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A Synthetic TLR4 Antagonist Has Anti-Inflammatory Effects in Two Murine Models of Inflammatory Bowel Disease

Madeline M. Fort,* Afsaneh Mozaﬀarian,* Axel G. Stöver,2* Jean da Silva Correia,† David A. Johnson,‡ R. Thomas Crane,‡ Richard J. Ulevitch,† David H. Persing,*§ Helle Bielefeldt-Ohmann,¶ Peter Probst,* Eric Jeffery,* Steven P. Fling,3* and Robert M. Hershberg4*§

Current evidence indicates that the chronic inﬂammation observed in the intestines of patients with inﬂammatory bowel disease is due to an aberrant immune response to enteric flora. We have developed a lipid A-mimetic, CRX-526, which has antagonistic activity for TLR4 and can block the interaction of LPS with the immune system. CRX-526 can prevent the expression of proinflammatory genes stimulated by LPS in vitro. This antagonist activity of CRX-526 is directly related to its structure, particularly secondary fatty acyl chain length. In vivo, CRX-526 treatment blocks the ability of LPS to induce TNF-α release. Importantly, treatment with CRX-526 inhibits the development of moderate-to-severe disease in two mouse models of colonic inﬂammation: the dextran sodium sulfate model and multidrug resistance gene 1a-deﬁcient mice. By blocking the interaction between enteric bacteria and the innate immune system, CRX-526 may be an effective therapeutic molecule for inﬂammatory bowel disease. The Journal of Immunology, 2005, 174: 6416–6423.

Inflammatory bowel disease (IBD)5 encompasses two distinct chronic inﬂammatory diseases of the intestine in humans: ulcerative colitis and Crohn’s disease. These are debilitating diseases of unknown etiology and for which there exist only limited therapeutic options (1, 2). Recently, there has been growing evidence that IBD results from abnormal immune responses to normal gut bacterial ﬂora. The human intestinal tract contains hundreds of different bacterial species that are largely tolerated by the host. It is hypothesized that this tolerance to enteric bacteria exists because of the effectiveness of the epithelial barrier in separating the mucosal immune system and enteric Ags and/or because of active or indirect suppression of mucosal immune responses to such Ags. There is some evidence that patients with IBD can have strong immune responses to enteric ﬂora, although these responses are absent in people without IBD (3, 4). In addition, allelic variation in the gene for NOD2, a pattern recognition receptor (PRR) that binds muramyl dipeptide, has been associated with susceptibility to Crohn’s disease (5–7). Although no single bacterial speciﬁes has been associated with the development of either Crohn’s disease or ulcerative colitis, probiotic therapies, which aim to decrease disease severity by changing the ﬂora of the gut, are currently being tested in various clinical trials and have shown some efﬁcacy (8). The strongest evidence for the role of bacteria in the development of IBD comes from the myriad of mouse models of colonic inﬂammation. Many of these murine models clearly show a dependence on the presence of enteric bacteria for the inﬂammatory process: when rendered germfree, these mice do not develop colitis (9). Therefore, it may be possible to ameliorate IBD by blocking the ability of the mucosal immune system from responding to bacterial Ags.

The innate immune system recognizes the presence of speciﬁc bacterial Ags through pattern recognition receptors (PRR). TLR4 is one of an extensive family of PRR that have been found in Drosophila melanogaster and mammals, and compelling research has shown that LPS, which is the major component of the outer membrane of Gram-negative bacteria, binds to TLR4 (10–12). The recognition of LPS requires a complex interaction of LPS with TLR4, MD-2, and CD14 (13–15). The triggering of TLR4R complex signaling by LPS results in a cascade of events that leads to the secretion of proinflammatory mediators from monocytes and dendritic cells, which ultimately leads to the activation of the acquired immune response (16, 17). In addition to LPS, there is evidence that other ligands may bind to the TLR4R complex, particularly molecules such as hyaluronic acid and heparan sulfate that are present during active inﬂammation (18). Thus, signaling through the TLR4R complex actively contributes to the development of inﬂammation and may help to maintain an ongoing inﬂammatory response. Therefore, in conditions of uncontrolled inﬂammation, blocking TLR4 signaling may be beneﬁcial.

