITC-conjugated anti-mouse CD14 antibody and Allophycocyanin (APC)-conjugated anti-mouse CD45, CD19, CD3, CD11, CD11c, F4/80 or with biotin-conjugated G8.8 antibody purified from hybridoma supernatants and streptavidin-phycoerythrin (PE) (all from BD Bioscience, San Jose, CA, USA). FACS analysis was replicated once.

Detection of sCD14

A ready-to-use ELISA kit (CD14 soluble mouse detection set for ELISA application, Alexis, Lausen, Switzerland) was applied according to the manufacturer's protocol to detect sCD14 in serum or supernatants of colonic tissue or colonic crypt cell cultures. Supernatants were taken after 6 h of culture and frozen at -80°C until use.

Real-time RT PCR analysis

Real-time RT PCR analysis was conducted as described previously.[14] RNA was extracted using the Nucleospin® RNAII kit (Macherey Nagel, Düren, Germany) and 1 µg was used for cDNA synthesis by Superscript II Reverse Transcriptase and oligo dT and random hexamer primers (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT PCR was performed in an ABI PRISM cycler (Applied Biosystems, Foster City, CA, USA) using a SYBR Green PCR kit from Stratagene (La Jolla, CA, USA) and specific primers. Relative mRNA levels were determined by using a standard curve and by further normalization to the house keeping gene *Rps9*. Primer sequences for *Cd14* were 5´-CAT-TTG-CAT-CCT-CCT-GGT-TTC-TGA-3´, 5´-GAG-TGA-GTT-TTC-CCC-TTC-CGT-GTG-3´ and those for *Rps9* were 5´-CTG-GAC-GAG-GGC-AAG-ATG-AAG-C-3´, 5´-TGA-CGT-TGG-CGG-ATG-AGC-ACA-3´. The annealing temperature was set to 55 °C.

Colonic tissue culture

Colons were rinsed with PBS and opened longitudinally. The proximal parts were cut into squares with a side length of 5 mm, washed in PBS containing 1 mM DTT (Fluka Riedel-de Haën, Seelze, Germany) in a shaker incubator at room temperature for 10 min and placed into a 48-well-plate (Greiner, Sigma-Aldrich, Munich, Germany) at five pieces per well containing 530 µl culture medium (DMEM [Biochrom, Berlin, Germany], 10% FCS, [Biochrom, Berlin, Germany], 200 U/ml penicillin G and 200 µg/ml streptomycin [GIBCO Invitrogen Corporation, Karlsruhe, Germany], 100 µM β-mercaptoethanol [GIBCO Invitrogen Corporation, Karlsruhe, Germany]), using six wells per mouse strain. After one hour of incubation at 37°C and 5% CO_2 wells were washed by changing the medium twice. After 6 h, culture supernatants were transferred into microcentrifuge tubes and frozen at -80°C.

Crypt cell culture

Intestines were processed and cells were isolated as described for FACS-analyses (see previously) with slight modifications (the first incubation step took 10 min instead of 15 min, and composition of the second incubation medium contained 2 mM EDTA). Crypts were resuspended in Quantum 286 (PAA, Cölbe, Germany) with 100 µg/ml Gentamycin (Biochrom, Berlin, Germany) and $1x10^6$ cells were transferred onto culture plate inserts (CM-0.4 µm, Millipore, Schwalbach, Germany), preincubated with collagen A (Biochrom, Berlin,

Germany). After 6 h of incubation at 37°C and 10% CO_2, medium was harvested and frozen at -80°C. Viability of cells was approved by fluorescine-diacetate (Sigma-Aldrich, Munich, Germany) before and after culture.

Stimulation of RAW264.7 cells

RAW264.7 cells were cultured in DMEM with 4.5 g D-glucose, stable L-Glutamine [Biochrom, Berlin, Germany], 10% FCS with ultra low endotoxin [PAA, Cölbe, Germany], and 100 U/ml penicillin and 100 µg/ml streptomycin [GIBCO Invitrogen Corporation, Karlsruhe, Germany] at 37°C and 5% CO_2. Stimulation was carried out using different concentrations of LPS (0.1, 1.0, 10.0, 100.0, and 1,000.0 ng/ml; from *Escherichia coli*, [Sigma-Aldrich, Munich, Germany]) or PGN (10.0, 100.0, 1,000.0, 10,000.0, and 100,000 ng/ml; from *Bacillus subtilis* [Fluka, Buchs, Switzerland]) in the presence of 50 ng/ml of recombinant mouse sCD14 (Cell Sciences, Canton, MA, USA) or in the absence of sCD14 in 96 well culture plates (TPP AG, Trasadingen, Switzerland) at a density of 100,000 cells per well. After 6 h of stimulation, supernatants were harvested and frozen at -80°C until use for detection of TNF by ELISA (TNF-ELISA kit Ready-set-go, eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

RESULTS

Modifying effect of CD14 in experimental colitis *in vivo*

A potential role of the CD14 protein in experimental IBD was investigated using *Cd14*$^{-/-}$ mice in the DSS-induced and *Il10*$^{-/-}$ colitis model. Typical histological lesions are shown in Figure 1.

