Treatment with a Urokinase Receptor-derived Cyclized Peptide Improves Experimental Colitis by Preventing Monocyte Recruitment and Macrophage Polarization

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Background: Leukocyte migration across the blood barrier and into tissues represents a key process in the pathogenesis of inflammatory bowel diseases. The urokinase receptor (urokinase-type plasminogen activator receptor) is a master regulator of leukocyte recruitment. We recently found that cyclization of the urokinase-type plasminogen activator receptor–derived peptide Ser-Arg-Ser-Arg-Tyr [SRSRY] inhibits transendothelial migration of monocytes. Now, we have explored the effects of [SRSRY] administration during experimental colitis.

Methods: The effects of [SRSRY] on cytokine profile, cytoskeletal organization, and cell migration were investigated using phorbol-12-myristate acetate–differentiated THP-1 cells exposed to polarizing stimuli. In vivo, [SRSRY] was intraperitoneally administered during dextran sodium sulfate– or 2,4,6-trinitrobenzene sulfonic acid–induced colitis in wild-type or urokinase-type plasminogen activator receptor knockout mice. Levels of pro-inflammatory cytokines and inflammatory monocytes in mucosal infiltrates were assessed by enzyme-linked immunosorbent assay and flow cytometry, respectively.

Results: [SRSRY] prevents M0 to M1 transition and migration of M1 polarized macrophages. In vivo, [SRSRY] reduces intestinal inflammation diminishing body weight loss and disease activity index. These beneficial effects are accompanied by a reduction of interleukin 1β, interleukin 6, and tumor necrosis factor α, an increase of interleukin 10, and an abridged recruitment of inflammatory monocytes to the inflamed tissue.

Conclusions: Altogether, these findings indicate that [SRSRY] may be considered as a new drug useful for the pharmacological treatment of chronic inflammatory diseases, such as inflammatory bowel diseases.

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Key Words: inflammatory bowel diseases, experimental colitis, urokinase receptor, monocytes, macrophages, peptides

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, refer to chronic inflammatory disorders that affect the gastrointestinal tract.[1] Even if their etiology is still not completely understood, it has been clarified that IBD have genetic and environmental components and seem to be immunologically mediated in part by enteric microbiota.[1,2] Indeed, intestinal immune cells, such as dendritic cells and macrophages, interact with an abnormal antigen loads, and their ability in sustaining microbial tolerance is lost during IBD.[3] Furthermore, the chronicity of the inflammation is prolonged and maintained by an aberrant recruitment and activation of intestinal leukocytes to the inflamed tissue.[1,2]

The urokinase-type plasminogen activator receptor (uPAR) regulates the activation and mobilization of leukocytes, being expressed by a wide variety of hematologic cells, including monocytes and macrophages.[4,5]

We recently proved that uPAR is able to control macrophage phagocytosis during experimental colitis.[6] Indeed, we observed that genetic deletion of uPAR augmented mouse susceptibility to experimental colitis models.[6] We and others have documented that uPAR is able to control cell migration through the assembly in composite regulatory units with transmembrane receptors, including formyl peptide receptors (FPRs) and the vitronectin receptor which, in turn, signal across the membranes.[7] Ligand-activated uPAR exposes on cell surface the minimal active ^{88}Ser-Arg-Ser-Arg-Tyr^{92} sequence, able to trigger cell migration and angiogenesis in vitro and in vivo, even in the form of synthetic linear peptide Ser-Arg-Ser-Arg-Tyr (SRSRY).[8–10]

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Mechanistically, $uPAR_{88–92}$ sequence interacts with the FPR types 1 and 2, henceforth inducing cell migration of several cell lines including monocytes and macrophages.[8,9,11]

We have recently documented that the cyclization of the Ser-Arg-Ser-Arg-Tyr peptide generates the peptide [SRSRY] exerting opposite effect on cell migration, as compared with its linear form. Unlike the linear peptide SRSRY, [SRSRY] blocks FPR1-mediated signaling by interfering with both internalization of FPR1 and ligand uptake. [SRSRY] inhibits monocyte locomotion with IC_{50} value of 0.01 nM and displays a long-time resistance to enzymatic digestion in serum. Notably, at 10 nM concentration, [SRSRY] prevents transendothelial migration of monocytes and causes a marked inhibition of their cytoskeletal reorganization occurring during locomotion.[12]

In the present study, we explored the effects elicited by the administration of [SRSRY] in 2 experimental models of colitis, characterizing the recruitment and the activation of monocytes and macrophages into the inflamed intestine. Herein, we show that [SRSRY] possesses anti-inflammatory properties, reducing the infiltration of inflammatory monocytes and influencing macrophage polarization.

MATERIALS AND METHODS

Ethics Statement

Animal experiments adhered to the requirements of the Commission Directive 86/609/EEC and to the Italian legislation (Decreto Legislativo 116; January 27, 1992). The studies were approved by the Animal Care and Use Committee (authorization no. 192/2012-B; Istituto Clinico Humanitas, Milan, Italy).

Peptide Synthesis and Purification

Cyclic peptides [Ser-Arg-Ser-Arg-Tyr] ([SRSRY]) and [Arg-Ser-Ser-Tyr-Arg] ([RSSYR]) were synthesized by Fmoc chemistry solid-phase approach and purified by reverse-phase HPLC, as previously described.[12]

Generation of M1 Polarized Macrophages

Human monocyte leukemia THP-1 cell line (American Type Culture Collection) was cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 U/mL). THP-1 monocytes were differentiated with 160 nM phorbol-12-myristate acetate (PMA; Sigma-Aldrich, Milan, Italy) for 2 days. M1 macrophage polarization was achieved adding 100 ng/mL lipopolysaccharide + 20 ng/mL interferon (IFN) γ (both from Sigma-Aldrich) to PMA-differentiated THP-1 cells for additional 2 days, as described.[13] In parallel experiments, M1 polarization occurred in the presence of 10 nM [SRSRY].

Cytokine Array

To confirm the polarization of macrophages into the M1 subtype after stimulation, conditioned media were collected and centrifuged at 1000 rpm for 5 minutes to remove cell debris. The relative levels of cytokines were analyzed using the dot blot Human Cytokine Array Kit panel A (no. ARY005; R&D Systems, Milan, Italy), according to the manufacturer's instructions. Briefly, 500 μL of supernatant was applied on each membrane, and the signals were detected using streptavidin–horseradish peroxidase and chemoluminescent detection reagents. The pixel density of each spot was measured using NIH Image J 2.0 software. The intensity of positive control spots was used to normalize results between the membranes. The intensity for each spot was then averaged over the duplicate spots.

