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Inhibition of CCL1-CCR8 Interaction Prevents Aggregation of Macrophages and Development of Peritoneal Adhesions

Akiyoshi Hoshino,* Yuki I. Kawamura,† Masato Yasuhara,§ Noriko Toyama-Sorimachi,‡ Kenji Yamamoto,‡ Akihiro Matsukawa,∥ Sergio A. Lira,# and Taeko Dohi2†

Peritoneal adhesions are a significant complication of surgery and visceral inflammation; however, the mechanism has not been fully elucidated. The aim of this study was to clarify the mechanism of peritoneal adhesions by focusing on the cell trafficking and immune system in the peritoneal cavity. We investigated the specific recruitment of peritoneal macrophages (PMΦ) and their expression of chemokine receptors in murine models of postoperative and postinflammatory peritoneal adhesions. PMΦ aggregated at the site of injured peritoneum in these murine models of peritoneal adhesions. The chemokine receptor CCR8 was up-regulated in the aggregating PMΦ when compared with naïve PMΦ. The up-regulation of CCR8 was also observed in PMΦ, but not in bone marrow-derived MΦ, treated with inflammatory stimulants including bacterial components and cytokines. Importantly, CCL1, the ligand for CCR8, is a product of both PMΦ and peritoneal mesothelial cells (PMCs) following inflammatory stimulation, was a potent enhancer of CCR8 expression. Cell aggregation involving PMΦ and PMCs was induced in vitro in the presence of CCL1. CCL1 also up-regulated mRNA levels of plasminogen activator inhibitor-1 in both PMΦ and PMCs. CCR8 gene-deficient mice or mice treated with anti-CCL1-neutralizing Ab exhibited significantly reduced postoperative peritoneal adhesion. Our study now establishes a unique autocrine activation system in PMΦ and the mechanism for recruitment of PMΦ together with PMCs via CCL1/CCR8, as immune responses of peritoneal cavity, which triggers peritoneal adhesions. The Journal of Immunology, 2007, 178: 5296–5304.

The serosal membrane of viscera and the peritoneal cavity are involved in numerous types of inflammation and surgical intervention. For example, in the case of surgery, postoperative adhesions occur in the majority of patients following laparotomy and laparoscopy (1, 2). Peritoneal adhesions cause significant signs and symptoms including intestinal obstruction, chronic pelvic pain and infertility, and eventually a second more serious surgery is often required. Thus, adhesions in the peritoneal cavity are both life-threatening and an enormous cost for patient care. For example, 34.6% of patients who had undergone intra-abdominal surgery were readmitted within the next 10 years for a disorder directly or possibly related to adhesions, or for abdominal or pelvic surgery that could be potentially complicated by adhesions (2). Despite the large number of surgical operations performed daily, the mechanism for peritoneal adhesions is not well-understood. Previous reports showed that peritoneal injury is triggered by leakage of plasma proteins, followed by formation of fibrinous deposits and proliferation of fibroblasts (3). A rapid and transient influx of neutrophils into the peritoneal cavity also occurs followed by an accumulation of mononuclear cells, largely macrophages (MΦ) (4, 5). CD4-positive T cells also play a significant role in peritoneal adhesions together with the T cell-derived proinflammatory cytokine, IL-17 (6), and the programmed death-1 inhibitory pathway (7). Although active roles for these cells in adhesions have been shown (8, 9), little is yet known about the cell origin or the dynamics of migration to help explain the peritoneal adhesion events. Inflammation such as appendicitis, endometriosis, and pelvic inflammatory disease can also cause peritoneal adhesion, which can lead to infertility and reproductive problems. In the case of Crohn’s disease, intestinal transmural ulcérations with fissures or fistulas are the most important pathological findings (10). These Crohn’s disease lesions involve the intestinal serosa and mesentery. The characteristic changes in the serosal surface, including fat wrapping, correlate directly with overall extent of inflammatory changes: the structure of the intestine (10, 11), the depth of lymphoid aggregate penetration, and the number of lymphoid aggregates in the underlying ileal wall (12). These observations suggest that inflammation of viscera is not limited to the organ, but provokes responses in the peritoneal cavity as well. Most importantly, pathological changes in the peritoneal cavity cause serious symptoms and directly affect the quality of life of patients.