DSS model

As C3/J mice (that were originally used for genetic analyses) carry the *Tlr4*$^{Lps\text{-}d}$ allele rendering them LPS hyporesponsive, we considered this background as not suitable for analysing CD14 effects on colitis, as this protein interacts with TLR4 for LPS signaling. Therefore, the LPS responsive C3/N substrain was used for this experiment, enabling us to focus on the effect of the CD14-deficiency without need to consider the defective TLR4 of C3/J mice as an additional variable. The C3/N substrain is often used as a control for the C3/J substrain; we have previously shown that C3/N mice also display high CD14 expression, which is therefore likely a characteristics of the C3-strain rather than a feature of certain C3-substrains or TLR4 status, respectively.[14]

In total, mice of four groups, B6- and C3/N-*Cd14*$^{-/-}$ as well as WT mice, were administered DSS, and sacrificed after 5 days of treatment. Control mice received water without DSS. In C3/N-*Cd14*$^{-/-}$ and WT mice, consistency of faeces became notably soft and first traces of blood appeared in the faeces and around the anus on day 3 of DSS-treatment. On day 4 and 5, all C3/N-*Cd14*$^{-/-}$ mice showed traces of blood around the anus (16 out of 16 mice), but only 11 of 16 C3/N WT mice (Fisher's exact test $p = 0.0434$). On necropsy, spleens were significantly larger in C3/N-*Cd14*$^{-/-}$ than in C3/N mice (mean spleen to body weight ratio: 0.47% vs. 0.39%, t-test; $p = 0.003$). Histological scores revealed significantly higher degrees of inflammation in C3/N-*Cd14*$^{-/-}$ mice than their WT counterparts in all parts of the large intestine (examplarily shown in Fig. 1A-F). These differences were significant in the caecum, mid colon, total colon, and total intestine as determined by Mann-Whitney-U tests (Fig. 2A). After separating sexes, Kruskal-Wallis and the *post-hoc* Dunn test revealed more severe inflammation in the proximal colon of female C3/N-*Cd14*$^{-/-}$ than in WT mice ($p < 0.05$). Otherwise, no differences were detected between both sexes.

Histological data were supported by significant higher levels of IL1β in serum of C3/N-*Cd14*$^{-/-}$ mice compared to C3/N mice upon DSS-treatment (Fig. 2B). Furthermore, extend of ulceration was measured (length of each ulcer in µm) in six randomly selected caeca of C3/N and C3/N-*Cd14*$^{-/-}$ mice, and total area of ulceration in the caecum of each animal as well as mean extend of each ulcer determined. As shown in Figure 2C, *Cd14*$^{-/-}$ mice displayed significantly more area of ulceration and larger mean ulcer sizes than WT mice. This might indicate that CD14-deficiency affects epithelial integrity.

In B6 mice, a significant difference in the severity of colitis was detected in the distal colon between *Cd14*$^{-/-}$ and WT mice (Fig. 2A). Blood appeared in faeces on day 4; on day 5, 11 of 17 B6-*Cd14*$^{-/-}$ mice were observed to have traces of blood compared to 7 out of 15 B6 WT mice (Fisher's exact test $p > 0.05$). No significant difference was observed when comparing spleen to body weight ratios of B6-*Cd14*$^{-/-}$ and B6 mice (means: 0.34% vs. 0.32%). As B6 mice express low levels of CD14 in the gut, less pronounced differences in colitis severity between WT and *Cd14*$^{-/-}$ mice were anticipated compared to the C3/N background.

Il10$^{-/-}$ model

The *Il10*$^{-/-}$ mouse was used as a second experimental IBD model, as original QTL-analyses were performed in these mice. Consistent with results obtained in the DSS model, IL10/CD14 double deficient (*Il10*$^{-/-}$*Cd14*$^{-/-}$) mice developed significantly more severe intestinal inflammation in the caecum and colon than *Il10*$^{-/-}$ controls as determined by earlier development of rectal prolapse (data not shown) and histological examination (determined at 12 and 25 weeks of age) (Fig. 1G-K; Fig. 3). Interestingly, *Il10*$^{-/-}$*Cd14*$^{-/-}$ mice showed already a pronounced inflammation in the large intestine at 12 weeks of age, which we do not observe in B6-*Il10*$^{-/-}$ controls (Fig. 1G-K). Degree of inflammation was identical in *Il10*$^{-/-}$ mice carrying one or two copies of the *Cd14* WT allele, therefore both were used as control groups (Fig. 3). Histological data were corroborated by significantly elevated secretion of TNF by MLN cells from *Il10*$^{-/-}$*Cd14*$^{-/-}$ mice compared to cells from *Il10*$^{-/-}$ controls ($p = 0.0056$), as well as significantly decreased numbers of red blood cells ($p = 0.04$) and hematocrit ($p = 0.03$) (data not shown).

