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Maresin 1, a Proresolving Lipid Mediator Derived from Omega-3 Polyunsaturated Fatty Acids, Exerts Protective Actions in Murine Models of Colitis

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It has been previously reported that dietary fish oils, which are rich in the polyunsaturated fatty acids eicosapentaenoic acid and docosahexaenoic acid, can exert beneficial effects in inflammatory bowel disease. In this study, we investigated the effects of docosahexaenoic acid–derived lipid mediator maresin 1 (MaR1) in dextran sulfate sodium (DSS)– and 2,4,6-trinitrobenzenesulfonic acid–induced colitis in mice. Systemic treatment with MaR1 significantly attenuated both DSS- and 2,4,6-trinitrobenzene sulfonic acid–induced colonic inflammation by improving the disease activity index and reducing body weight loss and colonic tissue damage. MaR1 treatment also induced a significant decrease in levels of inflammatory mediators, such as IL-1β, TNF-α, IL-6, and IFN-γ, in the acute protocol, as well as IL-1β and IL-6, but not TNF-α and INF-γ, in the chronic DSS colitis protocol. Additionally, MaR1 decreased ICAM-1 mRNA expression in both the acute and chronic protocols of DSS-induced colitis. Furthermore, the beneficial effects of MaR1 seem to be associated with inhibition of the NF-κB pathway. Moreover, incubation of LPS-stimulated bone marrow–derived macrophage cultures with MaR1 reduced neutrophil migration and reactive oxygen species production, besides decreasing IL-1β, TNF-α, IL-6, and INF-γ production. Interestingly, macrophages incubated only with MaR1 showed a significant upregulation of mannose receptor C, type 1 mRNA expression, an M2 macrophage phenotype marker. These results indicate that MaR1 consistently protects mice against different models of experimental colitis, possibly by inhibiting the NF-κB pathway and consequently multiple inflammatory mediators, as well as by enhancing the macrophage M2 phenotype.


Much experimental evidence indicates that the inflammation process is characterized by the migration of blood cells, mainly neutrophils and monocytes, in the initial phase, which release several inflammatory mediators (1) in the injured area. Under normal conditions the acute inflammation can be controlled by the activation of specific molecules that signal the end of inflammation (2–4). These endogenous mediators, which are biosynthesized in situ, such as lipoxins, resolvins, and protectins, are thought to exert a critical role in triggering the resolution of inflammation (1, 5), accelerating the uptake of apoptotic leukocytes by macrophages in a nonphlogistic fashion and switching from a proinflammatory macrophage to an anti-inflammatory phenotype (1, 6, 7).

Omega-6 fatty acids (such as linoleic acid) and omega-3 fatty acids (such as α-linolenic acid) are essential for mammalian systems (8). Omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the source of different biosynthetic products as resolvins and protectins mainly during the resolution of inflammation (1, 5, 9, 10). Additionally, DHA is the biosynthetic precursor of a new family of macrophage-derived lipid mediators, termed maresins (11). Maresins are biosynthesized via 12-lipoxygenase in humans to generate 14S-hydroperoxydocosahexaenoic acid, which undergoes further conversion via 13(14)-epoxidation and is subsequently converted to 7,14-dihydroxydocosahexaenoic acid, known as maresin 1 (MaR1) (11). Recently, in vitro and in vivo evidence has appeared indicating that MaR1 exerts potent anti-inflammatory and proresolution activities comparable to those already reported for resolvins, with such effects being largely linked with its ability to reduce neutrophil migration and increment in macrophage phagocytic activity (11–14).

The gut is regarded as being in a state of controlled inflammation (15, 16) and, consequently, the resolution of inflammation is critical to avoid excessive damage to host tissue. The breakdown of homeostasis in the intestine is considered an important event in the induction of the two most relevant human inflammatory bowel diseases (IBDs), Crohn’s disease and ulcerative colitis, which represent an important worldwide health problem (17, 18). Studies using experimental models of IBD such as dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) have pro-
vided substantial new information about the beneficial effects of the omega-3 polyunsaturated fatty acid (n-3 PUFA)-derived mediators resolvins E1 (RvE1), resolvin D2, 17(R)-hydroxy docosahexaenoic acid, and aspirin-triggered resolvin D1 in the resolution of intestinal inflammation (19–21). However, so far there are no reports about the effects and mechanisms underlying the actions of MaR1 in murine models of colitis. In this context, in this study we have evaluated the potential anti-inflammatory and proresolution properties of MaR1 in DSS- and TNBS-induced colitis in mice. Our results revealed that MaR1 exhibited a potent and marked anti-inflammatory activity in two models of intestinal inflammation, characterized by a significant reduction in disease activity, inhibition of colonic cell infiltration, and a decrease in proinflammatory mediators. Additionally, our data point to a possible switch in the macrophage phenotype from M1 to M2 after MaR1 treatment, as well as direct inhibition of neutrophil migration and reactive oxygen species (ROS) production, which could explain, at least in part, its beneficial actions in experimental colitis.