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Abbreviations used in this paper: MΦ, macrophage; BMΦ, bone marrow-derived MΦ; PMΦ, peritoneal MΦ; QD, quantum dot; PGN, peptidoglycan; pAb, polyclonal Ab; TNBS, 2,4,6-trinitrobenzene sulfonic acid; PTX, pertussis toxin; CIMA, chemokine-induced macrophage aggregation; PMC, peritoneal mesothelial cell; iPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1.

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However, the mechanism of peritoneal inflammation has not been fully understood at the cellular and molecular levels.

In this study, we postulated that there is a common serosal defense system that responds to both visceral inflammation and surgical stress. To clarify the molecular basis for peritoneal inflammation and tissue remodeling, we used two mouse models of postoperative and postinflammatory peritoneal adhesions. These models were used to study the traffic patterns of Mφ in the peritoneal cavity. In this study, we describe a chemokine system that is specific for peritoneal Mφ (PMφ) but not bone marrow-derived Mφ (BMφ), a system that plays a significant role in both postoperative and postinflammatory peritoneal adhesion events.

Materials and Methods

Mice

Male 6- to 7-week-old C57BL/6J mice obtained from CLEA Japan were maintained under pathogen-free conditions in a facility in the Research Institute, International Medical Center of Japan (IMCJ). Some experimentally CCR8 gene-deficient (CCR8−/−) mice of C57BL/6 background were used in this study. The CCR8−/− mice were purchased from RIKEN. All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and with the approval of the local ethics committee.

Materials

Fluorescent nanocrystal quantum dots (QDs) (red emission) were produced as described previously (13, 14). Recombinant mouse CCL1 and MCP1 were purchased from R&D Systems. Rat anti-mouse CCL1 mAb and a control rat IgG were purchased from R&D Systems. Rat anti-mouse CCL1 mAb and anti-F4/80 mAb (BD Pharmingen) were transferred by the i.p. routes 2 h before induction of colitis as described previously (17). Briefly, a laparotomy was performed through a midline incision and two ischemic buttons were created on both sides of the parietal peritoneum by grasping the peritoneum with a hemostat clamp and ligating the base of the segment with a 4-0 silk suture. In some experiments, adherent PMφ and BMφ were incubated with QDs and labeled with Alexa Fluor streptavidin (Invitrogen Life Technologies) or anti-F4/80 antibody (Southern Biotechnology Associates), followed by Alexa Fluor streptavidin (Invitrogen Life Technologies) or FITC-labeled goat anti-rabbit IgG pAb (Southern Biotechnology Associates). Laser capture microdissection

Frozen sections were prepared from the colonic tissues with colitis and then stained with the HistoGene LCM Frozen Section Staining kit (Arcturus Bioscience) or anti-F4/80 mAb (BD Pharmingen). The F4/80+ cells clustered at the serosal surface of the transmural ulcer of the colon were collected by use of the laser capture microdissection system (PixCell Ile LCM System; Arcturus Bioscience) to obtain an RNA fraction using the PicoPure RNA Isolation kit (Arcturus Bioscience). RT-PCR

Total RNA isolated from cells and organs was subjected to RT-PCR. Primer structures are shown in Table 1. Real-time quantitative PCR analysis was performed using a SYBR Green PCR Master Mix (Applied Biosystems) and the ABI 7700 Sequence Detector System (Applied Biosystems). Expression of mRNA was normalized to the levels of the GAPDH.

Table 1. List of primers for RT-PCR

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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>G7GGTCTCACATGCTAGTTCTGGG</td>
<td>GCTTTGAGAACTGTGTTTCTGTC</td>
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<tr>
<td>CCR7</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>GAPDH</td>
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Histological analysis

Tissues were snap-frozen and 6-μm sections were prepared and stained with H&E. For immunostaining, sections were fixed with cold acetone for 10 min, dried, and treated with Blockace (Dainippon Pharmaceuticals), incubated with indicated Abs followed by secondary Abs or fluorescent labeled streptavidin described in Materials. Images were captured with a fluorescence microscope (BX50/BXFLA; Olympus) equipped with a CCD camera. Merged images were produced using Adobe Photoshop CS2 (Adobe Systems).