CRX-526 is a synthetic lipid A mimetic molecule, also known as an aminoalkyl-glucosamine-phosphate (AGP) (Refs. 19 and 20; see Fig. 1). Lipid A is the active component of LPS that binds to TLR4, and AGP were developed to study the importance of structure on the function of lipid A (21, 22). We have established previously that CRX-526, unlike lipid A or other AGP, does not...
stimulate cytokine production or other gene expression in human peripheral blood monocytes in vitro or induce an inflammatory response in vivo and that this lack of proinflammatory activity is directly related to the length of its secondary fatty acyl chains (SAC) (22). In the present study, we show that CRX-526 is an antagonist for the TLR4R complex and can block the proinflammatory actions of LPS both in vitro and in vivo. This antagonist activity directly depends on the presence of a hexanoic SAC in the left and middle positions of the molecule. Furthermore, we were able to demonstrate the potential importance of the TLR4R complex in IBD as treatment with CRX-526 successfully inhibited the development of moderate-to-severe disease in the dextran sodium sulfate (DSS) and in the multidrug resistance gene 1a (MDR1a)-deficient mice.

Materials and Methods

CRX-526, CRX-567, CRX-568, and CRX-570

The synthesis of CRX-526 and other AGP has been described previously (19, 22). Briefly, the AGP were prepared by a highly convergent method, which allowed chemical differentiation of the hydroxyl and amino groups and sequential introduction of the (R)-3-n-alkanoyloxytetrahexanoyl residues. The AGP were purified by flash chromatography on silica gel (≥95% purity) and analyzed as a triethylammonium salt by standard analytical methods. For stimulation in vitro, the AGP were formulated in water containing 2/6 µg/ml dipalmitoylphosphatidyl choline (aqueous formulation (AF)), 0.2% triethanolamine (TEoA, pH 7.4), or in 2% glycerol (i.e. formulation).

Animals and reagents

Female BALB/cAnN mice (5–7 wk old) were obtained from Charles River Laboratories; female BALB/cJ mice (5–7 wk old) and C3H.Tlr4Ltpr-Lps-d/J mice (4–6 wk old) were obtained from The Jackson Laboratory; and female MDR1a-deficient mice (5–6 wk old) were obtained from Taconic Farms. LPS from Escherichia coli 0127:B8 (Sigma-Aldrich) was reconstituted in water at a concentration of 1 mg/ml and frozen at −20°C until use.

For cytokine-specific ELISA, the following mAb pairs (BD Biosciences) were used: IL-6 capture no. 18871D, detection no. 18882D; IL-12 detection no. 20512D, TNF-α capture no. 18631D, and detection no. 18642D. The IL-12p70 capture Ab no. 24910.1 was purchased from R&D Systems, and the MIP-1α CytoSource ELISA kit (no. CHC2204) was purchased from BioSource International. Murine TNF-α was determined with the mTNF-α Quantikine kit (R&D Systems).

Human monocyte-derived macrophages stimulation with LPS

Human PBMC were isolated by Ficoll-Hypaque 1.077 (Sigma-Aldrich) and centrifugation of leukapheresis product before aliquoting and freezing. Monocytes and monocyte-derived macrophages were prepared by an adherent step as described previously (22, 23). Phenotypic analysis of the cells revealed that >95% of the cells are CD14 positive. Stimulation of human monocyte-derived macrophages in the presence of LPS and/or AGP has been described previously in detail (22, 23). Briefly, monocyte-derived macrophages were exposed to CRX-526 at various concentrations. Thirty minutes later, LPS was added to the cultures. After 6 h of stimulation, the supernatant was removed and analyzed by ELISA for the presence of TNF-α. RNA was isolated from adherent cells as previously described for the generation of cDNA for microarray analysis (22).

Inhibition of LPS-induced cytokine production in monocytes

Human monocytes were generated from 4 × 10^6 PBMC by an adherent step and were cultured for 48 h in RPMI 1640 medium containing 2% human AB serum. For experiments determining IL-12p70 production, the culture medium was supplemented with IFN-γ (1000 U/ml) for the last 24 h. After incubating with CRX-526 for 30 min, monocytes were stimulated with 100 ng/ml E. coli LPS. Cytokines and MIP-1α were determined in supernatants taken after 18 h. Quantification of IL-6, IL-12p70, and TNF-α production was done by cytokine-specific ELISA as described previously (23). The working sensitivity of all ELISA was shown to be ~10 pg/ml. MIP-1α and murine TNF-α levels were determined by using ELISA kits, according to the manufacturer’s protocol. Cytokines were not detectable in the absence of stimulation.