Materials and Methods

Animals

Male CD1 mice (8–10 wk of age) were obtained from the animal house unit of the Laboratório de Farmacologia Experimental, Universidade Federal de Santa Catarina (Florianópolis, SC, Brazil). Animals were housed in collective cages at 22 ± 1°C under a 12 h light/dark cycle (lights on at 7:00 AM) with free access to food and water ad libitum. All experimental procedures were previously approved by the Ethical Committee for Use of Animals of Universidade Federal de Santa Catarina (Comissão de Ética no Uso de Animais/Universidade Federal de Santa Catarina protocol no. PP004906).

Induction and assessment of DSS colitis

To evaluate the involvement of MaR1 in intestinal inflammation, colitis was induced by DSS using two different protocols, as previously reported (22) with minor modifications. In the first protocol (acute protocol) animals received a solution of filtered water containing 2% DSS ad libitum during a 5-d period. Following this 5-d period, DSS was replaced by normal drinking water for 2 d and, at the end of day 7, the animals were euthanized. Control CD1 mice received only normal drinking water. In the acute protocol of DSS-induced colitis animals were treated endovaneously (e.v.) by the intracoelomic route with MaR1 (0.1, 0.3, and 1 μg/animal) once a day from day 0 to day 7. In the chronic colitis protocol, animals received two cycles of DSS. First, they were offered a solution of filtered water containing 2% DSS ad libitum during a 5-d period (DSS cycle 1). At the end of this 5-d period, DSS was replaced by normal drinking water for 10 d and, at the end of day 15, the animals received a new cycle of 2% DSS ad libitum during a 5-d period (DSS cycle 2). Following this period, DSS was replaced by normal drinking water for 2 d and the animals were euthanized on the day 22 (see scheme in Fig. 1). Control CD1 mice received only drinking water. In the chronic protocol of DSS-induced colitis, MaR1 (0.3 μg/animal) was given e.v. once a day from day 15 to day 22. Of note, in the chronic protocol animals did not receive MaR1 in the first cycle with DSS. The doses of MaR1 were chosen based on preliminary studies and previously published studies (11, 14, 19). MaR1 was solubilized in a 0.9% NaCl solution. Vehicle solutions were used for the respective control animal treatments. All animals were examined once a day and the disease activity index (DAI) was assessed as previously described (23). Briefly, the DAI combined scores for weight loss, stool consistency, and bleeding. At the end of the acute (day 7) or chronic (day 22) protocols, colons were removed and macroscopic scoring was performed as previously described (24) considering colon weight, length, gross macroscopic appearance, and stool consistency.

Induction and assessment of TNBS-induced colitis

Colitis was induced according to the methodology described previously (25) with minor modifications. Animals were deprived of food for 18–24 h with free access to a 5% glucose solution. Fasted animals were anesthetized by the administration of xylazine (10 mg/kg, i.p.) and ketamine (80 mg/kg, i.p.). Following this, animals received a new cycle of 2% DSS ad libitum during a 5-d period (DSS cycle 2). Following this period, DSS was replaced by normal drinking water for 2 d and the animals were euthanized on the day 22 (see scheme in Fig. 1). Control CD1 mice received only normal drinking water. In the chronic protocol of DSS-induced colitis, MaR1 (0.3 μg/animal) was given e.v. once a day from day 15 to day 22. Of note, in the chronic protocol animals did not receive MaR1 in the first cycle with DSS. The doses of MaR1 were chosen based on preliminary studies and previously published studies (11, 14, 19). MaR1 was solubilized in a 0.9% NaCl solution. Vehicle solutions were used for the respective control animal treatments. All animals were examined once a day and the disease activity index (DAI) was assessed as previously described (23). Briefly, the DAI combined scores for weight loss, stool consistency, and bleeding. At the end of the acute (day 7) or chronic (day 22) protocols, colons were removed and macroscopic scoring was performed as previously described (24) considering colon weight, length, gross macroscopic appearance, and stool consistency.

Histological analysis and evaluation of microscopic damage

Colon tissue samples were removed 7 d (acute protocol) or 22 d (chronic protocol) after DSS-induced colitis and 3 d after TNBS colitis and immediately fixed in 4% formaldehyde solution. Colons were embedded in paraffin, sectioned into 4-μm-thick slices, mounted on glass slides, and then deparaffinized. For histological analysis, slices were stained using the H&E technique. Distal portions of the colon were examined in cross-sections at × 200 magnification. Six random fields of view in each specimen were analyzed by two blinded observers using a Sight DS-5 M-L1 digital camera connected to an Eclipse 50i light microscope (both from Nikon, Melville, NY). Histological analyses were performed as previously described (24, 27).

Myeloperoxidase assay

Neutrophil infiltration into colonic tissue was assessed indirectly by measuring myeloperoxidase (MPO) activity. MPO was assayed as previously described (28). On day 7 (acute protocol) or day 3 (TNBS protocol), animals were killed using CO2 and colon tissue segments were homogenized in 5% EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 × g for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4) and the samples were frozen in liquid nitrogen and thawed three times. Upon thawing, the samples were similarly centrifuged and 25 μl supernatant was used for the MPO assay. The MPO enzymatic reaction was assessed by the addition of the 1.6 mM tetra- methylbenzidine (TMB), 80 mM NaPO4 and 0.3 mM hydrogen peroxide. The absorbance was measured spectrophotometrically at 690 nm and the results were expressed as OD per milligram tissue.