Preparation of QD-labeled Mφ and induction of postinflammatory and postoperative peritoneal adhesions

The cells collected from the peritoneal cavity were incubated in DMEM with 2% FCS for 45 min at 37°C on plastic dishes. After removal of the nonadherent cells by two washing steps, the adherent cells were gently scraped off with a silicon rubber scraper and used as naïve PMφ. Composition of this PMφ preparation was constantly 92.4 ± 3.4% (mean ± 1 SD of four preparations) of CD11b-positive cells (granulocytes and Mφ) and 7.5 ± 3.6% of F4/80-positive cells (Mφ). The BMφ were induced by M-CSF as described previously (15, 16). In some experiments, adherent PMφ and BMφ were incubated with QD solutions and labeled as reported previously (14), washed, and then scraped off. The labeling efficiency was 88%, and total cells were used for all experiments. We confirmed that the preparation and labeling process of PMφ did not cause significant alteration in the expression of chemokine receptors, surface markers, and cell viability.

A model for postoperative peritoneal adhesions was created in mice as previously described (17). Briefly, a laparotomy was performed through a midline incision and two ischemic buttons were created on both sides of the parietal peritoneum by grasping the peritoneum with a hemostat clamp and ligating the base of the segment with a 4-0 silk suture. In some experiments, the QD-labeled PMφ were injected i.p. after closing the abdominal wall. To induce colitis-associated peritoneal adhesions, a 2% solution of 2,4,6-trinitrobenzene sulfonic acid (TNBS; Research Organics)/ethanol 1:1 by volume was given rectally (4 μl/g body weight) (18). In some experiments, QD-labeled naive PMφ were transferred by the i.p. routes 2 h before the induction of colitis.
mRNA expressed. The step-cycle program was set for denaturing at 95°C for 15 s, annealing at 60°C, and extension at 72°C for 45 s, for a total of 40 cycles.

Chemotaxis assay
Aliquots of PMϕ or BMϕ (1 × 10^6 cells/ml) were prestained for 30 min at 37°C with 3 μg/ml 3′-O-Acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester (Molecular Probes) and then suspended at 1 × 10^6 cells/ml in DMEM containing 0.5% BSA and 20 mM HEPES. A chemotaxis assay was performed using a Chemo Tx-96 Chemotaxis Plate (NeuroProbe), as follows. Pretreatment of cells was performed by incubation with or without 50 ng/ml CCL1 for 4 h. Enhanced expression of CCR8 in CCL1-treated PMϕ at this time point was confirmed by flow cytometry. After washing, 65 μl of cell suspension was loaded onto the membrane plate and placed onto a flat-bottom microtiter plate with 96 wells containing 30 μl of serially diluted CCL1 solution in each well. The plate was then incubated at 37°C for 90 min and cells which had undergone migration were collected. These collected cells were counted using a fluorescence microplate reader (FluoroScan Ascent FL; Labsystems). Some experiments were performed in the presence of pertussis toxin (PTX; Calbiochem).

ELISA for CCL1 secretion into peritoneal cavity
To determine the levels of CCL1 in the peritoneal cavity, we collected peritoneal lavage fluid. PBS (1.5 ml) was injected into the peritoneal cavity of mice with or without TNBS-induced colitis as described below, and 1.2–1.4 ml of fluid was recovered. After clearing by centrifugation, the level of CCL1 was determined using paired Abs (Ab Mab8451 for capture and biotinylated Ab BAF845 for detection; R&D Systems) according to the manufacturer’s instructions. Bound Ab was detected with peroxidase-labeled avidin (Sigma-Aldrich) and tetramethyl benzidine was used as the substrate. Sensitivity of this assay was 0.2 ng/ml in our hands.