Microarray analysis

A detailed description of the microarray analysis can be found elsewhere (22). Briefly, ~1200 PCR products and controls were spotted onto 42 CMTR-GAPS2-coated slides in triplicate (Corning Glass) using an Affymetrix 417 arrayer (Affymetrix). Fluorescently labeled probes were generated from 1.5 µg of cDNA amplified as described above. The Cy3 (treated)- or Cy5 (untreated)-labeled probes were generated using the amino-allyl method as previously described (22) and included in the microarray resources section of the Institute of Genomic Research web site: (http://pga.tigr.org/PDF/BiotechniquesCook-book, II.pdf). Hybridizations and posthybridization washes were conducted as described by the slide manufacturer (Corning Glass) for DMSO arrays using a formamide hybridization buffer. Slides were scanned using an Affymetrix 418 microarray scanner and saved as 16-bit TIFF files (Affymetrix). The Cy3 and Cy5 images for each array were overlaid, gridded, and quantified using Imagene software (Biodiscovery). Hybridization data quality was evaluated using the mean signal-to-noise ratios for all cDNA spots, the mean signal-to-noise ratios for control spots, treated-to-untreated ratios for control and spike-in spots, and spot morphology. Normalization and additional data analysis was performed using GeneSpring (Silicon Genetics). The hybridization intensity of each spot was normalized to the median intensity of all noncontrol spots on the array.

HeLa cell transfection and reporter assay

A previously described HeLa transient transfection system was used to test the ability of CRX-526, CRX-567, CRX-568, and CRX-570 to act as an antagonist/agonist for the TLR4-MD2-CD14R complex (14, 22). Briefly, HeLa cells were transfected with 50 ng of IL-8 promoter-derived luciferase reporter and 10 ng each of TLR4, MD-2, and CD14 plasmids. Eighteen hours after transfection, cells were stimulated with varying concentrations of AGP in the presence of vehicle or 100 ng/ml LPS for 6 h, lysed, and then analyzed for luciferase and β-galactosidase activity.

DSS model of intestinal inflammation

Two separate protocols were used to cause DSS-induced intestinal inflammation, depending on the strain of mice and the dose of DSS (ICN Biomedicals) used. For experiment no. 1 (see Fig. 1), BALB/cAnN females were given 4% DSS (lot no. 43379F) in their water ad libitum starting on day 0, with all mice sacrificed on day 7. Negative control animals remained on standard facility water. Starting on day 0, mice were given s.c. injections of either AF vehicle or varying doses of CRX-526-AF every other day for

FIGURE 1. The chemical structures of MPL and CRX-526.
a total of four doses (n = 5/group). For experiment no. 2 and Fig. 5, BALB/cJ females and C57Bl6 Tlr4<sup>-/-</sup>/J mice were given 3.0% DSS (lot no. 8296F) in their drinking water from days 0 to 7 and were s.c. injected daily with either AF-vehicle or 50 μg of CRX-526-AF from days 3 through 6 for a total of nine doses (n = 10/group). Disease activity index (DAI), a clinical score that factors in weight loss, changes in stool consistency, and the presence of blood in the stool was assessed by a previously published method (24). Paraffin-embedded sections of the large intestines, prepared as “Swiss rolls” (25), were stained with H&E, and histological scoring of disease in the cecums and colons was determined in a blinded fashion by a certified veterinary pathologist (H. Bielefeldt-Ohmann). Briefly, the cecum, proximal colon, mid-colon, distal colon, and rectum were assessed for loss of mucosal architecture (0–4), the presence of mucoid crypt cysts (0–4), goblet cell depletion (0–4), erosion (0–4), edema (0–4), mononuclear cell infiltration (0–4), polymorphonucleocyte and/or eosinophil infiltration (0–4), and transmural inflammation (0–4). To obtain a total score for each mouse, the total scores for each segment were added together; the maximal possible score is 160.

**Treatment and assessment of colitis in MDR1α-deficient mice**

Starting at 5–6 wk of age, MDR1α-deficient female mice were injected s.c. on a weekly basis with 50 μg (experiment no. 1) or 100 μg (experiment no. 2) of CRX-526-TeoA or with an equal volume of TeoA alone (vehicle treated). Treatment continued for 5–6 wk, and then, mice were sacrificed. Treatment and assessment of colitis in MDR1α-deficient mice were assessed for loss of mucosal architecture (0–4), the presence of mucoid crypt cysts (0–4), goblet cell depletion (0–4), erosion (0–4), edema (0–4), mononuclear cell infiltration (0–4), polymorphonucleocyte and/or eosinophil infiltration (0–4), and transmural inflammation (0–4). To obtain a total score for each mouse, the total scores for each segment were added together; the maximal possible score is 160.