CD14 Expression

As *in vivo* analyses corroborated our previous study[14] and confirmed a protective effect of CD14 against experimental IBD, we subsequently searched for the source of differential CD14 expression in B6 and C3/J intestines.

CD14 in the gut: mCD14

Low expression of mCD14 was detected on cells isolated from colon and ileum of B6-*Il10*$^{-/-}$ and C3/J-*Il10*$^{-/-}$ mice by FACS-analysis. mCD14-positive cells were not positive for any of the surface markers used to differentiate the cell-types; however, after depleting CD45^{+} cells and gating for size and granularity on intestinal epithelial cells (IEC), low expression of CD14 was detected on the surface of a minority of IECs (Fig. 4A). Slightly more cells were CD14^{+} in the ileum than in the colon in C3/J-*Il10*$^{-/-}$ and B6-*Il10*$^{-/-}$ mice, but no strain differences were detected. Freshly isolated peritoneal macrophages (harvested by peritoneal

lavage without subsequent culture)[14] served as a postive control (Fig. 4B). Using immunofluorescence microscopy no mCD14 was detected in normal tissue of either B6-*Il10*$^{-/-}$ or C3/J-*Il10*$^{-/-}$ mice but in inflamed tissue that served as a positive control. Therefore, mCD14 expression did not explain expression differences detected previously in not inflamed mucosa of both mouse strains.

CD14 in the gut: sCD14

Primary colonic tissue and crypt cell cultures were used to detect the soluble form of CD14. Supernatants of C3/J-*Il10*$^{-/-}$ colonic tissue cultures contained significantly more soluble CD14 than those of B6-*Il10*$^{-/-}$ cultures after 6 hours of incubation (Fig. 4C). Furthermore, higher levels of sCD14 were detected in supernatants of primary crypt cells cultured from C3/J-*Il10*$^{-/-}$ than from B6-*Il10*$^{-/-}$ mice for 6 hours (Fig. 4C). FACS analysis prior to culture ensured that less than 3% of the isolated cells were leukocytes. In concordance with the data presented above, neither G8.8 positive cells nor the few CD45^{+} cells showed surface expression of mCD14. These experiments strongly suggested that differential *Cd14* expression between C3/J and B6 mice is due to differences in the release of sCD14 by IEC.

Extraintestinal CD14 expression

In the liver (which is the primary production site of serum CD14) no differences in *Cd14* gene expression were detected by real-time RT PCR between C3/J- and B6-*Il10*$^{-/-}$ mice; however, two-way ANOVA revealed significant more expression in livers of SPF than of germ-free mice (Fig. 4D). The latter was also seen previously in the gut. In addition, serum levels of sCD14 were not significantly different between B6- and C3/J- mice under SPF conditions (B6 mice, mean: 41.2 ng/ml, SD: 15.4; C3/J mean: 32.7 ng/ml, SD: 16.3, t-test, $p = 0.4$). Therefore, different intestinal protein levels observed in this and our previous[14] study were unlikely due to carry over of serum CD14.

Relevance of sCD14 on TLR2 and TLR4 signalling *in vitro*

To investigate whether sCD14 concentrations detected in supernatants of colonic tissue cultures are of biological relevance, RAW264.7 cells were stimulated with different concentrations of LPS or PGN in absence or presence of sCD14 (at a concentration [50 ng/ml] that resembled the amount of sCD14 secreted by colonic tissue of C3/J mice) for 6 h, and TNF was measured in the supernatant of the cell culture by ELISA. The presence of sCD14 resulted in an increase of TNF secretion from RAW264.7 cells, that was significant for LPS concentrations of 0.1 and 1.0 ng/ml as well as for PGN concentrations of 10.0 and 100.0 ng/ml (Fig. 5), indicating that sCD14 in the amount detected in the gut likely is of biological relevance.

Sequence and promoter analyses of the *Cd14* gene

To identify the genetic cause of differential CD14 expression, coding and promoter sequences of the *Cd14* genes were analysed in B6 and C3/J mice. No polymorphisms were detected in the protein coding region of the *Cd14* gene. We confirmed an already known insertion/deletion polymorphism in the intron of the gene (insertion of actg in C3/J mice) and detected 12 single nucleotide polymorphisms (SNPs) in the region 1067 bp upstream of the start codon (B6>C3/J sequence: -1041 t>c; -1034 g>a, -876 c>t, -837 t>c, -823 a>g, -596 c>t, -360 c>t, -328 g>t, -260 t>c, -244 g>c, -121 a>del, -49 c>t). *In silico* analysis[26] revealed that 11 SNPs potentially alter binding sites for transcription factors, including sites for STAT1 (present in C3/J at position -823 but not in B6), for BCL6 (present only in B6 mice at position -244), SP2 (present only in the C3 promoter at -244), and for PPARγ (present only in B6 at -360; present in C3 at -876).