Fluorescence Microscopy

To analyze FPR1 expression, M1 polarized THP-1 cells were incubated with 2 μg/mL rabbit anti-FPR1 antibody (Santa Cruz Biotechnology, Milan, Italy) for 2 hours at 23°C and then with 1:800 goat Alexa Fluor 488 anti-rabbit immunoglobulin G (Molecular Probes, Milan, Italy) at 23°C for 45 minutes. Nuclear staining was performed with 4-6-diamidino-2-phenylindole dye. To analyze the effect of [SRSRY] on FPR1 internalization, cells were incubated with the indicated unlabeled peptides for 30 minutes at 37°C and then exposed to 10 nM *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FITC-fMLF; Molecular Probes) for 30 minutes at 37°C, as described.[14] To analyze cytoskeletal organization, cells were fixed and permeabilized with 2.5% formaldehyde–0.1% Triton X-100 in phosphate-buffered saline for 10 minutes at 4°C, washed in phosphate-buffered saline, and then incubated with 0.1 μg/mL rhodamine-conjugated phalloidin (Invitrogen, Milan, Italy) at 23°C for 45 minutes. In all cases, coverslips were mounted using 20% (wt/vol) mowiol and analyzed by a fluorescence-inverted microscope connected to a video camera or by the confocal 510 META LSM microscope (Carl Zeiss, Milan, Italy).

DUNN Chamber Assay

M1 polarized THP-1 cells were seeded on 20 × 20 mm coverslips for 24 hours. Before inverting the coverslip on top of a double-concentric DUNN chamber, cells on the coverslip covering the outer chamber were carefully scraped away, as previously described.[15] A gradient of a chemoattractant was created by placing serum-free medium in the inner chamber and 10 nM fMLF with or without 10 nM [SRSRY] or 10 nM [RSSYR] in the outer chamber. The ring separating the inner and outer chambers permits slow diffusion between the chambers. For control experiments, both wells were filled with serum-free medium. After 6 hours of incubation at 37°C with 5% CO_2, the coverslip was removed from the chamber and the cytoskeleton was visualized by staining with rhodamine-conjugated phalloidin. A total of 100 cells per sample, which translocated to the area corresponding to the ring separating the inner and outer chambers, were counted, and their cytoskeletal organization was examined with a fluorescence-inverted microscope connected to the video camera.

Cell Viability

The viability of LPS/IFNγ-stimulated THP-1 macrophages was investigated using the CellTiter 96 AQueous Cell Proliferation Assay Kit (Promega, Milan, Italy), according to the manufacturer's

instructions. PMA-differentiated THP-1 cells (2×10^4 cells per well) were seeded in 96-well tissue culture plates, exposed to polarizing stimuli for 48 hours, and then incubated with 10 μM [SRSRY], 10 μM [RSSYR], or diluents. Medium with or without peptides was replaced every 24 hours. After 24, 48, and 72 hours, suspended cells were removed and the adherent cells stained with tetrazolium/formazan for 3 hours at 37°C. The absorbance was detected at 490 nm using a microplate reader (Bio-Rad Laboratories, Segrate [MI], Italy).

Mice

Wild-type C57BL6 mice were purchased by Charles River Laboratories (Calco, Lecco, Italy). uPAR knockout (KO) mice[16] were kindly provided by Nicolai Sidenius (IFOM-IEO, Milan, Italy) and maintained on C57BL6/N genetic background. Animals were kept under specific pathogen-free conditions. Six-to 10-week-old, age-matched, and sex-matched wild-type (WT) and KO littermates were used for all experiments. Typically, 4 to 6 mice were used for each group of treatment, and the experiment was repeated at least 3 times.

Mouse Models of Colitis and Colitis Evaluation

Colitis was induced in C57BL/6 WT and uPAR KO littermates. For dextran sodium sulfate (DSS)–induced colitis, mice received 2.5% DSS in drinking water ad libitum for 5 days. Body weights were recorded and referred as percent value of the initial weight. To assess disease activity index (DAI), feces were collected from each mouse of the experimental groups and evaluated for consistency and the presence of occult blood (Hemoccult SENSA; Beckman Coulter, Indianapolis, IN).

The DAI was then calculated as average of 3 different parameters: body weight loss (score 1: 1%–5%; score 2: 5%–10%; score 3: 10%–15%; score 4: more than 15%), blood in the feces (score 0: no blood; score 2: occult blood; score 4: visible blood), and feces consistency (score 0: well formed; score 2: soft; score 4: diarrhea). At the end of treatment, mice were killed, colons were excised, and colon length from the end of the cecum to the anus was recorded.

For 2,4,6-trinitrobenzene sulfonic acid (TNBS)–induced colitis, mice were anesthetized and received an intrarectal injection of 2.5 mg/mouse of TNBS in 15% ethanol or 15% ethanol alone as a control. After instillation, mice were held in an upright position for 3 minutes to avoid reflux. Weight loss was monitored daily. At the end of treatment, mice were killed and colons were excised.

For both models, mice were divided into 3 experimental groups. The first group was treated with intraperitoneal injection of [SRSRY] at 3 mg/kg every 2 days, starting from 2 days before DSS administration or TNBS instillation. The control group received the same amount of physiological solution, whereas the third group was left untreated. Colons from colitic and healthy mice were histologically evaluated using 4 μM paraffin sections stained with hematoxylin and eosin. A pathologist blinded to treatments and genotype of the tissue evaluated the degree of inflammatory cell infiltration and mucosal damage.

In parallel experiments, colonic tissues were employed for the isolation of lamina-propria (LP) mononuclear cells (LPMCs) or immediately frozen on dry ice for protein extraction and enzyme-linked immunosorbent assay (ELISA) analysis.

Characterization of Murine LPMCs

LPMCs were obtained from healthy and inflamed colonic tissues, as previously described.[6] Briefly, colons were cut longitudinally and washed in phosphate-buffered saline containing 1% fetal bovine serum and 1 mM dithiothreitol for 15 minutes at 23°C. Enzymatic digestion was then performed in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.5 mg/mL collagenase type VIII (Sigma-Aldrich), 20 mg/mL DNase I (Roche Diagnostics, Milan, Italy), 100 U/mL penicillin, and 100 μg/mL streptomycin for 60 minutes at 37°C with gentle shaking. The cell suspension obtained was passed through a 100-μm filter and clarified over 70-μm nylon mesh. The total amount of live cells was determined by Trypan blue exclusion count on Neubauer chamber.

To characterize the different myeloid populations, we stained murine LP cells with the following fluorochrome-conjugated antibodies: PerCP-conjugated anti-mouse CD45, Pacific Blue–conjugated anti-mouse CD11b, FITC-conjugated anti-mouse Ly6C, APC-conjugated anti-mouse F4/80, and PE-Cy7.7-conjugated anti-mouse CD11c (all from BD Biosciences-Pharmingen, Mountain View, CA). Fluorochrome-conjugated, isotype-matched control antibodies were included to assess background fluorescence. All incubations were done in 3% bovine serum albumin, supplemented with the corresponding immunoglobulin G to minimize nonspecific binding.

ELISA

Proteins were extracted from colons of healthy and colitic mice, as previously described.[6] The presence of interleukin (IL) 1β, IL-6, IL-10, and tumor necrosis factor (TNF) α was quantified by ELISA following the manufacturer's instructions (Duo Set ELISA; R&D systems) and normalized over the total amount of proteins extracted.

Statistical Analyses

The results are expressed as mean ± SD of the number of indicated determinations. Data were analyzed by 1-way analysis of variance and post hoc Bonferonni's modified *t* test for multiple comparisons. $P < 0.05$ was accepted as significant.