**Results**

**CRX-526 inhibits the activation of human monocytes by LPS in vitro**

We have established previously that CRX-526, though structurally similar to lipid A (Fig. 1), did not by itself induce the production of proinflammatory cytokines from human monocytes (22). To determine whether CRX-526 interacts with the TLR4R complex, we tested whether CRX-526 could antagonize the proinflammatory properties of a known TLR4 ligand, LPS. Human monocyte-derived macrophages were preincubated with increasing concentrations of CRX-526 and then exposed to various concentrations of LPS. Preincubation of macrophages with CRX-526 resulted in an inhibition of TNF-α production after exposure to LPS (Fig. 2A). In agreement with our previous studies, incubation of CRX-526 alone with macrophages did not result in any TNF-α production (Ref. 22; data not shown). A minimum w/w ratio of 5:1 CRX-526: LPS was necessary to see a clearly detectable inhibition of TNF-α production; a w/w ratio of 50:1 was required to completely abolish TNF-α production in response to LPS. Preincubation of human monocytes with CRX-526 also resulted in inhibition of IL-6, MIP-1α, and production after exposure to LPS (Fig. 2B). Because

**FIGURE 2.** CRX-526 inhibits LPS stimulation of human monocytes and monocyte-derived macrophages. A, Dose dependency of CRX-526 inhibition of LPS. TNF-α produced by human monocyte-derived macrophages exposed to 10 ng/ml LPS alone ( ), LPS + 1 μg/ml ( ), 5 μg/ml ( ), or 10 μg/ml ( ) CRX-526. The data of one of three separate experiments with similar results is shown. B, Inhibition of IL-6 and Mip-1α. Supernatants from monocytes incubated with CRX-526 (30 min) and then stimulated with 100 ng/ml E. coli LPS were analyzed by ELISA for IL-6 ( ), Mip-1α ( ), and TNF-α ( ). Data are presented as percent inhibition of CRX-526 treatment compared with LPS stimulation without AGP (medium). Results are shown as means ± SD for the two donors tested. C, CRX-526 inhibition of IFN-γ-activated monocytes. Human monocytes were cultured with IFN-γ (1000 U/ml for 24 h) and then incubated with CRX-526 for 30 min before stimulation with 100 ng/ml LPS in the presence IFN-γ. IL-6 ( ), IL-12p70 ( ), and TNF-α ( ) were determined in supernatants taken after 18 h. D, Microarray analysis of changes in gene expression in human monocyte-derived macrophages stimulated with LPS in the presence or absence of CRX-526 or with CRX-526 alone.

**Statistics**

For statistical analyses of in vivo experiments, data were analyzed by the Mann-Whitney nonparametric test using Prism software (GraphPad Software). Values of p ≤ 0.05 were deemed statistically significant.
IFN-γ activation of monocytes not only enhances the secretion of TNF-α but also is required for the production of biological active IL-12p70 in response to LPS (23). We determined the inhibitory effect of CRX-526 on the LPS-induced cytokine secretion in IFN-γ-activated monocytes (Fig. 2C). In contrast to resting monocytes where the secretion of IL-6 and TNF-α were inhibited at similar levels (Fig. 2B), CRX-526 had a differential inhibitory effect on the cytokine secretion of IFN-γ-activated monocytes in response to LPS (Fig. 2C). A 10:1 w/w ratio of CRX-526 to LPS inhibited IL-12p70 and TNF-α production by 79 and 57%, respectively, but had no effect on the LPS-induced IL-6 production. In a murine system, CRX-526 inhibited the LPS-induced production of IL-6, IL-12p70, and TNF-α by murine splenic dendritic cells (data not shown), indicating that CRX-526 inhibits LPS signaling through either human or murine TLR4R complexes. Antagonism of LPS signaling by CRX-526 in human dendritic cells has also been demonstrated (P. Probst, unpublished observations).

To confirm that CRX-526 is a pure antagonist and does not induce any gene transcription by signaling through the TLR4R complex, we used a custom microarray containing ~300 inflammatory gene targets arranged in triplicate to look at mRNA expression in human monocytes after exposure to CRX-526 alone and/or in the presence of LPS (22). Exposure of monocytes to LPS alone (Fig. 2D, left) stimulated the expression of >60 array elements >2-fold, while exposure to CRX-526 alone (Fig. 2D, right) did not induce expression of any genes found on the microarray. Furthermore, pretreatment of monocytes with increasing amounts of CRX-526 before exposure to LPS completely suppressed all LPS-induced gene transcription (Fig. 2D). NF-κB is known to be involved in the transcriptional regulation of many of the elements on this microarray (22). The level of inhibition by CRX-526 of representative NF-κB-regulated genes is summarized in Table I. Thus, CRX-526 can act as an antagonist for the TLR4R complex and can inhibit the ability of LPS to signal through the TLR4R complex.