Whether the detected promoter polymorphisms affect gene expression was investigated in RAW264.7 cells transfected with a *Cd14*-promoter/luciferase reporter vector construct and stimulated with LPS (0.1 ng/ml). After 48 hours, a higher basal activity as well as higher activity upon LPS stimulation of the C3 promoter was observed (Fig. 6), which is consistent with our expression data of the *Cd14* gene.

DISCUSSION

The mouse *Cd14* gene was identified as a potential candidate gene for modification of colitis susceptibility in experimental IBD by a combination of QTL mapping and microarray analyses in previous studies.[14] *Cd14* is located within the interval of the B6-derived QTL *Cdcs6* on chromosome 18, the human homologue within a susceptibility region for human IBD on chromosome 5q (*IBD5*). *Cd14* was considered to be a good candidate gene because of expression differences between B6- (low *Cd14* expression, presumably conferring susceptibility) and C3/J-*Il10*$^{-/-}$ mice (high *Cd14* expression, presumably conferring resistance) as well as between the respective WT strains in microarray analyses, real-time RT PCR, and at protein level in the intestine. Furthermore, CD14 was found to be differentially expressed on peritoneal macrophages after antigen stimulation. This study was designed to investigate if *Cd14* expression in the gut modifies IBD susceptibility.

Although the role of *Cd14* in normal gut was undetermined, there was evidence that murine colonic epithelial cells do express mCD14,[21] and this protein was even detected on a minority of resident macrophages in normal human intestine.[22] Human epithelial cell lines were shown to produce sCD14.[27] Under inflammatory conditions, CD14 expression increases in the gut in humans[28] and mice[21]. We have observed only weak expression of mCD14 in normal colon and ileum mucosa by FACS, exclusively on epithelial cells, but no strain

difference was detectable. By immunohistochemistry, mCD14 positive cells were detected only in inflamed intestinal tissue. As this was contradictory to our previous results, we investigated if the soluble form of this protein is present in the gut, which was not done to our best knowledge before. Therefore, colonic tissue and crypt cells of B6 and C3/J mice were cultured and sCD14 was measured in the supernatant by ELISA. Significantly higher levels of sCD14 were secreted by colonic tissue and isolated crypt cells of C3/J mice, explaining our previous observations. Serum levels of sCD14 and *Cd14* expression in the liver (the main production site of serum sCD14) did not differ between B6 and C3/J mice excluding the possibility that a carry-over of serum sCD14 caused different sCD14 concentrations measured in culture supernatant.

In humans, a -260C>T (also known as -159C>T) polymorphism in the promoter of the *CD14* gene was shown to be associated with Crohn's disease[1-3] and ulcerative colitis[3-5], or showed interaction with polymorphisms in the *CARD15* gene[2,6]; however, in other studies an association to IBD was not detected,[7,29] underlining genetic heterogeneity in IBD patients. LeVan and colleagues showed that the CD14/-159 T allele decrease binding of the repressor SP2;[30] however, the effect of this polymorphism is still not fully elucidated. We have detected 13 SNPs in the 5'-flanking region of the *Cd14* gene between the B6 and C3/J mouse strains. *In silico* analysis revealed that some SNPs affect putative transcription factor-binding sites; the presence of activating elements (binding site for STAT1[31]) in C3/J and inhibitory elements (binding sites for PPARγ[32] and BCL6[33] near the start codon) in B6 are in concordance with the enhanced activity of the C3 promoter as determined by luciferase assay. A potential binding site for SP2 protein is given in C3/J but not in B6 mice. If SP2 indeed decreases *Cd14* expression, other TF are likely of greater importance in our model.