Culture and stimulation of neutrophils

Bone marrow–derived neutrophils (BMDNs) were obtained as described previously (29). Briefly, the BMDNs were isolated from femurs and tibias. The cells were removed from bones by inserting a 25-gauge needle with a syringe filled with HBSS without Ca2+ and Mg2+. Afterward, the cells were resuspended in 45% Percoll solution in HBSS. The samples were then washed and resuspended in medium (RPMI 1640) containing glucose, supplemented with 2 mmol/l l-glutamine, 10% FCS, 100 μmol/l penicillin, and 100 μmol/l streptomycin (all from Sigma-Aldrich, St. Louis, MO). Following this, the cells were incubated at 37°C in a 5% CO2-enriched atmosphere and were used for the migration chamber assay and ROS assay. Cell death was assessed using the trypan blue assay.

In vitro neutrophil migration assay

Murine BMDNs were assessed for their ability to migrate across poly-carbonate membrane Transwell filters with a pore size of 3 μm (Corning, Cambridge, MA). In brief, filters were impregnated with RPMI 1640 medium overnight. Neutrophils (105 cells/well) were added to the upper chamber and incubated with MaR1 (300 nM) or vehicle for 30 min. After this period, the cells were stimulated with the chemotractant mediator (CXCL1/keratinocyte-derived chemokine [KC], 30 ng/ml) in the lower chamber. The cells were incubated at 37°C in a 5% CO2-enriched atmosphere for 2 h and the number of migrated cells in the lower chamber was quantified by flow cytometry (number of gated events in 60 s).

ROS assay

ROS production was measured using 2,7'-dichlorofluorescin diacetate (DCFH-DA) as previously described (30, 31). In brief, neutrophils (2 × 105 cells/well) were incubated with MaR1 (300 nM) or vehicle for 30 min. After this period, cells were incubated with 100 μM DCFH-DA and stimulated with 5 μl PMA (10 ng/ml) at 37°C and protected from light for 15 min. Flow cytometric analysis was performed with a FACScan Canto II flow cytometer (BD Biosciences, San Diego, CA). The data were analyzed using FACSDiva software (BD Biosciences) and the mean fluorescence intensity (MFI) was used to quantify the responses.

Culture and stimulation of macrophages

Bone marrow–derived macrophages (BMDMs) were obtained as described previously (32). Briefly, bone marrow mononuclear phagocyte precursor...
cells were propagated in suspension by culturing in macrophage medium (DMEM–containing glucose, supplemented with 2 mmol/l L-glutamine, 10% FCS, 10 mmol/l HEPES, 100 μg/ml streptomycin, and 100 U/ml penicillin (all from Sigma-Aldrich) supplemented with 20% L929 cell–conditioned medium (as a source of M-CSF). Cells were incubated at 37 °C in a 5% CO2-enriched atmosphere and fed on day 5 by replacing the supplemented medium with 20% L929 cell–conditioned medium. The cells were harvested on day 7 and 2 × 10^5 cells/well were cultured in a 96-well cell culture plate for 24 h. Afterward, adherent cells were stimulated for 24 h with LPS (1 μg/ml) in the presence or absence of MaR1 (300 nM) in a final volume of 250 μl/well. After stimulation, the plate was centrifuged (200 × g, 10 min) the cell-free supernatant was collected for cytokine determination, and the adherent cells were used for real-time PCR analyses. In another set of experiments, 2 × 10^5 cells/well were cultured in a 96-well cell culture plate for 24 h. Thereafter, the cells were pretreated for 1 h with GW9662 (1 μM), a peroxisome proliferator–activated receptor γ antagonist; BOC-1 (10 μM), a lipoxin A4 receptor (formyl peptide receptor 2/ALX) antagonist; AM251 (1 μM), a cannabinoid receptor type 1 antagonist; or RU486 (10 μM), a glucocorticoid receptor antagonist. After incubation with the antagonists, the cells received MaR1 (300 nM) for 24 h. Following this, the plate was centrifuged (200 × g, 10 min) and the adherent cells were used for RT-PCR analyses to quantify mannose receptor C, type 1 (MRC1) mRNA expression. In vitro concentrations of MaR1 were based on previously published studies (11) and our pilot study.

Cytokine quantification by cytometric bead array
Colon tissue samples were removed 7 d (acute protocol) or 22 d (chronic protocol) after the beginning of DSS treatment and were homogenized in phosphate buffer containing 0.05% Tween 20, 0.1 mmol/l PMSF, 0.1 mmol/l benzthiazonium chloride, 10 mmol/l EDTA, and 20 μl aprotinin A. The homogenate was centrifuged at 3000 × g for 10 min and the supernatants were used for cytometric bead array (CBA) analyses. Furthermore, cell culture supernatants were collected 24 h after LPS stimulation as described earlier and used for cytokine measurement of TNF-α, IL-1β, IL-6 and IFN-γ secretion using CBA (BD Biosciences) as described before (19). The amount of protein in colonic samples was measured by the method of Lowry et al. (33).