RESULTS

The Peptide [SRSRY] Inhibits Agonist-dependent FPR1 Activation in M1 Polarized THP-1 Cells and Prevents M1 Polarization

PMA-differentiated THP-1 cells responded to LPS/IFNγ, resulting in M1 polarization, as expected. Indeed, PMA-differentiated THP-1 macrophages showed a rounded and spindle-shaped morphology, whereas M1 polarized macrophages adopted a "dendritic"-like morphology with the appearance of large

filopodia (Fig. 1A). Because chemokine repertoires of mononuclear phagocytes exposed to polarizing stimuli are profoundly different,[13] M1 polarization was ascertained by determining their cytokine profile. Accordingly, M1 polarized cells secreted higher levels of CXCL-10, CXCL-11, IL-1β, IL-6, and TNF-α as compared with M0 and showed a significant increase of CCL5 and CD54 (Fig. 1B), confirming the M1 activation status of the macrophages.

We have previously shown that the peptide [SRSRY] inhibits fMLF-directed migration of monocytes and macrophages by interfering with both ligand uptake and internalization of FPR1.[12] According to Gemperle et al,[17] we found that M1 polarized macrophages express high levels of FPR1 on cell surface (Fig. 1C). FPR1 is internalized in response to agonist stimulation, this process being a prerequisite for receptor activation.[18] Incubation of M1 macrophages with *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-(fMLF)-fluorescein resulted in the appearance of numerous intracytoplasmic green fluorescent spots (arrows), which disappeared in M1 macrophages preincubated with excess of nonfluorescent fMLF, as expected. We found that, like fMLF, 100 nM [SRSRY] prevented agonist-dependent FPR1 internalization, whereas the scrambled control peptide [RSSYR] did not exert such effect (Fig. 1D), indicating that [SRSRY] is able to inhibit FPR1 activation also on polarized M1 macrophages.

It has been documented that LPS upregulates FPR1 mRNA levels promoting both enhanced transcription and mRNA stability[19] and that LPS and fMLF regulate the induction of inflammation in a synergistic manner both in vitro and in vivo,[20] raising the possibility that [SRSRY] may interfere with LPS-induced signaling. Therefore, we investigated the effects of [SRSRY] on M1 polarization. Interestingly, when PMA-differentiated THP-1 cells were exposed to polarizing stimuli in the presence of 10 nM [SRSRY], a general reduction of chemokine repertoires was observed, whereas the control scramble peptide [RSSYR] did not exert such effect (Fig. 1B). In particular, [SRSRY] dramatically reduced secretion of CXCL-10, CXCL-11, IL-1β, IL-6, IL-8, and TNF-α (Fig. 1B). These findings indicate that the peptide [SRSRY] blocks agonist-dependent FPR1 internalization in M1 polarized macrophages and prevents M0 to M1 transition.

The Peptide [SRSRY] Inhibits M1 Polarized Macrophage Migration Affecting Cytoskeletal Reorganization

The mechanisms by which cells move when subjected to a chemoattractant gradient involve changes in cytoskeletal organization, which provide both the protrusive and contractile forces necessary for cell migration. To analyze the effects of

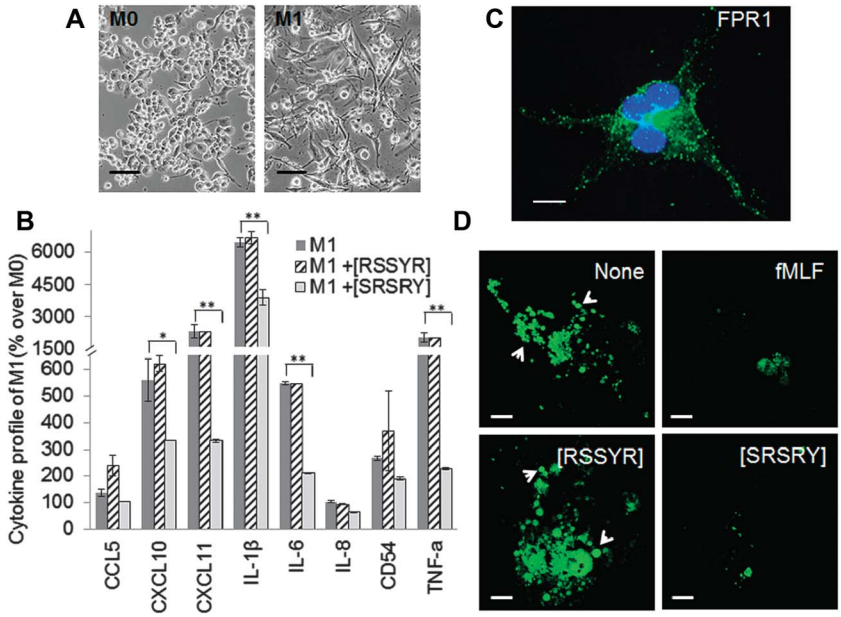

FIGURE 1. The peptide [SRSRY] reduces cytokine levels secreted by FPR1 expressing M1 polarized macrophages. A, Representative images of PMA- (M0) and INFγ/LPS (M1)-stimulated THP-1 cells analyzed by phase contrast microscopy at ×200 (scale bar: 100 μm). B, Cytokine levels secreted by PMA-differentiated THP-1 cells exposed to LPS/IFNγ (M1), LPS/IFNγ + 10 nM [SRSRY], or LPS/IFNγ + 10 nM [RSSYR] for 48 hours, expressed as percentage of cytokines secreted by M0 macrophages. The pixel density of each spot was measured using NIH Image J 2.0 software. The intensity of positive control spots was used to normalize results between the membranes. Data were averaged over the duplicate spots. *$P < 0.05$, **$P < 0.01$. C, Representative images of M1 polarized THP-1 cells double stained for FPR1 and 4-6-diamidino-2-phenylindole dye. Original magnification: ×400, scale bar: 10 μm. D, Representative images of M1 polarized THP-1 cells incubated with diluents (none), 100 nM fMLF, 100 nM [SRSRY], or 100 nM [RSSYR] for 30 minutes at 37°C, exposed to 10 nM *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein for additional 30 minutes at 37°C and then visualized using a Zeiss 510 META LSM microscope. Arrows indicate the intracytoplasmic green fluorescent spots. Scale bar: 10 μm. Original magnifications: ×630.