Our results obtained in the acute DSS and the *Il10*$^{-/-}$ models strongly indicate that *Cd14* plays a role in mediating susceptibility to experimental IBD, with high CD14 expression being protective. This is in concordance with previous mapping data, as *Cd14* is located within the B6-derived susceptibility locus *Cdcs6* and B6 mice display low expression of CD14, irrespectively of the *Il10* status. As sCD14 at the concentration measured in the intestine had an effect on TLR2 and TLR4 signalling, it might enhance known effects mediated by these innate immune receptors in the gut. TLR signaling and subsequent NFκB activation by commensal bacteria induce homeostatic effects in the intestine, as shown for epithelial NEMO (IkappaB kinase-gamma)-deficient mice that are characterized by an increased apoptosis of colonic epithelial cells, decreased expression of antimicrobial peptides, and bacterial translocation into the mucosa; subsequently these mice develop IBD spontaneously due to the lack of NFκB activation in epithelial cells.[34] However, under proinflammatory conditions, detrimental effects have been observed by TLR activation. This contrasting effects were investigated in detail for bacterial DNA motifs.[35,36] Furthermore,

germ-free mice develop more severe colitis upon DSS-treatment than conventional mice,[37,38] while therapeutical treatment with antibiotics was protective in the same colitis model.[39] Further observations on TLR2-,[40] TLR4-,[41-43] TLR9-,[44] and MyD88-[41,45]-deficient mice corroborate these findings. Although the outcome of TLR-deficiency in experimental colitis seems to depend also on the model under investigation (and probably on microflora), mechanistic approaches were already provided by groups that observed increase in colitis susceptibility by TLR-deficiency. These groups showed that MyD88, TLR2, and TLR4 influence intestinal barrier functions including apoptosis and regeneration of epithelial cells as well as bacterial translocation,[40-42] which is in line with the findings in epithelial NEMO-deficient mice.[34] MyD88/TLRs even affect T cell function.[25,46-48]

Effects of TLR, especially TLR4, on epithelial barrier function are already known since long, as *Tlr4*$^{Lps\text{-}d}$ mice are very susceptible to Gram-negative infections[49,50] and even die upon monoassociation with the probiotic *E. coli* Nissle due to massive bacterial translocation.[51] Similarly, blocking of CD14 with antibodies during experimental *Shigella flexneri* infection resulted in a 50-fold increase in bacterial invasion and more severe tissue injury of the intestinal mucosa.[52] Our data also indicate that epithelial barrier function might be affected by CD14-deficiency, as extend of ulceration was significantly increased in *Cd14*$^{-/-}$ compared to WT mice in the DSS model combined with elevated IL1β-levels in the serum and because of the very early onset of intestinal inflammation in *Il10*$^{-/-}$*Cd14*$^{-/-}$ mice. As *Il10*$^{-/-}$ deficient mice need environmental/chemical triggers to develop colitis,[53,54] an impact of the *Cdcs6*-QTL and *Cd14*, respectively, on mucosal barrier functions might explain similar findings in both the DSS-induced model, that is primarily based on a toxic effect on epithelial cells,[55] and the *Il10*$^{-/-}$ model, in which colitis is induced by a dysregulation of adaptive immune functions.[56]

As described above, sCD14 leads to a pro-inflammatory response in cells that do not express mCD14,[17] but also shows anti-inflammatory effects, e.g. during sepsis; this anti-inflammatory effect of sCD14 was observed upon very high concentrations of sCD14, up to 100-fold of what we have detected in intestinal mucosa.[18] We tested *in vitro* whether sCD14 at concentrations measured in intestinal mucosa affects the pro-inflammatory response of RAW264.7 cells upon stimulation with LPS or PGN. Our data showed that an increase in TNF secretion by RAW264.7 cells occurred in the presence of sCD14, especially at low concentrations of LPS and PGN. Furthermore, a dose dependent effect of CD14 was observed when RAW264.7 cells were stimulated with LPS (data not shown). These findings correspond to data from LPS-stimulated peritoneal macrophages from transgenic mice with low and high expression of human recombinant CD14.[19] Together with our finding that CD14 plays a protective role in experimental IBD, these data are in line with the previously

mentioned studies showing that TLR and NFκB activation is not only detrimental but also exert protective effects because of their requirement for intestinal homeostasis.[34,45]

In summary, we have shown that differential expression of CD14 in the B6 and C3 background in normal intestine is likely explained by the soluble form of this protein secreted by intestinal epithelial cells, that expression differences are caused by strain specific promoter sequences, and that sCD14 alters the TNF-response of a macrophage cell line upon stimulation with low doses of LPS and PGN. Furthermore, we have demonstrated that presence of CD14 is protective in experimental IBD by using two different mouse models. This observation corroborates the hypothesis that expression differences contribute to strain specific susceptibilities to experimental IBD and support the concept that human *CD14* promoter polymorphisms contribute to disease development. Most likely, CD14 mediates its effect through innate immune mechanisms, possibly by enhancing intestinal barrier functions. Thereby, this study underlines the importance of innate mechanisms in aetiology and development of therapeutic strategies in IBD.

Acknowledgements*:* This work was supported by grants from the DFG (SFB621, HH*)* and Hannover Medical School (HILF, AB). The authors gratefully acknowledge the excellent technical assistance of A. Smoczek and E. Wiebe.