Isolation of RNA and quantitative real-time PCR
Total RNA was extracted from BMDMs 24 h after LPS stimulation using the TRIzol reagent as described by the supplier’s protocol (Invitrogen, Carlsbad, CA). Additionally, total RNA was isolated from colon obtained from control mice (not treated with DSS) and from mice subjected to acute or chronic colitis models and treated with vehicle or MaR1 (0.3 μg/animal) using the SV Total RNA Isolation System Z3100 (Promega, Madison, WI) according to the manufacturer’s recommendations. The concentration of total RNA was determined using a NanoDrop 1100 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quantities of 10 (cells) and 50 ng (tissue) total RNA were used for cDNA synthesis. A reverse transcription assay was performed using the Moloney murine leukemia virus reverse transcriptase protocol according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). cDNA was amplified in duplicate using the TaqMan universal PCR Master Mix kit with specific TaqMan gene expression target genes, the 3′ quencher MGB- and FAM-labeled probes for mouse MRC1 (Mm00485148_m1), NO synthase 2 (NOS2; Mm01309898_m1), ICAM-1 (Mm005616024_g1), and GAPDH (NM_008084.2), which was used as an endogenous control for normalization. The amplifications were carried out in a thermal cycler (StepOne Plus; Applied Biosystems) for 50 cycles; the fluorescence was collected for each amplification cycle and the data were analyzed using the 2^-ΔΔCt method for relative quantification of expression. The expression of the target genes was calibrated against conditions found in control animals, that is, without treatment.

Western blot analysis
Colon tissue samples were removed 7 d (acute protocol) or 22 d (chronic protocol) after DSS-induced colitis and were processed in complete RIPA buffer. Equal amounts of protein for each sample (20 μg) were loaded per lane and electrophoretically separated using 10% denaturing PAGE (SDS-PAGE). Afterward, the proteins were transferred to nitrocellulose membranes using a Mini Trans-Blot Cell System (Bio-Rad Laboratories, Hercules, CA) following the manufacturer’s protocol. Western blot analysis was carried out using monoclonal mouse anti–phospho-p65 (NF-κb subunit) (1:1000; Cell Signaling Technology, Danvers, MA) incubated overnight. Following washing, the membrane was incubated with a secondary Ab conjugated to HRP (1:10,000; Cell Signaling Technology). The immunocomplex was visualized using the SuperSignal West Femto chemiluminescent substrate detection system (Thermo Fischer Scientific, Rockford, IL) and densitometric values were normalized using monoclonal mouse β-actin Ab (1:500; Cell Signaling Technology). Protein levels were quantified by Odyssey using ImageJ software and expressed as the ratio to β-actin represented by arbitrary units.

Drugs and reagents
DSS (molecular weight, 36,000–50,000 Da) and BOC-1 were obtained from MP Biomedicals (Solon, OH). MaR1 was obtained from Cayman Chemical (Charlotte, NC). Primers and probes for mouse MRC1 (Mm00485148_m1), NOS2 (Mm01309898_m1), ICAM-1 (Mm005616024_g1), GAPDH (NM_008084.2), and the TaqMan Universal PCR Master Mix kit were purchased from Applied Biosystems. The SV Total RNA Isolation System and Moloney murine leukemia virus reverse transcriptase were purchased from Promega. AM251 was obtained from Tocris Bioscience (Ellisville, MO). TRizol was purchased from Invitrogen. CXCL1/KC was purchased from R&D Systems (Minneapolis, MN). Hydrogen peroxide, Tween 20, 80, EDTA, Percoll, DMEN, PMA, DCCF-DA, aprotinin A, RU-486, DAB, RPMI 1640, TNBS, PBS, hematocycin, eosin, tetramethylrhodamine, L-glutamine, FCS, HEPES, streptomycin, Corning Transwell, penicillin, and LPS were purchased from Sigma-Aldrich. GW9662 was obtained from Merck (Darmstadt, Germany). The CBA inflammation kit was purchased from BD Biosciences. Primary Abs for monoclonal anti–phospho-p65, HRP-conjugated goat anti-mouse IgG secondary Ab, and anti–β-actin were obtained from Cell Signaling Technology.