[SRSRY] on the motility of M1 polarized macrophages, cells grown adherent onto a glass slide were exposed to diluents, 10 nM fMLF gradient ± 10 nM [SRSRY] or 10 nM [RSSYR] in a DUNN chamber for 6 hours, and then stained with rhodamine-phalloidin. First, migrating cells, located on the ring separating the inner and outer chambers, were analyzed for their cytoskeletal organization (Fig. 2). In the absence of fMLF gradient (CTRL), cells exhibited a flattened morphology with multiple unpolarized F-actin–rich protrusions. When subjected to the fMLF gradient alone or mixed to the control peptide [RSSYR], M1 macrophages exhibited an elongated morphology and recognizable aligned protrusions associated to locomotion in the 83% and 80% of cell population, respectively (Fig. 2A). Vice versa, the addition of [SRSRY] peptide to the fMLF gradient reduced cell elongation and alignment with the appearance of F-actin linear distribution along the plasma membranes in the 63% of M1 cell population (Fig. 2A). Next, to assess whether [SRSRY] affects their motility, cells located on the ring were visualized by an inverted microscope and counted. M1 macrophage exposure to 10 nM fMLF elicited a considerable cell migration (Fig. 2B, C), reaching 294% of the basal cell migration, which was unchanged by the presence of 10 nM [RSSYR]. On the contrary, the addition of 10 nM [SRSRY] to the outer chambers drastically reduced M1 polarized macrophage migration below the basal level (Fig. 2B, C). The inhibitory effect exerted by [SRSRY] on cytoskeletal organization and migration of M1 macrophages was not due to a reduced cell viability as the number of macrophages exposed to polarizing stimuli did not change in the presence of 10 μM [SRSRY] as assessed by a colorimetric method for quantification of viable cells (Fig. 2D). Together, these results indicated that the peptide [SRSRY] inhibits motility of M1 polarized macrophages.

In Vivo Administration of [SRSRY] Reduced Colitis Susceptibility, Independent of Endogenous uPAR Expression

Given the effects elicited by [SRSRY] in vitro and the important role of macrophages in acute and chronic inflammation, we investigated whether [SRSRY] might affect the development of experimental colitis. To this end, colitis was initially induced by the administration of DSS for 5 days, as described in the Materials and Methods. Furthermore, mice were injected

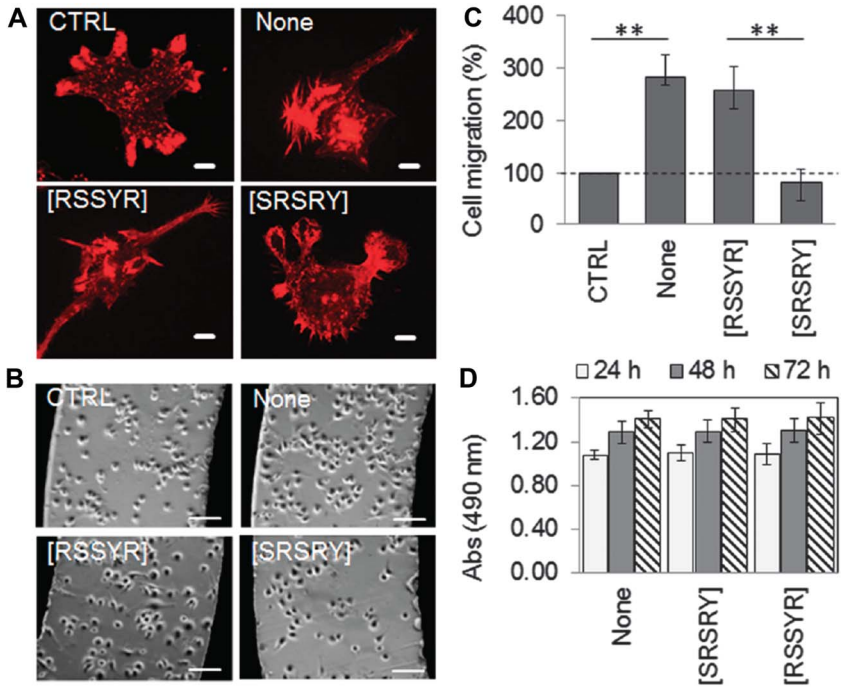

FIGURE 2. The peptide [SRSRY] inhibits migration of M1 polarized macrophages causing a marked inhibition of cytoskeletal reorganization. M1 polarized THP-1 cells were allowed to migrate for 6 hours in a DUNN chamber toward diluents (CTRL), or a fMLF chemotactic gradient, in the absence (none) or in the presence of 10 nM [SRSRY] or 10 nM [RSSYR]. A, Representative images of M1 polarized THP-1 cells located on the ring separating the inner and outer chambers and stained with rhodamine-phalloidin. Scale bar: 5 μm. Original magnifications: ×400. B, Representative images of cells located on the ring separating the inner and outer of the DUNN chamber. Scale bar: 100 μm. Original magnifications: ×50. C, For quantitative analysis of cell migration, M1 polarized cells located on the ring were counted. The arbitrary value of 100% was given to the basal cell migration, assessed in the absence of chemoattractant (CTRL: ——), and the results were expressed as percentage of the basal cell migration. $^{**}P < 0.001$. D, M1 polarized THP-1 cells were exposed to diluents (none), 10 μM [SRSRY] or 10 μM [RSSYR]. Medium with or without peptides was replaced every 24 hours. At the indicated times, the absorbance of adherent cells stained with tetrazolium/formazan was assessed with a Microplate Reader.

intraperitoneally with 3 mg/kg [SRSRY] every 2 days, starting 2 days before the administration of DSS.

Interestingly, [SRSRY] treatment reduced body weight loss (Fig. 3A), and overall, all the signs of acute intestinal inflammation, including anal bleeding and stool consistency, were significantly diminished in [SRSRY]-treated mice (DAI, Fig. 3B).

We have previously shown that [SRSRY] inhibitory effects on monocyte locomotion are due to its ability to inhibit FPR1 signaling.[12] Thus, we assessed whether the endogenous expression of uPAR is required to elicit the beneficial effects exerted by [SRSRY] administration. To this end, uPAR KO littermates were subjected to DSS-induced colitis. uPAR KO mice experienced a severe colitis compared with WT littermates, as observed by body weight loss and DAI (Fig. 3C, D), confirming our previous results.[6] In keeping with the previously reported ability of [SRSRY] to inhibit FPR1 activation,[12] we found that intraperitoneal administration of [SRSRY] significantly improved body weight loss and DAI, also in the absence of uPAR expression (Fig. 3C, D). Indeed, both histological and endoscopic analyses showed a reduced intestinal inflammation not only in WT but also in uPAR KO treated mice, which displayed a lower number of edema and a decreased mucosal infiltrate as compared with untreated mice (Fig. 3E). Accordingly, analyses of the colon length and histological and endoscopic scores assessed at the end of the treatment indicate that beneficial effects exerted by intraperitoneal administration of [SRSRY] does occur and are independent of endogenous uPAR expression (Fig. 3F).

[SRSRY] Administration Reduces the Severity of TNBS-induced Colitis

We next investigated whether [SRSRY] could reduce the severity of TNBS model of experimental colitis, which induces severe colonic inflammation resembling clinical and histopathological features of Crohn's disease.[21]

Because we previously observed that the endogenous expression of uPAR is not required to exert the protective effects by [SRSRY] administration at least in the DSS-induced colitis model, we compared the severity of TNBS-induced colitis in both WT and uPAR KO littermates.

As expected, uPAR KO mice experienced a serious colonic inflammation compared with WT littermates, as demonstrated by body weight loss and histopathological signs of inflammation (Fig. 4). Interestingly, [SRSRY] administration was able to reduce the severity of inflammation in both WT and uPAR KO littermates. However, [SRSRY]-treated mice displayed a significant reduction of the body weight loss exclusively in the absence of endogenous uPAR expression, whereas WT treated mice showed a similar trend even if the differences were not significant (Fig. 4A, B).