FIGURE LEGENDS

FIGURE 1. Representative histological lesions in ceaca of DSS-treated (A-F) and *Il10*$^{-/-}$ (G-K) mice. Pronounced oedema and inflammatory cell infiltration in mucosa and submucosa, complete loss of crypts and large ulcers were typically observed in DSS-treated C3/N-*Cd14*$^{-/-}$ mice (A-C), while less severe loss of crypts, milder infiltration of inflammatory cells and only small ulcers were detected in caeca of C3/N WT mice (D-F). Arrows indicate edges of ulcers. In 12-week-old *Il10*$^{-/-}$*Cd14*$^{-/-}$ double deficient mice (G-H), severe hyperplasia, ulceration, and massive transmural inflammatory cell infiltration with abundant polymorphonuclear cells were typically observed, while age matched *Il10*$^{-/-}$ mice carrying the WT *Cd14* gene (J-K) showed only small, focal lesions with no ulceration, only mild hyperplasia and few inflammatory (mainly mononuclear) cells limited to the mucosa. Note that sections in G and J were taken at the same magnification. Original magnifications in A/D/G/J, B/E, and C/F/H/K 50x, 100x, and 200x, respectively. Bars in A, B, D, E, G, J = 100 µm, in C, F, H, K = 50 µm.

FIGURE 2. Grading severity of DSS-induced inflammation. **A)** Histological scores for DSS-induced lesions in the large intestine (p-values determined by Mann-Whitney U-tests). *Cd14*$^{-/-}$ mice showed more severe lesions than WT mice in the caecum (C3/N background), mid colon (significant differences in the C3/N background), and distal colon (significantly different in the B6 background). Furthermore, C3/N-*Cd14*$^{-/-}$ showed significantly higher scores for the total intestine (including caecum and all parts of the colon). **B)** Levels of IL1β were significantly increased in C3/N-*Cd14*$^{-/-}$ compared to C3/N WT mice upon DSS treatment (t-test). **C)** Extent of caecal ulceration and mean ulcer size in *Cd14*$^{-/-}$ and WT mice. *Cd14*$^{-/-}$ mice displayed more area of ulceration in their caeca and ulcers in *Cd14*$^{-/-}$ mice were larger than in WT mice (p-values determined by t-test).

FIGURE 3. Intestinal inflammation in B6-*Il10*$^{-/-}$*Cd14*$^{-/-}$ mice. *Il10*$^{-/-}$ mice develop significant more severe typhlitis and colitis when they carry the *Cd14* null-mutation in contrast to one or two copies of the *Cd14* WT allele after 12 and 25 weeks (Mann-Whitney U-tests; n = 5-8).

FIGURE 4. *Cd14* gene expression in intestine and liver. **A)** Detection of mCD14 on cells isolated from the colon and the ileum by FACS. mCD14 was only expressed by a fraction of intestinal epithelial cells (IEC). **B)** Freshly isolated peritoneal cells served as a positive control for anti-CD14 staining and FACS analysis, respectively, and showed the expected fluorescence signal. **C)** Detection of sCD14 in supernatant of colonic tissue and crypt cell cultures. Colonic tissue and crypt cells of C3/J-*Il10*$^{-/-}$ mice secreted significantly higher levels of sCD14 than those of B6-*Il10*$^{-/-}$ mice after 6h of culture (t-test; n = 5 tissue cultures, n = 6

crypt cell cultures). **D)** *Cd14* mRNA levels in the liver of germ-free (GF) or SPF mice. Strain differences were not significant (p=0.021), but SPF mice expressed significantly more *Cd14* than GF mice (determined by two-way ANOVA; n = 5).

FIGURE 5. Effect of sCD14 on TLR2 and TLR4 responses of RAW264.7 cells. TNF-secretion of RAW264.7 cells was determined after 6 h stimulation with LPS or PGN in presence (50 ng/ml) or absence of sCD14. At low LPS (0.1, 1.0 ng/ml) and PGN (10.0, 100.0 ng/ml) concentrations, sCD14 enhanced TNF response significantly (t-tests; n = 4 cultures).

FIGURE 6. Activity of the B6 and C3/J *Cd14*-promoters in RAW264.7 cells cultured for 48 h. The C3/J promoter showed significant more basal acitvity and more activity upon LPS stimulation than the B6 promoter ($p<0.0001$, determined by ANOVA; n = 4). Activity of the control vector pGL4.10 (pGL) is shown.