Chemokine-induced Mϕ aggregation (CIMA) assay
Mouse peritoneal mesothelial cells (PMCs) were isolated from omental tissue as described previously (19, 20). The PMCs (1 × 10^5 cells/well) were plated and cultured on the collagen-coated 24-well dish until they had reached confluence. The QD-labeled PMϕ were added to PMC cultures at a concentration of 1 × 10^5 cells/well in 10% FCS-DMEM. After addition of serial dilutions of CCL1 or other stimulants, the plates were incubated at 37°C and examined by fluorescent microscopy at the indicated time points. The formation of aggregates was quantified by capturing and analyzing images using NIH ImageJ (National Institutes of Health, Bethesda, MD). The cell aggregates which occurred in >10-μm^2 areas were picked and the total aggregation area in the field was summed. Three fields in each well were randomly chosen and analyzed.

Prevention of postinflammatory and postoperative peritoneal adhesions
In the colitis-associated adhesion model, anti-CCL1-neutralizing mAb or control rat IgG (150 μg) was administered 1 h before the colonic administration of TNBS. Mice were sacrificed at the indicated time point and the severity of adhesion was evaluated according to a standard scoring system reported previously (6) as follows: 0, no adhesion; 1, thin filmy adhesion; 2, more than one thin adhesions; 3, thin adhesion with focal point; 4, thick adhesion with plantar attachment or more than one thick adhesion with focal point; 5, very thick vascularized adhesions of more than one plantar adhesion. In some experiments, the removed colon was observed with a Realtime In Vivo MacroImaging System (Relyon) equipped with a long passed red-viewing filter (>610 nm wavelength) and a CCD camera (Hamamatsu Photonics). The area of fluorescent red color was extracted using Adobe Photoshop CS2 from captured images and quantified using ImageJ. In a model for postoperative peritoneal adhesions, 150 μg of anti-CCL1 mAb or control rat IgG was administered i.p. immediately after surgery and 3 days later. All mice were sacrificed at day 6 and the severity of adhesions to each ischemic button was scored according to the following system: 0, no adhesion; 1, thin filmy adhesion; and 2, thick planter adhesion.

Statistics
Data are expressed as mean ± SD. Statistical analysis was performed using the Statview II statistical program (Abacus Concepts) adapted for the Macintosh computer. The Student t, Tukey Kramer’s honestly significant difference and Mann-Whitney U tests were used as indicated in the figure legends. Statistical interpretation of the results is indicated in the
Results

PM\(\phi\) trafficking in postoperative and postinflammatory peritoneal adhesions

We first used a postoperative peritoneal adhesion model where peritoneal ischemic buttons were induced by grasping and ligation of the parietal peritoneum. In this system, peritoneal adhesions were constantly formed within 6 days following the operation (17). Cells at the site of adhesion included neutrophils, CD3\(^+\) lymphocytes, and CD11c\(^+\) cells as reported previously (4–6); however, the infiltration of these cells was rather scattered. In contrast, we found that F4/80\(^-\) PM\(\phi\) formed their own large aggregates (Fig. 1). In the TNBS hapten-induced colitis model, perforating colonic ulcers were constantly formed and always associated with the adhesions to adjacent tissue. We also found that adhesions to the colon were associated with the presence of M\(\phi\) aggregates at the serosal side (Fig. 1B).

To investigate the possibility that PM\(\phi\) actually represent the source of these aggregating cells associated with peritoneal adhesions, we labeled naïve PM\(\phi\) with fluorescent nanocrystal QDs and transplanted them into the peritoneal cavity of mice. QD-labeled PM\(\phi\) transferred to peritoneal cavity of naive mice resided in the omentum 24 h after transfer (data not shown). When QD-labeled PM\(\phi\) were transferred at the time of induction of postoperative or postinflammatory peritoneal adhesions, they accumulated in the cell aggregates at the serosal site of adhesions and perforating ulcers.
However, the cell infiltrates into the inflamed colonic wall hardly contained QD-labeled cells (Fig. 1B, arrowhead). These results indicated that peritoneal adhesions were associated with the massive recruitment of PM/H9278 to the serosal membrane.