Statistics
All data are expressed as the means ± SEM. For nonparametric data, a Kruskal–Wallis test followed by a Dunn test was used. For parametric data, the statistical differences between groups were determined by one-way ANOVA followed by a Student–Newman–Keuls test. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.
Results

MaR1 protects mice against acute DSS-induced colitis

Previous reports have shown the relevance of MaR1 as a potent antinociceptive and anti-inflammatory lipid mediator with proresolving properties (11, 14). Therefore, we aimed to investigate the potential beneficial effects of MaR1 in two experimental colitis models. Initially, we used the well-established DSS chemical model of intestinal inflammation. Oral DSS administration to mice for 5 d induced a severe illness characterized by bloody diarrhea, culminating in a significant increase in DAI score (Fig. 2A) and sustained weight loss (Fig. 2B). However, the animals that received MaR1 at the two higher doses (0.3 and 1 μg/animal) showed a significant protection against the change in DAI score (Fig. 2A) associated with a significant improvement in body weight loss (Fig. 2B). It is generally accepted that colon length is inversely associated with the severity of DSS-induced colitis. Morphological examination of the colonic tissue 7 d after the beginning of the DSS regimen revealed a significant shortening of colon length in the DSS-treated mice (Fig. 2C). Interestingly, the systemic pretreatment with MaR1 (0.3 and 1 μg/animal) markedly prevented colon length reduction (Fig. 2C). Furthermore, our results showed that DSS administration resulted in colon inflammation associated with hyperemia, ulceration, and bowel wall thickening, leading to an increase in macroscopic colon damage (Fig. 2D). Notably, MaR1 (0.3 and 1 μg/animal) treatment markedly reduced macroscopic damage in the acute protocol of DSS-induced colitis (Fig. 2D).

MaR1 treatment reduces cellular infiltration and improves microscopic colonic tissue damage in acute DSS-induced colitis

Several reports have suggested that tissue damage and inflammatory signs in DSS-induced colitis are mainly mediated by PMN infiltration (34, 35). For this reason, we next assessed the effect of systemic MaR1 treatment on the regulation of colonic neutrophil infiltration indirectly by MPO activity. As shown in Fig. 2E, the DSS-treated animals displayed a marked increase in colonic MPO activity when compared with control animals. Notably, the treatment with MaR1 (0.3 and 1 μg/animal) significantly reduced MPO levels in mouse colon tissue (Fig. 2E). Furthermore, systemic treatment with MaR1 (0.3 and 1 μg/animal, e.v.) consistently restored the histological appearance of the mucosa and submucosa and decreased the loss of epithelial cells and mucosal ulceration when compared with the DSS-treated group (Fig. 2F, 2G).
Interestingly, the doses of 0.3 and 1 \( \mu g/\text{animal} \) were similarly effective (Fig. 2) and, for this reason, MaR1 at 0.3 \( \mu g/\text{animal} \) was used for subsequent experiments.

**MaR1 protects mice against chronic DSS-induced colitis**

As previously described (22), oral administration of two cycles of 2% DSS (Fig. 3) starts the chronification process of colitis. As shown in Fig. 3A, a great increase in DAI was observed following both cycles of DSS, with this increment being accompanied by body weight loss (Fig. 3B) and significant colon shortening (Fig. 3C). Following the first DSS cycle, animals were subjected to a recovery period during which they received filtered water (days 5–15), but at the end of this period (day 15) the DAI score still had not reached the basal values. At that time, therapeutic treatment with MaR1 was initiated. Animals were treated with MaR1 (0.3 \( \mu g/\text{animal, e.v.} \)) once a day from the days 15–22. MaR1 treatment significantly prevented the second increase in DAI (Fig. 3A) and significantly protected mice from the body weight loss observed in the second cycle of DSS (Fig. 3B). Additionally, similarly to the acute period, systemic MaR1 treatment in the chronic protocol consistently prevented colon length reduction (Fig. 3C) and macroscopic colonic damage (Fig. 3D). The histological changes in the colons of the mice at the end of the chronic protocol showed a severe mucosal inflammation with massive infiltration of inflammatory cells, loss of goblet cells, destruction in the crypts, and fibrosis. Of note, systemic treatment with MaR1 (0.3 \( \mu g/\text{animal, e.v.} \)) significantly improved these signs, restored the histological appearance of the mucosa and submucosa, and decreased the loss of epithelial cells and mucosal ulceration when compared with the DSS-treated group (Fig. 3).

**MaR1 changes colonic protein levels of inflammatory mediators**

Previous reports have suggested that cytokines such as IL-1\( \beta \), TNF-\( \alpha \), IL-6, and INF-\( \gamma \) are critically involved in the recruitment and activation of inflammatory cells during the progression of intestinal inflammation (36). Because MaR1 decreased cell infiltration and damage in colonic tissue in both the acute and...
chronic protocols, we asked whether treatment with MaR1 could also modulate the levels of proinflammatory mediators. Our results showed that DSS administration resulted in a pronounced increase in colonic IL-1β, TNF-α, IL-6, and INF-γ protein levels with both the acute (Fig. 4A–D) and chronic (Fig. 4E–H) protocols. Interestingly, pretreatment with MaR1 (0.3 μg/animal, e.v.) resulted in significant decreases in the inflammatory cytokines IL-1β, TNF-α, IL-6, and INF-γ, as observed in the acute protocol, whereas the therapeutic treatment with MaR1 reduced only IL-1β and IL-6, but not TNF-α and INF-γ levels, as observed in the chronic protocol of intestinal inflammation (Fig. 4).