Furthermore, histopathological analysis confirmed that [SRSRY] administration was effective in reducing colonic inflammation principally in uPAR KO mice, whereas WT mice showed a slight improvement of the colon length and the histological score (Fig. 4C, D).

Altogether, these results clearly indicate that [SRSRY] is able to ameliorate in vivo the severity of experimental colitis.

[SRSRY] Modulates the Secretion of Pro-inflammatory Cytokine During Experimental Colitis

Because the DSS and TNBS models of colitis are associated with an unbalanced cytokine profile,[21] we evaluated whether [SRSRY] could affect the secretion of pro- and anti-inflammatory cytokines.

To accomplish this goal, we quantified by ELISA the amounts of IL-1β, IL-6, TNF-α, and IL-10 produced by colonic tissues under steady-state conditions (before colitis induction) and after intestinal injury. Comparable levels of cytokines were detected in healthy mice, treated or not with [SRSRY] (data not shown). By contrast, treatment with either DSS or TNBS induced a greater increase of IL-1β, IL-6, and TNF-α, as expected[21] (Fig. 5).

According to the finding that PMA-differentiated THP-1 cells exposed to polarizing stimuli in the presence of 10 nM [SRSRY] secrete in vitro lower levels of IL-1β, IL-6, and TNF-α as compared with untreated cells (Fig. 1B), we found that [SRSRY] treatment significantly reduced the release of IL-1β (Fig. 5A), IL-6 (Fig. 5B), and TNF-α in colonic tissues (Fig. 5C). IL-10 has been prompted as a master regulator of intestinal inflammation because it has been recognized as an immune modulatory cytokine that is capable of dampening T helper cells type 1-like responses.[22] Intriguingly, [SRSRY] administration was able to significantly augment the mucosal levels of IL-10 after either DSS administration or TNBS instillation (Fig. 5D).

Altogether these results clearly indicated that [SRSRY] dampened intestinal inflammation. Furthermore, they also suggest that other and more complex immune modulatory events affecting Th1 responses and promoting IL-10 release may occur during [SRSRY] administration.

[SRSRY] Reduces the Mucosal Infiltrate, Decreasing the Recruitment of Inflammatory Monocytes

We previously reported that [SRSRY] is a potent inhibitor of monocyte transendothelial migration.[12] Given the unbalanced cytokine profile observed, we reasoned that the beneficial effect of [SRSRY] administration might be due to an alteration of the monocyte/macrophage infiltrate. Because uPAR plays an important role in leukocyte recruitment after inflammation,[4,23] to better appreciate the effect of [SRSRY] treatment on monocyte/macrophage trafficking, only uPAR KO mice were employed in this set of experiments.

LPMCs were isolated from the healthy and inflamed colon of uPAR KO mice, treated or not with [SRSRY]. We then characterized myeloid cells ($CD45^+CD11b^+$) by the expression of specific surface markers to distinguish between LP-dendritic cells ($F4/80^-CD11c^+$), LP-macrophages ($F4/80^+CD11c^{+/-}Ly6C^-$), LP-monocytes/macrophages ($F4/80^+CD11c^{+/-}Ly6C^+$), and monocytes ($SSC\text{-}A^{low}F4/80^-CD11c^-Ly6C^+$).

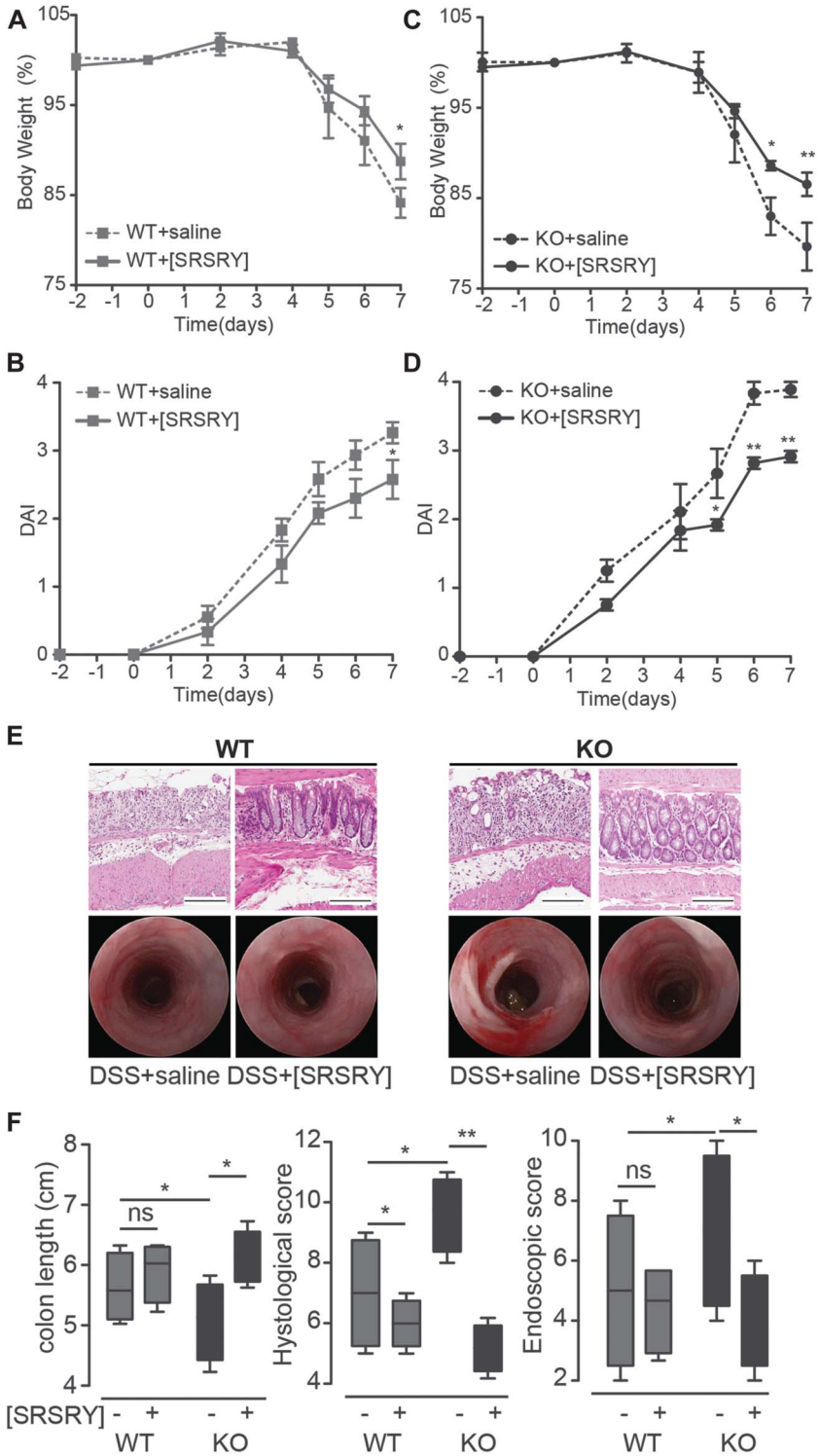

FIGURE 3. In vivo administration of the peptide [SRSRY] reduces the severity of DSS-induced colitis. A, The severity of DSS-induced colitis was assessed by monitoring body weight loss in WT mice, treated or not with [SRSRY]. B, The DAI was calculated as indicated in the Materials and Methods. C and D, DSS-induced colitis was induced in uPAR KO mice and the severity assessed by body weight loss and DAI. E, Representative images of the histological and endoscopic analyses, carried at the end of the experimental procedure in both WT and uPAR KO mice. F, Histogram bars were plotted to represent colon length, histological score, and endoscopic score. Values were calculated as mean ± SD of 3 independent experiments. *$P < 0.05$, **$P < 0.01$.