The antagonistic activity of CRX-526 is dependent on SAC length

The structure of CRX-526 differs significantly from monophosphoryl lipid A (MPL) and other TLR4-agonist AGP in the length of its SAC: CRX-526 contains 3 SAC of 6 carbons in length, whereas MPL and other AGP, which signal through the TLR4R complex by CRX-526, contain SAC of ≥10 carbons in length (Ref. 22; Fig. 1). To confirm whether hexanoic SAC are a requirement for the antagonistic activity of CRX-526, we synthesized molecules that are variants of CRX-526, containing either hexanoic or decanoic SAC at three different positions (Fig. 3). CRX-576 differs from CRX-526 by only a decanoic SAC at the right position, whereas CRX-568 has a decanoic SAC in the middle position, and CRX-570 has a decanoic SAC at the left position (compare Fig. 1 vs Fig. 3). These molecules were then tested for their ability to inhibit LPS signaling through the TLR4R complex. Using a previously described model system (14), HeLa cells were transfected transiently with DNA encoding TLR4, MD-2, and CD14, along with human IL-8 promoter-driver luciferase reporter plasmid and β-galactosidase vector. These transfected cells were stimulated with increasing concentrations of either AGP alone (○) or AGP + 100 ng/ml LPS (■), and luciferase activity was monitored (Fig. 3). CRX-526 inhibited, in a dose-dependent manner, the ability of LPS to signal through the TLR4/MD2/CD14 complex. LPS was not able to stimulate HeLa cells that were transfected with a control vector nor did CRX-526 alone induce a signal in the TLR4/MD2/CD14-transfected cells (Ref. 14; Fig. 3). CRX-576 has antagonistic activity for the TLR4/MD2/CD14R complex similar to CRX-526 (Fig. 3). However, CRX-568 has less antagonistic activity compared with CRX-568 and CRX-569 and even some partial agonist activity. CRX-570 has almost no antagonistic activity and significant agonistic activity. These findings show that a shortened (hexanoic) SAC on the left and middle position of the molecule are required for the inhibition of LPS signaling through the TLR4/MD2/CD14 complex by CRX-526.

CRX-526 can inhibit LPS-induced TNF-α release in vivo

Our data clearly show that CRX-526 can inhibit LPS signaling through the TLR4R complex in vitro. To test whether CRX-526 can also inhibit LPS activity in vivo, we injected LPS i.v. in the presence or absence of CRX-526 into mice and 1 h later measured TNF-α in the serum. We were able to detect significant amounts of TNF-α in the serum of mice given as little as 5 ng of LPS (data not shown). Therefore, we injected mice with either 5 ng of LPS alone or with increasing amounts of CRX-526. Significant inhibition of TNF-α release was seen with as little as 80 ng of CRX-526 (data not shown), and background levels of TNF-α were reached in the presence of 10 μg of CRX-526 (Fig. 4). These data confirm that CRX-526 acts as an antagonist for LPS in vivo as well as in vitro.

Table I. CRX-526 suppression of LPS-induced gene transcription for representative NF-κB-regulated gene elements

<table>
<thead>
<tr>
<th>Element</th>
<th>Gene Description</th>
<th>Treatment (expression level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCYB10</td>
<td>Small inducible cytokine subfamily B member 10</td>
<td>LPS 29.8 LPS/CRX526 0.96</td>
</tr>
<tr>
<td>IL-2Rα</td>
<td>IL-2 receptor, α (IL2RA)</td>
<td>LPS 12.4 LPS/CRX526 0.04</td>
</tr>
<tr>
<td>PTX3; TSG-14</td>
<td>Pentraxin-related gene</td>
<td>LPS 8.14 LPS/CRX526 0.41</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2 (COX-2)</td>
<td>LPS 7.09 LPS/CRX526 0.43</td>
</tr>
<tr>
<td>TAP1</td>
<td>Peptide transporter</td>
<td>LPS 6.27 LPS/CRX526 1.53</td>
</tr>
<tr>
<td>IL-1Rα</td>
<td>IL-1 receptor antagonist</td>
<td>LPS 5.89 LPS/CRX526 1.00</td>
</tr>
<tr>
<td>GRO1</td>
<td>GRO1 oncogene (SCYB1)</td>
<td>LPS 5.33 LPS/CRX526 1.88</td>
</tr>
<tr>
<td>IL-1B</td>
<td>IL-1, β</td>
<td>LPS 3.77 LPS/CRX526 2.15</td>
</tr>
<tr>
<td>CD48</td>
<td>Pan-leukocyte antigen</td>
<td>LPS 3.13 LPS/CRX526 1.04</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>Nuclear factor of κ light polypeptide gene enhancer</td>
<td>LPS 2.75 LPS/CRX526 1.27</td>
</tr>
<tr>
<td>CD95</td>
<td>Apo-1 Fas</td>
<td>LPS 2.41 LPS/CRX526 0.61</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6</td>
<td>LPS 2.18 LPS/CRX526 0.26</td>
</tr>
</tbody>
</table>

*Values were determined from the analysis shown in Fig. 2 for specific microarray elements that have been identified by literature searches as having NF-κB-regulated transcription.