MaR1 treatment changes NF-κB protein activation in colonic tissue

The transcription factor NF-κB controls several genes involved in inflammation (37), and its inhibition is able to prevent experimental colitis (38). To further define some of the signaling systems modulated by MaR1 in experimental colitis, we assessed the effects of pre- and therapeutic MaR1 treatment on NF-κB activation. We observed a pronounced NF-κB activation in colons from animals subjected to both acute (Fig. 5A) and chronic (Fig. 5B) protocols of DSS-induced colitis. Nevertheless, MaR1 treatment significantly reduced NF-κB activation in both DSS protocols (Fig. 5).

MaR1 treatment reduces adhesion molecule ICAM-1 expression in colonic tissue

Cell adhesion molecules such as ICAM-1 allow adherence of leukocytes to the endothelium and permit their subsequent transmigration into the inflammatory site (39). To verify whether the effect of MaR1 on PMN reduction is associated with expression of cell adhesion molecules in epithelial cells, we next assessed ICAM-1 mRNA expression after DSS-induced colitis. The DSS administration resulted in a pronounced upregulation of colonic ICAM-1 mRNA expression in both the acute and chronic protocols (Fig. 6). Of note, treatment with MaR1 (0.3 μg/animal) resulted in a significant decrease in ICAM-1 mRNA in both analyzed periods (Fig. 6).

MaR1 incubation reduces migration of neutrophils and ROS production in vitro

Previous reports have shown that neutrophils are involved in a wide range of inflammatory diseases (40–42), especially colitis (28, 43). Neutrophil transmigration is a key event in the subsequent action of neutrophils, such as phagocytosis and degranulation (44). Initially, to investigate a direct effect of MaR1 on neutrophil influx, we performed a transwell migration assay. As observed in Fig. 7A, MaR1 alone did not induce any migration of PMNs. However, CXCL1/KC, a potent chemoattractant mediator, induced strong neutrophil migration (Fig. 7A). Of note, MaR1 (300 nM) consistently reduced neutrophil migration to close to basal levels (Fig. 7A). Production of ROS is directly linked to destruction of foreign agents that have been engulfed by neutrophils; thus, its production has been used to document neutrophil activation (44, 45). For this reason, we investigated the possible effect of MaR1 in reduced neutrophil activation using an ROS marker, DCFH-DA. Our results showed that control cells presented low ROS production as observed by basal dichlorofluorescein (DCF) fluorescence intensity, which was markedly increased after PMA stimulation (Fig. 7B, 7C). Basal levels of DCF fluorescence did not change in the MaR1 control group. Interestingly, MaR1 significantly abolished ROS production after PMA stimulation (Fig. 7B, 7C).

MaR1 incubation reduces macrophage cytokine levels and upregulates expression of the M2 macrophage cell marker in vitro

Recently, several studies have suggested that polarization of proinflammatory M1 macrophages toward the M2 macrophage phenotype plays a critical role in the resolution of inflammation and tissue repair (46, 47). Moreover, high levels of proinflammatory M1 macrophage have been shown in the colonic tissue of IBD patients (46, 48, 49). For this reason, we assessed the effect of MaR1 incubation on the secretion of these M1 macrophage cytokines. The data presented in Fig. 8A–D show that LPS stimulation (1 μg/ml, for 24 h) produced a prominent increase in M1 macrophage cytokine levels for IL-1β, IL-6, TNF-α, and INF-γ, compared with the control group. Surprisingly, our results showed that

FIGURE 4. Systemic treatment with MaR1 prevents increases in colonic proinflammatory mediator levels. (A–H) Mice were treated with MaR1 (0.3 μg/animal) or vehicle once per day in the acute and chronic protocols of DSS-induced colitis. The control group was treated with vehicle and received only filtered water. The systemic treatment with MaR1 (0.3 μg/animal) reduced the colonic levels of IL-1β (A, E) and IL-6 (C, G) in both protocols. MaR1 treatment reduced TNF-α (B) and INF-γ (D) levels only in the acute protocol. Data are reported as means ± SEM of six to eight mice per group. #p < 0.05 versus control group, *p < 0.05 versus DSS group.
MaR1 (300 nM) incubation significantly reduced IL-1β, IL-6, TNF-α, and INF-γ secretion from LPS-stimulated macrophages (Fig. 8A–D). Additionally, it is well known that M1 macrophage polarization is characterized by an increase in NOS2 expression, whereas the M2 macrophage phenotype is associated with up-regulation of proteins such as MRC1 (50). Thus, we next assessed the expression of NOS2 and MRC1 mRNAs in cultured adherent BMDMs stimulated with LPS. Our results showed that LPS incubation for 24 h induced a marked downregulation of MRC1 mRNA (Fig. 8E) and upregulation of NOS2 mRNA (Fig. 8F). Incubation of these cells with MaR1 did not change MRC1 or NOS2 mRNA expression after stimulation with LPS (Fig. 8E, 8F). However, MaR1 incubation in macrophages without LPS stimulation significantly increased MRC1 mRNA expression, suggesting a possible MaR1 effect on M2 macrophage polarization (Fig. 8E, 8F). However, this effect did not seem to be associated with stimulation of the peroxisome proliferator-activated receptor γ, cannabinoid receptor type 1, lipoxin A4 receptor (formyl peptide receptor 2/ALX), and glucocorticoid receptor, because pharmacological antagonism of these receptors did not cause any significant change in MCR1 mRNA expression induced by MaR1 incubation (data not shown).