REFERENCES

1. Klein W, Tromm A, Griga T, et al. A polymorphism in the *CD14* gene is associated with Crohn disease. Scand J Gastroenterol. 2002;37:189-191

2. Gazouli M, Mantzaris G, Kotsinas A, et al. Association between polymorphisms in the Toll-like receptor 4, *CD14*, and *CARD15/NOD2* and inflammatory bowel disease in the Greek population. World J Gastroenterol. 2005;11:681-685

3. Baumgart DC, Buning C, Geerdts L, et al. The c.1-260C>T promoter variant of *CD14* but not the c.896A>G (p.D299G) variant of toll-like receptor 4 (*TLR4*) genes is associated with inflammatory bowel disease. Digestion. 2007;76:196-202

4. Obana N, Takahashi S, Kinouchi Y, et al. Ulcerative colitis is associated with a promoter polymorphism of lipopolysaccharide receptor gene, *CD14*. Scand J Gastroenterol. 2002;37:699-704

5. Wang F, Tahara T, Arisawa T, et al. Genetic polymorphisms of *CD14* and Toll-like receptor-2 (*TLR2*) in patients with ulcerative colitis. J Gastroenterol Hepatol. 2007;22:925-929

6. Klein W, Tromm A, Griga T, et al. Interaction of polymorphisms in the *CARD15* and *CD14* genes in patients with Crohn disease. Scand J Gastroenterol. 2003;38:834-836

7. Peters KE, O'Callaghan NJ, Cavanaugh JA. Lack of association of the *CD14* promoter polymorphism--159C/T with Caucasian inflammatory bowel disease. Scand J Gastroenterol. 2005;40:194-197

8. Torok HP, Glas J, Tonenchi L, et al. Polymorphisms of the lipopolysaccharide-signaling complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis. Clin Immunol. 2004;112:85-91

9. Canto E, Ricart E, Monfort D, et al. TNF alpha production to TLR2 ligands in active IBD patients. Clin Immunol. 2006;119:156-165

10. Baldini M, Lohman IC, Halonen M, et al. A Polymorphism in the 5' flanking region of the *CD14* gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am J Respir Cell Mol Biol. 1999;20:976-983

11. Griga T, Klein W, Epplen JT, et al. CD14 expression on monocytes and soluble CD14 plasma levels in correlation to the promotor polymorphism of the endotoxin receptor CD14 gene in patients with inactive Crohn's disease. Hepatogastroenterology. 2005;52:808-811

12. Farmer MA, Sundberg JP, Bristol IJ, et al. A major quantitative trait locus on chromosome 3 controls colitis severity in IL-10-deficient mice. Proc Natl Acad Sci U S A. 2001;98:13820-13825

13. Mähler M, Most C, Schmidtke S, et al. Genetics of colitis susceptibility in IL-10-deficient mice: backcross versus F2 results contrasted by principal component analysis. Genomics. 2002;80:274-282

14. de Buhr MF, Mähler M, Geffers R, et al. *Cd14*, *Gbp1*, and *Pla2g2a*: three major candidate genes for experimental IBD identified by combining QTL and microarray analyses. Physiol Genomics. 2006;25:426-434

15. Bleich A, Mähler M, Most C, et al. Refined histopathologic scoring system improves power to detect colitis QTL in mice. Mamm Genome. 2004;15:865-871

16. Gupta D, Kirkland TN, Viriyakosol S, et al. CD14 is a cell-activating receptor for bacterial peptidoglycan. J Biol Chem. 1996;271:23310-23316

17. Pugin J, Schurer-Maly CC, Leturcq D, et al. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A. 1993;90:2744-2748

18. Kitchens RL, Thompson PA, Viriyakosol S, et al. Plasma CD14 decreases monocyte responses to LPS by transferring cell-bound LPS to plasma lipoproteins. J Clin Invest. 2001;108:485-493

19. Jacque B, Stephan K, Smirnova I, et al. Mice expressing high levels of soluble CD14 retain LPS in the circulation and are resistant to LPS-induced lethality. Eur J Immunol. 2006;36:3007-3016

20. Lee JW, Paape MJ, Elsasser TH, et al. Recombinant soluble CD14 reduces severity of intramammary infection by Escherichia coli. Infect Immun. 2003;71:4034-4039

21. Ortega-Cava CF, Ishihara S, Rumi MA, et al. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. J Immunol. 2003;170:3977-3985

22. Rogler G, Hausmann M, Vogl D, et al. Isolation and phenotypic characterization of colonic macrophages. Clin Exp Immunol. 1998;112:205-215

23. Nicklas W, Baneux P, Boot R, et al. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim. 2002;36:20-42

24. Liedtke C, Groger N, Manns MP, et al. The human caspase-8 promoter sustains basal activity through SP1 and ETS-like transcription factors and can be up-regulated by a p53-dependent mechanism. J Biol Chem. 2003;278:27593-27604

25. Obermeier F, Strauch UG, Dunger N, et al. In vivo CpG DNA/toll-like receptor 9 interaction induces regulatory properties in CD4+CD62L+ T cells which prevent intestinal inflammation in the SCID transfer model of colitis. Gut. 2005;54:1428-1436