*Hybridization intensities were quantified using Imagene software and normalized and analyzed using Genespring software. Expression levels (fold scale) are expressed for treated relative to untreated controls.
CRX-526 can inhibit the development of disease in two different mouse models of colitis

Because LPS is the major inflammatory portion of the cell membrane of Gram-negative bacteria, we hypothesized that CRX-526 may be able to inhibit the ability of Gram-negative bacteria to cause or perpetrate the inflammation seen in IBD. Therefore, we tested the ability of CRX-526 to inhibit the development of disease in two different mouse models of colonic inflammation: dextran sodium sulfate-induced colitis, and MDR1a-deficient mice.

Exposure to DSS in drinking water causes an acute colitis in mice due to damage to the epithelial layer. DSS-exposed animals show distinct clinical signs, including weight loss, changes in stool consistency, and blood in the stool and/or gross intestinal bleeding, that correlate directly with histological changes of the large intestine (24). Mice that are deficient in TLR4 expression do develop acute colitis when exposed to DSS. However, Lange et al. (26) have observed that TLR4-mutant mouse strains, such as C3H/HeJ and C57BL/10ScN, show delayed onset of intestinal bleeding compared with strain-matched TLR4-wild-type mice and show increased survival upon exposure to DSS for ≥10 days. Their data indicate that TLR4 may play a minor, but detectable, role in DSS-induced colitis. Therefore, we exposed BALB/c females to DSS for 7 days and simultaneously treated the mice with CRX-526. Mice were assessed for clinical signs of colitis (DAI) (24) and for histological changes in the large intestine on day 7. As shown in Fig. 5, exposure to DSS caused the development of significant clinical and histological changes in these mice. Furthermore, in agreement with previous studies, DAI correlated with the histological score. Cotreatment with CRX-526 significantly decreased the histological score at all doses tested but decreased DAI only at the 10- and 50-μg doses (experiment no. 1, Fig. 5A). These data show that blocking TLR4R complex signaling can decrease disease severity in an acute model of colitis and that a decrease in DAI is a rigorous measure of a decrease in disease severity in our model system. A repeat experiment confirmed the ability of 50 μg of CRX-526 to significantly decrease the DAI score in DSS-exposed mice (experiment no. 2, Fig. 5B). To confirm that our results were due to inhibition of signaling through the TLR4R complex, we compared the ability of CRX-526 to affect disease severity in TLR4-wild-type (BALB/c) and TLR4-mutant (C3H.Tlr4Δ/Δ) mice. Both TLR4-wild-type and TLR4-mutant mice developed acute disease when exposed to DSS, although the TLR4-mutant mice had a significantly lower DAI (mean DAI of 1.9 ± 0.4 and 1.4 ± 0.2, respectively, for TLR4-wild-type and TLR4-mutant mice; p = 0.004; Fig. 6). Treatment with 50 μg of CRX-526 by s.c. injection significantly decreased the DAI score in TLR4-wild-type but not TLR4-mutant mice (Fig. 6). These data confirm that...
the decreased disease severity seen in CRX-526-treated animals is due to blocking of TLR4R complex signaling. Interestingly, treatment of TLR4-wild-type mice with CRX-526 brought the DAI score to a level similar to that of the TLR4-mutant mice (mean DAI of 1.4 ± 0.3 for TLR4-mutant mice and 1.3 ± 0.8 for CRX-526-treated TLR4-wild-type mice), suggesting that at the 50-µg dose we obtained an almost complete inhibition of TLR4R complex signaling (Fig. 6).

As DSS exposure induces acute colitis, we wanted to test the efficacy CRX-526 in a chronic model of colitis. MDR1a-deficient mice spontaneously develop chronic inflammation of the colon with age. As the colitis seen in MDR1a-deficient mice can be prevented by treatment with oral antibiotics, this model is clearly dependent on the presence of bacterial Ags in the gut (27). Furthermore, evidence suggests that MDR1a-deficient mice develop colitis due to leakiness in the epithelial barrier of the intestine, which may allow bacterial Ags to stimulate the immune system (27). Therefore, we hypothesized that Gram-negative bacteria may potentially play a role in the development of colitis in MDR1a-deficient mice and that inhibition of signaling through the TLR4R complex could decrease disease severity. We injected s.c. 5- to 7-wk-old MDR1a-deficient female mice, which were free of any clinical signs of colitis, with 50 µg (experiment no. 1) or 100 µg (experiment no. 2) of CRX-526 or with vehicle control for 5 wk and then sacrificed the mice 1 wk after the last dose. The cecum, colon, and rectum of each animal were examined histologically for the extent and severity of inflammation. As shown in Fig. 7 (data pooled from experiment nos. 1 and 2), the majority of vehicle-treated animals (Fig. 7B, iii and iv) had developed moderate to severe colitis at this time point (mean histological score = 32.3). However, the majority of the mice that had been given CRX-526 had mild or no disease (Fig. 7B, i and ii) (mean histological score = 16.3; p = 0.0565). These data demonstrate that the TLR4R complex is at least partially involved in the development of colitis in MDR1a-deficient mice and that blocking TLR4R complex signaling can prevent the development of moderate-to-severe disease in this model.