**Treatmet with MaR1 attenuates the severity of colitis induced by TNBS**

It has been well established that a single intracolonic administration of TNBS results 72 h later in formation of colonic granulomas associated with infiltration of inflammatory cells in all layers, strong thickening of the intestinal wall, hyperplasia of the epithelium, and ulceration (27, 51). Thus, to evaluate the possible anti-inflammatory effect of MaR1 in another model of colitis, we investigated its effect against TNBS-induced gut inflammation. In agreement with previous data, we observed that TNBS induced a severe colitis, which was characterized by marked body weight loss (Fig. 9A). Alternatively, previous treatment of the mice with MaR1 (0.3 μg/animal, e.v.) significantly ameliorated the loss in body weight (Fig. 9A). Additionally, TNBS instillation caused thickening hyperemia, necrosis, and inflammation, leading to an increase in macroscopic damage (Fig. 9B), accompanied by increased MPO activity (Fig. 9C). Histological examination of TNBS-induced colitis was characterized by significant enhancement of mucosal and submucosal cell infiltration, as well as greater loss of architecture and disruption of the epithelial barrier, resulting in elevated histopathological scores (Fig. 9D, 9E). Likewise, MaR1 (0.3 μg/animal, e.v.) treatment significantly reduced the macroscopic colon damage (Fig. 9B) and MPO activity (Fig. 9C). Additionally, MaR1 markedly improved the intestinal structure, restoring the histological appearance of the mucosa when compared with the TNBS-treated group (Fig. 9D, 9E).

**Discussion**

The incidence and prevalence of IBD are increasing with time and in different regions around the world (18). Although a recent systematic review does not allow firm recommendations about the usefulness of n-3 PUFA dietary supplements in IBD (52), other works, especially at the preclinical stage, suggest that n-3 PUFAs or their lipid-derived mediators might have a therapeutic effect (19–21, 53). Indeed, the n-3 PUFAs DHA and EPA, found in fish oils, are extensively used currently as dietary supplements and are thought to exert beneficial effects in a number of inflammation-related diseases (54, 55) and in different experimental models (53, 56, 57). More recently, EPA and DHA lipid-derived mediators known as resolvins and protectins have been described as key mediators in the resolution of inflammation and regulators of

**FIGURE 5.** MaR1 treatment modulates the activation of NF-κB in colonic tissue. Mice were treated with MaR1 (0.3 μg/animal) or vehicle once per day in acute and chronic protocols of DSS-induced colitis. Treatment with MaR1 (0.3 μg/animal, e.v.) inhibited p65 (NF-κB) (A, B) activation compared with the DSS-treated group. Data are reported as means ± SEM of six to eight mice per group. *p < 0.05 versus control group, **p < 0.05 versus DSS group.

**FIGURE 6.** MaR1 treatment reduced the colonic ICAM-1 mRNA expression induced by DSS. Mice were treated with MaR1 (0.3 μg/animal) or vehicle once per day in the acute and chronic protocols of DSS-induced colitis. The systemic MaR1 treatment (0.3 μg/animal) reduced ICAM-1 mRNA expression in both the acute (A) and chronic protocols (B) after DSS administration. A real-time PCR assay was performed in duplicate, and GAPDH mRNA was used to normalize the relative amount of mRNA. Data are reported as means ± SEM of six to eight mice per group. *p < 0.05 versus control group, **p < 0.05 versus DSS group.
normal homeostasis (5, 9). It has been demonstrated that DHA lipid-derived mediators, such as resolvin D2 and aspirin-triggered resolvin D1 (19), as well as mediators such as RvE1 from EPA (20, 21), are quite effective in preventing gut inflammation. MaR1, a new DHA-derived mediator, was recently described as an important modulator of inflammation resolution (11); however, so far little is known about its mechanism of action or its possible beneficial effects on IBD. In this study, we report for the first time, to the best of our knowledge, the effects of MaR1 in murine IBD models and demonstrate that both pre- and therapeutic treatment with very low doses of MaR1 consistently protected animals from DSS-induced colitis. Our data also give mechanistic insights into its protective actions, some closely related to other lipid mediators, but also new and relevant direct actions upon neutrophils and macrophages, such as a role in neutrophil activation and macrophage switching from the M1 to the M2 phenotype.

A number of lines of experimental evidence suggest that, following chemokine (40, 44) and cytokine stimuli (58, 59), an excessive leukocyte recruitment into colonic tissue plays an important role in the progression of gut inflammation (60, 61), whereas inhibition of PMN migration ameliorates colon damage (28, 43, 62). Previous data have described the role of MaR1 in murine IBD models and demonstrate that both pre- and therapeutic treatment with very low doses of MaR1 consistently protected animals from DSS-induced colitis. Our data also give mechanistic insights into its protective actions, some closely related to other lipid mediators, but also new and relevant direct actions upon neutrophils and macrophages, such as a role in neutrophil activation and macrophage switching from the M1 to the M2 phenotype.