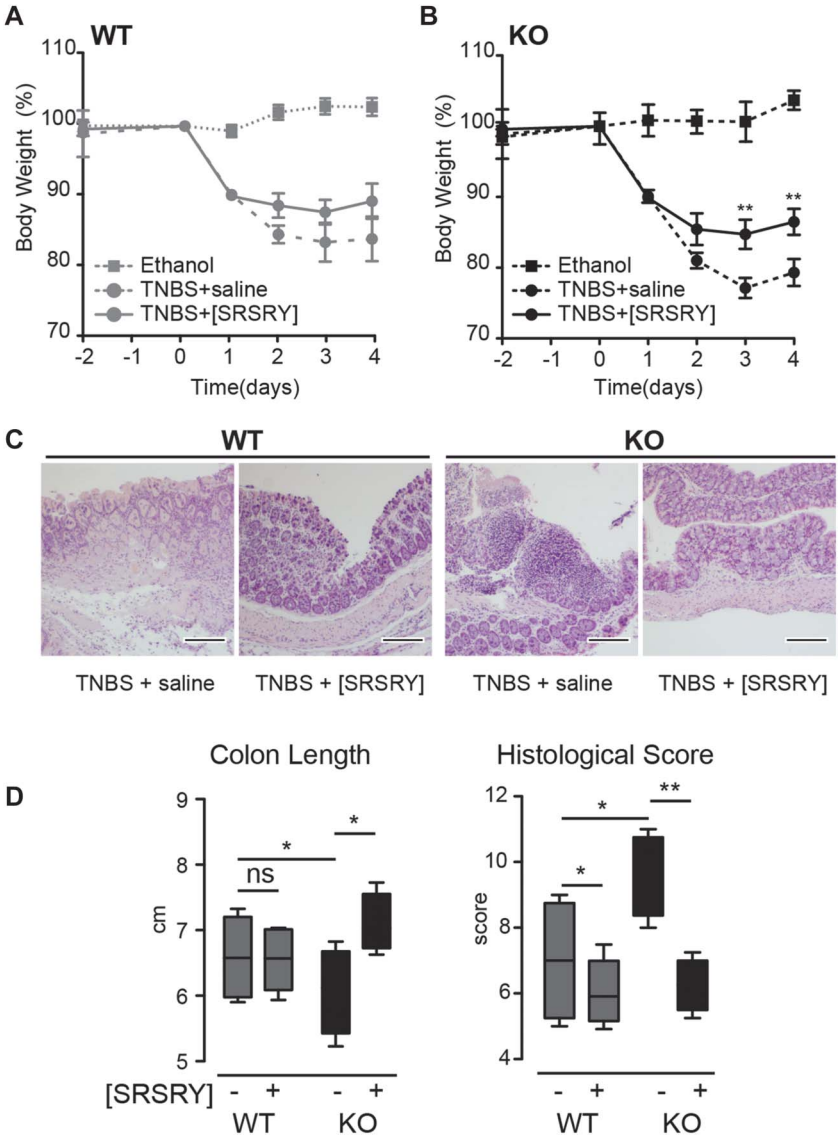

FIGURE 4. [SRSRY] administration reduces the severity of the TNBS model of experimental colitis. A, C57BL/6 WT mice received intrarectal administration of TNBS. Colitis severity was evaluated by recording body weight loss and compared with mice intrarectally injected with ethanol (control) or with mice injected with [SRSRY]. B, TNBS-induced colitis was also induced in uPAR KO mice and the severity evaluated by body weight recording. C, Representative images of the histological analysis, carried at the end of the experimental procedure in both TNBS-treated WT and uPAR KO mice, injected with saline or [SRSRY]. Histogram bars were plotted to represent colon length and histological score. Values were calculated as mean ± SD of 3 independent experiments. *$P < 0.05$, **$P < 0.01$.

As expected, healthy mice possessed few leukocytes in the uninflamed mucosa ($CD45^+$ cells: 10.3% ± 2.5% over total vital cells). Furthermore, myeloid cells (Fig. 6A) were mainly represented by residential macrophages (47.4% ± 2.5% over $CD11b^+$ cells), whereas only a small amount of LP-monocytes/macrophages (1.2% ± 1.5% over $CD11b^+$ cells) and monocytes were detected (7.2% ± 3.8% over $CD11b^+$ cells).

In contrast, inflammation driven by DSS administration induced a prominent recruitment of LP-leukocytes in the inflamed mucosa, as expected ($CD45^+$ cells: 40.1% ± 9.3% over total vital cells). Furthermore, we noticed a significant increase in the relative percentage of both inflammatory monocytes (23.5% ± 8.5% over $CD11b^+$ cells) and LP-monocytes/macrophages (21.5% ± 5.5% over $CD11b^+$ cells, Fig. 6B). Interestingly, [SRSRY] treatment minimally affected the relative percentage of LP-leukocytes ($CD45^+$ cells: 38.3% ± 6.5% over total vital cells). Nevertheless, [SRSRY] was able to significantly reduce the relative amount of both inflammatory monocytes and LP-monocytes/macrophages (Fig. 6B, D). Similar results were obtained in the TNBS experimental model of colitis (Fig. 6C), even if a prevalent recruitment of myeloid cells was evident, along with a huge increment of inflammatory monocytes and LP-monocytes/macrophages