26. Cartharius K, Frech K, Grote K, et al. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics. 2005;21:2933-2942

27. Funda DP, Tuckova L, Farre MA, et al. CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: lipopolysaccharide activation of epithelial cells revisited. Infect Immun. 2001;69:3772-3781

28. Rogler G, Andus T, Aschenbrenner E, et al. Alterations of the phenotype of colonic macrophages in inflammatory bowel disease. Eur J Gastroenterol Hepatol. 1997;9:893-899

29. Arnott ID, Nimmo ER, Drummond HE, et al. *NOD2/CARD15*, *TLR4* and *CD14* mutations in Scottish and Irish Crohn's disease patients: evidence for genetic heterogeneity within Europe? Genes Immun. 2004;5:417-425

30. LeVan TD, Bloom JW, Bailey TJ, et al. A common single nucleotide polymorphism in the *CD14* promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. J Immunol. 2001;167:5838-5844

31. Schreiber S, Rosenstiel P, Hampe J, et al. Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. Gut. 2002;51:379-385

32. Dubuquoy L, Jansson EA, Deeb S, et al. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. Gastroenterology. 2003;124:1265-1276

33. Takeda N, Arima M, Tsuruoka N, et al. Bcl6 is a transcriptional repressor for the IL-18 gene. J Immunol. 2003;171:426-431

34. Nenci A, Becker C, Wullaert A, et al. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature. 2007;446:557-561

35. Obermeier F, Dunger N, Strauch UG, et al. CpG motifs of bacterial DNA essentially contribute to the perpetuation of chronic intestinal inflammation. Gastroenterology. 2005;129:913-927

36. Bleich A, Janus LM, Smoczek A, et al. CpG Motifs of Bacterial DNA Exert Protective Effects in Mouse Models of IBD by Antigen-Independent Tolerance Induction. Gastroenterology. 2009;136:278-287

37. Kitajima S, Morimoto M, Sagara E, et al. Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. Exp Anim. 2001;50:387-395

38. Greweling MC, Rozell B, Bleich A, et al. Commensal Gut Flora Reduces Susceptibility to DSS Colitis Most Likely Via T-cell derived Interleukin-10 (Abstract). Wien Klin Wochenschr. 2008;120 (Supplement 1):360-368

39. Hans W, Schölmerich J, Gross V, et al. The role of the resident intestinal flora in acute and chronic dextran sulfate sodium-induced colitis in mice. Eur J Gastroenterol Hepatol. 2000;12:267-273

40. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology. 2007;132:1359-1374

41. Fukata M, Michelsen KS, Eri R, et al. Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. Am J Physiol Gastrointest Liver Physiol. 2005;288:G1055-1065

42. Fukata M, Chen A, Klepper A, et al. Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine. Gastroenterology. 2006;131:862-877

43. Fine S, Gonzalez Navajas J, Shen C, et al. The protective role of TLR4 signaling in IL-10 dependent colitis. Gastroenterology. 2007;132:A124

44. Lee J, Mo JH, Katakura K, et al. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol. 2006;8:1327-1336

45. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, et al. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004;118:229-241

46. Fukata M, Breglio K, Chen A, et al. The Myeloid Differentiation Factor 88 (MyD88) Is Required for CD4+ T Cell Effector Function in a Murine Model of Inflammatory Bowel Disease. J Immunol. 2008;180:1886-1894

47. Liu G, Zhao Y. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells. Immunology. 2007;122:149-156

48. Bleich A, Janus LM, Smoczek A, et al. CpG Motifs of Bacterial DNA Exert Protective Effects in Mouse Models of IBD by Antigen-Independent Tolerance Induction. Gastroenterology. 2008

49. Branger J, Knapp S, Weijer S, et al. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect Immun. 2004;72:788-794

50. Bleich A, Kirsch P, Sahly H, et al. *Klebsiella oxytoca*: opportunistic infections in laboratory rodents. Lab Anim. 2008;42:369-375

51. Bleich A, Sundberg JP, Smoczek A, et al. Sensitivity to *Escherichia coli* Nissle 1917 in mice is dependent on environment and genetic background. Int J Exp Pathol. 2008;89:45-54

52. Wenneras C, Ave P, Huerre M, et al. Blockade of CD14 aggravates experimental shigellosis. J Endotoxin Res. 2001;7:442-446

53. Bleich A, Mähler M. Environment as a critical factor for the pathogenesis and outcome of gastrointestinal disease: experimental and human inflammatory bowel disease and *Helicobacter*-induced gastritis. Pathobiology. 2005;72:293-307

54. Berg DJ, Zhang J, Weinstock JV, et al. Rapid development of colitis in NSAID-treated IL-10-deficient mice. Gastroenterology. 2002;123:1527-1542

55. Okayasu I, Hatakeyama S, Yamada M, et al. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology. 1990;98:694-702

56. Kühn R, Löhler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993;75:263-274