Discussion

We have shown that CRX-526 is an antagonist for the TLR4R complex and prevents the development of colonic inflammation in mice. CRX-526 blocks the ability of LPS to trigger proinflammatory responses from human monocytes both in vitro and in vivo. This antagonistic activity of CRX-526 is strictly dependent on the molecule’s structure: changes in the length of SAC led to a decrease in antagonist activity and an increase in agonist activity.

FIGURE 5. CRX-526 inhibits the development of moderate-to-severe clinical and histological disease in the DSS model of colitis. A. Experiment no. 1: BALBcAnN females were exposed to DSS for 7 days and treated every other day with various doses of CRX-526 or vehicle control. n = 5/group. Upper graph shows DAI (clinical score), and lower graph shows histological score for the large intestine. See Materials and Methods for details on treatment with DSS, CRX-526, DAI, and histological scoring. B. Experiment no. 2: BALB/cJ females were exposed to DSS for 7 days and treated daily with CRX-526 or vehicle control. See Materials and Methods for details on treatment with DSS, CRX-526, and DAI scoring; histological scores were not determined. n = 10/group. A and B. Water control groups were not exposed to DSS. Error bars indicate SD, and p values for CRX-526 treatment groups vs vehicle treatment groups are indicated in parentheses.

FIGURE 6. CRX-526 inhibits the development of clinical disease in wild-type, but not TLR4-deficient, mice exposed to DSS. Treatment of BALB/cJ and C3H Tlr4<sup>−/−</sup>/J mice with DSS and CRX-526 and assessment of DAI as described in Materials and Methods. n = 10/group. The water control group was not exposed to DSS. Error bars indicate SD; values of p for CRX-526 treatment group vs vehicle treatment group indicated in parentheses.
Mean histological score for each group.  Figure 7. CRX-526 inhibits the development of colitis in MDR1a-deficient mice. Data is pooled from two separate experiments. A. Histological scores for individual mice treated with vehicle control (■) or CRX-526 (▲) are shown. ■ Mean histological score for each group. B. Representative histological sections of colonos from mice treated with either: i, 50 μg of CRX-526 (no disease); ii, 50 μg of CRX-526 (mild disease); iii, vehicle control (ulcerative colitis); and iv, vehicle control (severe proliferative lymphoplasmocytic colitis).

for the TLR4R complex. Most importantly, treatment with CRX-526 was able to block the development of moderate-to-severe colitis in two independent mouse models. In both DSS-induced colitis and MDR1a-deficient mice, exposure to enteric Ags is mediated via defects in the epithelial barrier of the large intestine. Although not all enteric bacteria are Gram negative, these results suggest that signaling through the TLR4R complex may be critical for the development of colonic inflammation because LPS and/or other TLR4 ligands are important factors in the induction of colitis in these models. Thus, a TLR4R antagonist, such as CRX-526, may be an effective treatment for IBD as well other chronic inflammatory diseases where TLR4 plays a significant role.

Little is known about the role of TLR4 in the most of the mouse models of colitis. In the DSS model, evidence suggests that TLR4 may play a partial role in the development of severe disease, at least in certain mouse strains (26). Our results confirm these findings in this DSS model. However, a genetic variant of the TLR4-mutant C3H/HeJ mice, C3H/HeJ-Bir mice, is susceptible to the spontaneous development of chronic colitis (28), suggesting that genetic factors can influence the relative importance of TLR4 in the development of colitis. A role for TLR4 in the development of chronic colitis has been demonstrated clearly in one mouse model of colitis. Mice that have a myeloid-specific deletion of Stat3 (conditional Stat3 knockout (KO) mice) have enhanced Th1 responses and develop chronic colitis, probably due to the inability of myeloid cells to respond to IL-10 (29). Kobayashi et al. (30) have demonstrated that the chronic colitis seen in conditional Stat3 KO mice is dependent on the presence of TLR4, IL-12p40, and T cells because conditional Stat3 KO mice that are also deficient in any of these molecules or cells do not develop colitis. They hypothesize that enteric bacterial products signaling through the TLR4R complex induce the production of IL-12 and/or IL-23 that leads to the development of Th1 cells, which drive the intestinal inflammation seen in this model (30). Our observations confirm that blocking TLR4R complex signaling can result in decreased intestinal inflammation in another model of colitis that is characterized by the presence of Th1-type T cells, MDR1a-deficient mice (27).