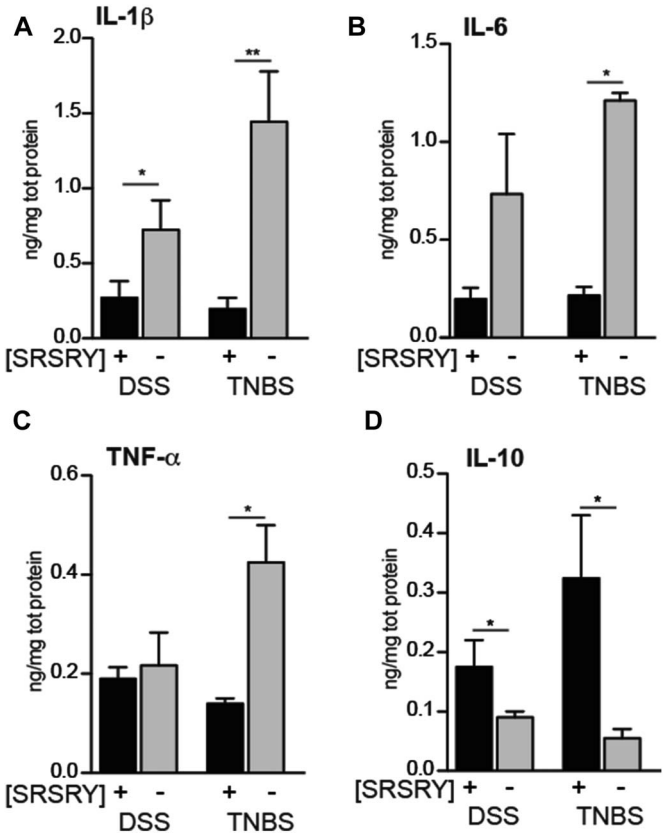

FIGURE 5. [SRSRY] treatment reduces the severity of experimental colitis through effect on the release of pro- and anti-inflammatory cytokines. Colitis was induced in uPAR KO mice treated or not with [SRSRY] by DSS or TNBS administration, as described in the Materials and Methods. A, The levels of IL-1β in the mucosal protein extracts obtained from inflamed colonic tissues were quantified by ELISA assay. B–D, Similarly, the levels of IL-6, TNF-α, and IL-10 were assessed after colitis induction in mucosal protein extracts. Histogram bars represented the mean ± SD of 3 independent experiments. *$P < 0.05$, **$P < 0.01$.

(69.5% ± 10.5% and 14.5% ± 3.5% over $CD11b^+$ cells, respectively). As for the DSS experimental model of colitis, [SRSRY] was able to reduce the relative amount of inflammatory monocytes and partially of LP-monocytes/macrophages (Fig. 6C, D).

Altogether these results clarified that intraperitoneal injection of the peptide [SRSRY] is able to reduce intestinal inflammation diminishing body weight loss and the DAI. These beneficial effects were due to an abridged recruitment of inflammatory monocytes to the inflamed tissue.

DISCUSSION

Leukocyte migration across the blood barrier and into tissues is one of the first events during inflammation, immune response against infection, and tissue remodeling and broadly represents a key process in the pathogenesis of chronic inflammatory diseases. Although molecules that mediate leukocyte motility have resulted in a significant number of new targets that hold promise for new therapies,[24,25] we still need to refine our understanding on the molecules that are important for the trafficking of specific lymphocyte subsets in humans.

In this article, we propose the cyclized peptide [SRSRY] as a new drug for treating chronic inflammatory diseases sustained by increased motility of inflammatory monocytes and macrophages, such as IBD.

uPAR plays an important role in the regulation of leukocyte trafficking.[26] The capability of uPAR to trigger cell migration depends on its ^{88}Ser-Arg-Ser-Arg-Tyr^{92} chemotactic sequence that, even in the form of a synthetic linear peptide, promotes cell migration by interacting with the G protein–coupled FPR1.[9] We have recently found that the cyclization of this sequence confers stability to the peptide and reverts its function providing the ability to inhibit transendothelial migration of monocytes by blocking fMLF-triggered FPR1 activation.[12]

FPR1 is recognized as a key molecule in sustaining inflammatory environment.[27] Activation of FPR1 results in increased cell migration, phagocytosis, and release of pro-inflammatory mediators, and the signaling cascade culminates in heterologous desensitization of other receptors including chemokine receptors CCR1, CCR5, and CXCR4.[28–30] Thus, by interacting with a variety of exogenous and host-derived agonists, FPR1 constitutes a novel pharmacological target.

During intestinal infection or inflammation, blood monocytes accumulate in the LP and actively pursue invading microorganisms through uptake and degradation of the organism and release of inflammatory mediators.[31] In particular, M1 polarized macrophages that are characterized by the secretion of high levels of pro-inflammatory cytokines, high production of reactive nitrogen and oxygen intermediates, are implicated not only in initiating but also in sustaining intestinal inflammation.[32]

In this study, we show that the peptide [SRSRY] inhibits cytoskeletal reorganization and migration of M1 polarized macrophages toward an fMLF chemotactic gradient. Mechanistically, as previously documented for monocytes and macrophages,[12] the peptide [SRSRY] blocks fMLF-dependent migration of M1 polarized macrophages by interfering with both internalization and ligand uptake of FPR1. When PMA-differentiated THP-1 cells were exposed to polarizing stimuli (LPS/IFNγ) in the presence of [SRSRY], a general reduction of pro-inflammatory cytokine repertoire was observed, suggesting that [SRSRY] also interferes with M0 to M1 transition. This finding is not surprising because LPS and fMLF regulate both in vitro and in vivo the induction of inflammation in a synergistic manner[20] and FPR1 expression is rapidly upregulated through both transcriptional and posttranscriptional mechanisms in mouse macrophages in response to a wide number of inflammatory stimuli, including LPS and TNF-α.[19] FPR1 regulates macrophage activation through the activation of nuclear factor kB (NF-kB), which in turn increases cytokine gene transcription inducing the secretion of IL-1α, IL-1β, and IL-6 from human peripheral blood mononuclear cells.[29,33] Intriguingly, NF-κB is a recognized mediator of LPS-triggered TLR4 signaling.[33] Thus, it