A number of lines of experimental evidence suggest that, following chemokine (40, 44) and cytokine stimuli (58, 59), an excessive leukocyte recruitment into colonic tissue plays an important role in the progression of gut inflammation (60, 61), whereas inhibition of PMN migration ameliorates colon damage (28, 43, 62). Previous data have described the role of MaR1 in reducing PMN infiltration (11, 14) in zymosan-induced peritonitis. Our study corroborates these data by demonstrating that MaR1 attenuates PMN infiltration in colon tissue. Nonetheless, the mechanisms that underlie this process are not fully known and could involve reductions in chemokines/cytokines, change in expression of cell adhesion molecules, and/or a direct effect on PMNs. It is now well documented that intestinal immune and epithelial cells are responsible for producing a potent combination of broadly active proinflammatory cytokines, including IL-1β, TNF-α, IL-6, and INF-γ, as well as the expression of adhesion molecules during intestinal inflammation (22, 63, 64). Our present data have shown that beneficial effects of MaR1 in DSS-induced colitis were seen to be associated with its ability to decrease colonic IL-1β, TNF-α, IL-6, and IFN-γ levels, as well as ICAM-1 adhesion molecule expression, which could, at least in part, explain the reduction in PMN infiltration and consequent colon damage. In fact, the protective action of MaR1 appears to be similar to the activities of some resolvins, which also reduce inflammatory mediators such as TNF-α, IL-6, INF-γ, and CXCL1/KC as well as adhesion molecules such as ICAM-1 (13, 19–21).

Besides an indirect action possibly involved in PMN infiltration reduction, we presently demonstrated that MaR1 abolished in vitro CXCL1/KC-induced neutrophil migration, suggesting a direct effect of this DHA-derived mediator on PMN recruitment to the site of inflammation. Interestingly, a very similar action was already reported for other lipid mediator, RvE1 (65). Neutrophils are an important source of ROS, which normally exerts a key role in microbial defense, but may also induce tissue damage (44, 66). In this study, we observed that MaR1 treatment significantly inhibited ROS production by neutrophils in vitro after PMA stimulation, suggesting a direct role in modulating activation in these cells, and a possible mechanism involved in vivo damage reduction. Interestingly, the relevance of lipid mediators, such as RvE1 and RvD1, in inhibiting ROS production has recently been reported (67, 68).
It is now well recognized that ROS can regulate NF-κB activity in many ways, whereas this transcription factor in turn plays a central role in inflammation and immunity (69) by regulating the expression of a number of enzymes and cytokines implicated in several inflammatory diseases, including IBD (70). Because we observed a decrease in cytokine expression and a reduction in

**FIGURE 8.** MaR1 induces a switch in macrophage polarization from the M1 to the M2 phenotype. BMDMs from naive mice were stimulated with LPS (1 μg/ml) in the presence or absence of MaR1 (300 nM/well) for 24 h. MaR1 incubation significantly reduced IL-1β (A), IL-6 (B), TNF-α (C), and INF-γ levels (D). MaR1-treated macrophages did not reduce MRC1 (E) and NOS2 (F) mRNA expression, but per se enhanced MRC1 mRNA expression (E). A real-time PCR assay was performed in duplicate, and GAPDH mRNA was used to normalize the relative amount of mRNA. Data are reported as means ± SEM (n = 6/group). *p < 0.05 versus the control group, *p < 0.05 versus the LPS-treated group.

**FIGURE 9.** MaR1 treatment prevents TNBS-induced colitis. Mice received 100 μl TNBS (1 mg/animal in 35% ethanol) into the colon. Systemic treatment with MaR1 (0.3 μg/animal) per day from days 0–3 significantly protected the mice from body weight loss (A), colonic macroscopic damage (B), reduced MPO activity (C), and microscopic damage (D), when compared with the TNBS-treated group. (E) Representative paraffin sections of colons from control mice (control), TNBS-treated mice (TNBS), and mice treated with MaR1 (0.3 μg/animal, e.v.) were stained with H&E. The arrows indicate the crypt damage, inflammatory cell infiltration, and edema. Original magnification ×200. Data are reported as means ± SEM of six to eight mice per group. *p < 0.05 versus vehicle-treated control group, *p < 0.05 versus TNBS-treated group.
effects exerted by data support the notion that MaR1 contributes to the beneficial is, preventive and therapeutic, were effective. Therefore, the present mediators, as well as possibly inducing a switch to the M2 macrophages to the M2 phenotype (12). Interestingly, this same study maresin, an MaR1 precursor, promoted conversion of M1 macrophages to maresin1 (MaR1), inhibits leukotriene A₄ hydrolyase (LTA₄H), sulfite sodium-induced colitis, *FASER J.*, 27: 2575–2580.