There is increasing evidence in the literature that LPS is not the only ligand for the TLR4R complex. Heparan sulfate, the extra-domain-A of fibronectin, hyaluronic acid, fibrinogen, and Streptococcus pneumoniae-derived pneumolysin, has been shown to signal through the TLR4R complex (18). It is possible that an antagonist for LPS that binds to the TLR4R complex may be an antagonist for other TLR4 ligands. Therefore, by treating with a TLR4 antagonist such as CRX-526, one has the potential of blocking more than LPS-induced inflammatory responses. Currently, studies are ongoing to test the ability of CRX-526 to antagonize other TLR4 ligands. If CRX-526 can block multiple TLR4 ligands, this could explain why TLR4 antagonism is effective in preventing an inflammatory disease as complex as the colitis seen in DSS-treated and MDR1a-deficient mice.

The exact role for TLR4 in intestinal inflammation remains controversial. Although most studies show a role for TLR4 in inflammatory responses, Rakoff-Nahoum et al. (31) have demonstrated recently an important role for TLR in the maintenance of intestinal epithelial homeostasis and protection from direct epithelial injury. Their results show that mice deficient in TLR signaling, due to a deficiency in MyD88, are more susceptible than wild-type mice to intestinal epithelial damage due to exposure to DSS or to gamma irradiation. Furthermore, their data argue that constitutive signaling through TLR in intestinal epithelial cells results in the production of tissue protective factors, such as IL-6, KC-1, and TNF (31). Although their evidence suggests that complete inhibition of all TLR signaling may be more injurious than beneficial under circumstances of acute epithelial injury, it remains possible that inhibition of TLR4 signaling by an antagonist may be beneficial under circumstances of chronic inflammatory disease.

Although the exact mechanism of action of CRX-526 is still under investigation, we hypothesize that CRX-526 binds directly to the TLR4R complex and sterically inhibits the ability of LPS and, perhaps, other ligands to bind. We have demonstrated previously that MPL and other AGP molecules do not induce cytokine production from cells lacking TLR4 expression, suggesting these lipid A-derived molecules are strictly ligands for TLR4 (22). Recently, it has been shown by Akashi et al. (15) that LPS binds directly to the TLR4-MD-2 complex, while CD14 can augment this binding. They also tested a TLR4R complex antagonist, E5531, and showed it also bound directly to TLR4-MD-2. As E5531 has very similar LPS-antagonist activity compared with E5531, and showed it also bound directly to TLR4-MD-2 complex, while CD14 can augment this binding. They also tested a TLR4R complex antagonist, E5531, and showed it also bound directly to TLR4-MD-2. As E5531 has very similar LPS-antagonist activity compared with E5531, and showed it also bound directly to TLR4-MD-2. Although their evidence suggests that complete inhibition of all TLR signaling may be more injurious than beneficial under circumstances of acute epithelial injury, it remains possible that inhibition of TLR4 signaling by an antagonist may be beneficial under circumstances of chronic inflammatory disease.

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Clearly, additional investigation is necessary to elucidate the role of TLR4R signaling in human IBD. In normal intestinal tissue, epithelial cells express little TLR4/MD-2/CD14 and are relatively unresponsive to LPS (34, 35). However, m-Iccl2 cells derived from the crypt epithelium of the murine small intestine express...
TLR4 internally, and internalization of LPS is required for TLR4R complex signaling (36). Furthermore, human patients with IBD have increased TLR4, but not TLR2, expression on their intestinal epithelial cells and increased TLR4 and TLR2 expression on lamina propria monocytes (37, 38). These data confirm the expression of TLR4 in sites of inflammation in the intestine, although it is not yet clear whether expression of TLR4 on the intestinal epithelium is a cause and/or effect of the development of IBD. Although the definitive role for TLR4 in human IBD remains to be determined, the evidence we present here clearly suggest that TLR4 may be a critical therapeutic target for this disease.

Acknowledgments
We thank Drs. Sally M ossman, Mark Alderson, and Ken Grabstein for helpful discussions of this manuscript and Andrea Reitsma for expert technical assistance.

Disclosures
A. Mozaffarian, D. A. Johnson, D. H. Persing, P. Probst, E. Jeffery, and S. P. Fling are current employees with stock or equity interests in Corixa Corporation. A. G. Stover, R. T. Crane, R. M. Hershberg, and M. M. Fort were previous (within the past 5 years) employees of Corixa Corporation. H. Bielefeldt-Ohmann was paid as a consultant for these studies.

References