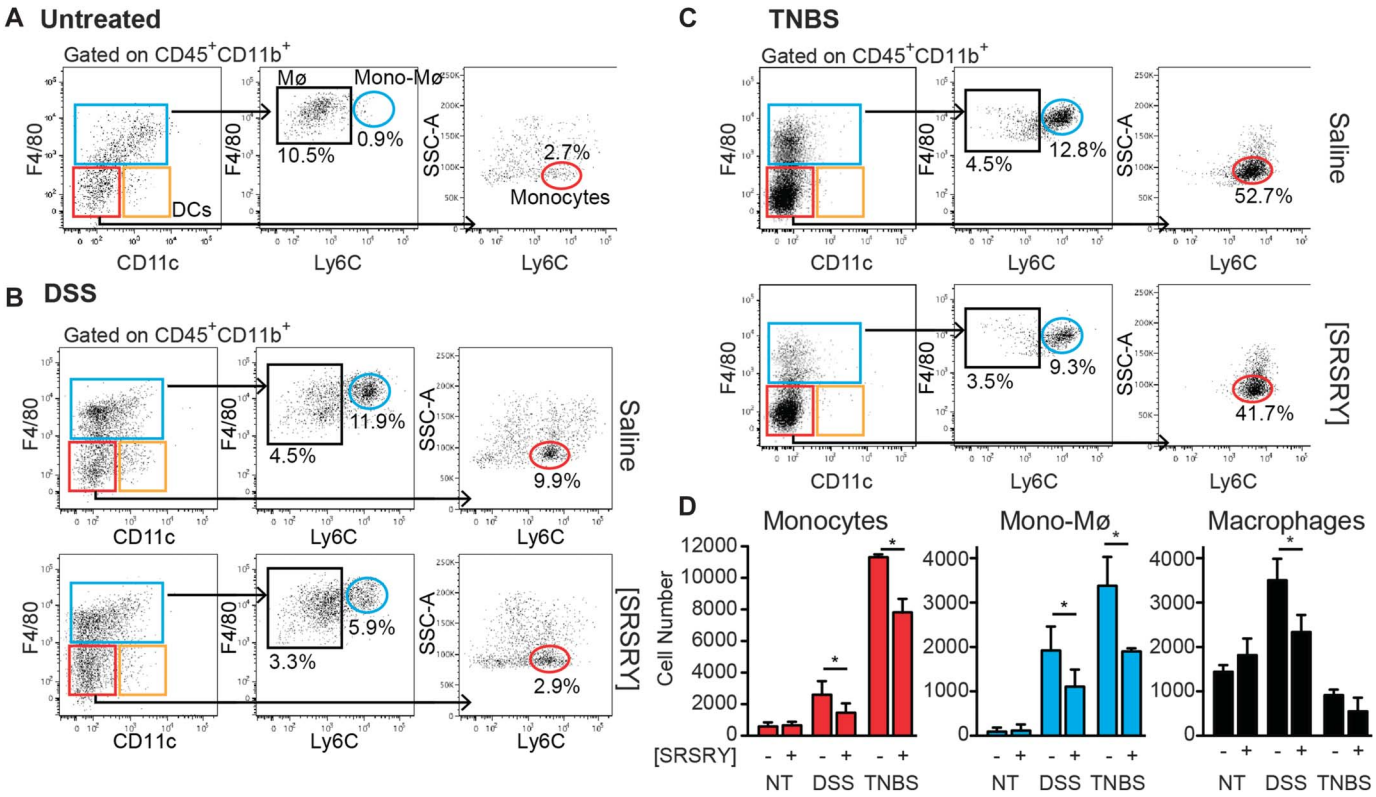

FIGURE 6. The recruitment of inflammatory monocytes is significantly affected by [SRSRY] treatment during experimental colitis. Colitis was induced in uPAR KO mice treated or not with [SRSRY] by DSS or TNBS administration, as described in the Materials and Methods. A, LPMCs were initially evaluated in steady-state conditions by flow cytometry analysis. Representative dot plots are showing the gating strategy used to identify among $CD45^+CD11b^+$ myeloid cells: LP-dendritic cells ($CD11c^+F4/80^-$), LP-macrophages ($CD11c^{+/-}Ly6C^-F4/80^+$), LP-mono-macrophages ($CD11c^{+/-}Ly6C^+F4/80^+$), and inflammatory monocytes ($SSCA^{low}CD11c^-F4/80^-Ly6C^+$). B, LPMCs were assessed after DSS-induced colitis both in saline-treated and [SRSRY]-treated mice. Representative dot plot showed a reduction both in LP-mono-macrophages and inflammatory monocytes, whereas LP-macrophages slightly increased after [SRSRY] treatment. C, Representative dot plot of flow cytometry analysis conducted in TNBS-treated uPAR KO mice, comparing saline versus [SRSRY]-treated mice. [SRSRY] treatment affected the massive recruitment of both LP-mono-macrophages and inflammatory monocytes. D, The absolute cell number of the population analyzed was calculated over the total number of live cells. Histogram bars represented the mean ± SD of 3 independent experiments. The percent values in the dot plot are calculated among the total number of $CD45^+$ leukocytes. $^*P < 0.05$, $^{**}P < 0.01$.

is difficult to pinpoint the exact sequence of events and the contribution of each subset of mediators in the inhibitory effects exerted by [SRSRY]. It is conceivable to hypothesize that [SRSRY] inhibitory effect is mediated by FPR1 that, in turn, regulates macrophage activation by modulating directly or indirectly multiple signaling pathways. Moreover, THP-1 macrophages express high level of uPAR, which interacts with the FPR1 through its $uPAR_{88–92}$ sequence.[8,9,11] Thus, in vitro, inhibitory effect exerted by [SRSRY] on M1 polarization may be due to the inhibition of $uPAR_{88–92}$-dependent FPR1 activation. In vivo, *N*-formyl peptides derived from bacterial and mitochondrial sources have been detected in intestinal mucosa from patients with IBD and have been suggested to support the development and progression of IBD.[34] Probably, during DSS- or TNBS-induced colitis in WT or uPAR KO mice, [SRSRY] blocks both $uPAR_{88–92}$- and fMLF-dependent FPR1 activation. The exact mechanism by which [SRSRY] counteracts M0 to M1 transition will be the subject of a future research.

When administered in mice in which colitis had been induced by DSS or TNBS, [SRSRY] exerts beneficial effects, most likely attenuating the recruitment of inflammatory monocytes to the inflamed tissue. [SRSRY]-treated mice displayed a reduced body weight loss and DAI. In agreement with its anti-inflammatory property, [SRSRY] administration during DSS- or TNBS-induced colitis caused a strong reduction of the cytokines IL-1β, IL-6, and TNF-α and a statistically significant increase of IL-10, which is recognized as a master regulator of intestinal inflammation in virtue of its ability to dampen Th1-like responses.[22]

Formyl peptides, which are released in the intestinal microenvironment by all bacteria, and other ligands, such as sex pheromones from *Enterococcus faecalis* and *Enterococcus faecium*, promote directional migration of monocytes, neutrophils, and other leukocytes by binding to FPR1.[35] We cannot exclude that neutrophils and other subset of leukocytes expressing FPR1 may concur to the beneficial effects exerted by [SRSRY] on

experimental colitis. Furthermore, because it is documented that beside FPR1 also the related FPR2 is involved in leukocyte recruitment for sustaining inflammatory environment[19] and that the ^{88}Ser-Arg-Ser-Arg-Tyr^{92} chemotactic sequence of uPAR is also able to bind FPR2,[8] it will be interesting to explore the possibility that FPR2 activity is also inhibited by [SRSRY].

We had previously shown that uPAR expression is significantly augmented on intestinal macrophages during experimental colitis. This physiological response is required to dampen intestinal inflammation because the absence of uPAR leads to an increased production of inflammatory cytokines by macrophages that showed an impaired phagocytosis and M1 polarization.[6]

Our data confirmed the involvement of uPAR in controlling macrophage polarization and underlined the important role of its ^{88}Ser-Arg-Ser-Arg-Tyr^{92} sequence in the uPAR/FPR1 interaction. Furthermore, we provide novel evidence regarding the in vivo activity harbored by the cyclization of the peptide. Indeed, [SRSRY] ameliorates the severity of experimental colitis, with a pronounced effect in the absence of endogenous uPAR expression. In this context, [SRSRY] could restore the balance between M0 and M1 polarized macrophages, thus reducing the severity of colonic inflammation. Moreover, it is reasonable to expect that [SRSRY] easily accesses to FPR1 avoiding competition with endogenous uPAR. The effects elicited become even more appreciable and likely revert the uPAR KO phenotype to the WT condition.

Furthermore, although FPR1 expression has been found on intestinal epithelial cell surface to promote intestinal epithelial repair by inducing phosphatidylinositol 3-kinase–dependent Rac1 and Cdc42 activation,[36] it is well established that during inflammation FPR1 expression is strongly and rapidly upregulated in mouse macrophages and neutrophils,[19] acting as a potent enhancer of the inflammatory responses. However, the specific effect of [SRSRY] on tissue repair remains to be investigated.

Given the finding that peptide [SRSRY] is able to ameliorate in vivo the severity of experimental colitis, it may be considered as a new FPR1 inhibitor that may be useful for the generation of new pharmacological treatments for chronic inflammatory diseases.

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