Specific-induction chemokine receptors in PM/H9278

To clarify the mechanism of aggregation of PM/H9278, we next investigated the chemokine receptor expression patterns in PM/H9278 aggregates at the serosal surface of the inflamed colon using laser capture microdissection. In contrast to expression of mRNA for all chemokine receptors examined in naive PM/H9278, aggregating F4/80+ cells expressed only limited numbers of receptors, i.e., CCR8, CCR9, and CCR10 (Fig. 2). The results of real-time RT-PCR revealed that expression of mRNA for CCR8 was specifically high in aggregated cells (Fig. 2). Expression of CCR8 in serosal-aggregated PM/H9278 was also confirmed by immunohistological staining in both colitis and postoperative models (data not shown).

Up-regulation of CCR8 in PM/H9278 is induced by proinflammatory stimuli and by CCL1

We next investigated what types of stimuli might up-regulate mRNA for CCR8 in PM/H9278. We found that significant up-regulation of CCR8 mRNA in PM/H9278 was induced by bacterial components, including LPS, PGN, and CpG, and by the proinflammatory cytokines TNF-α and IL-1-β (Fig. 3A). Notably, obvious up-regulation of CCR8 mRNA was induced by CCL1, the ligand for CCR8. In contrast, this degree of CCR8 mRNA up-regulation was not induced in BM/H9278 by any of stimuli tested (Fig. 3A). Up-regulation of

### FIGURE 4

Chemokine-induced aggregate formation of PM/H9278 on monolayers of PMCs (CIMA assay). A, QD-labeled pooled PM/H9278 were placed on the PMC monolayer and stimulated with CCL1 (10 ng/ml) for 1, 3, and 6 h. Pictures are shown for one of three independent experiments with similar results. *, This column shows pictures taken under visible light, which were identical samples as the column using CCL1. Arrowheads indicate the traces of the detached PMC monolayer. B, Cell aggregates involve PMCs. The CCL1-induced, QD (red)-labeled PM/H9278 aggregates as in A were collected under a stereomicroscope after 24 h of incubation. Frozen sections were prepared and stained with 4,6 diamidino-2-phenylindole (DAPI, blue, **top**) or with anti-pancytokeratin Ab (green, **bottom**). Original magnification, ×400. C, Quantification of CIMA assays. The PM/H9278 (**) or BM/H9278 (□) were cultured on PMC monolayers with inflammatory stimulants at the concentrations described in the legend of Fig. 3A or at various concentrations of CCL1 for 24 h. The areas of aggregation in captured images were measured. Data are the mean aggregation area ± 1 SD of triplicate experiments. *, Statistically significant differences from cells without stimulation (p < 0.01) by the Student t test. D, Inhibitory effect of anti-CCL1-neutralizing mAb and PTX on aggregate formation. Coculture of PM/H9278 and PMCs was stimulated with 5 ng/ml CCL1 for 24 h in the presence of various concentrations of inhibitors. Data are the mean aggregation area ± 1 SD of triplicate experiments. *, The difference from controls (without anti-CCL1 mAb, blank column) were statistically significant (p < 0.01) by the Student’s t test. E, Expression levels of CCL1 and CCR8 in PMCs after addition of proinflammatory stimuli or CCL1. The PMCs were incubated with stimulants at the concentrations described in the Fig. 3A legend or 50 ng/ml CCL1 for 6 h, and subjected to RT-PCR for CCL1 and CCR8. One representative result from four experiments, all giving an identical result, is shown. F, CCL1 altered expression of tPA and PAI-1. PM/H9278 and PMCs were left without stimulation (□) or stimulated with 50 ng/ml CCL1 for either 2 (□) or 4 (■) h and subjected for quantitative RT-PCR. Results are the mean relative expression when compared with unstimulated cells ± 1 SD of six RNA preparations. *, Statistically significant difference from cells without stimulation (p < 0.05) by the Student t test.
CCL1 mRNA was also induced in PMφ by LPS, PGN, TNF-α, IL-1β, and CCL1 itself (Fig. 3A). A 3-fold increase of CCL1 in PMφ cultures stimulated with LPS was also detected by ELISA (data not shown).

Specific up-regulation of CCR8 in various chemokine receptors was seen in PMφ treated with CCL1. In contrast, CCL1 did not induce particular chemokine receptors in BMφ (Fig. 3B). Immunostaining for CCR8 after stimulation with CCL1 or LPS showed up-regulated expression of CCR8 in the PMφ together with enhanced expression of the integrin CD49d (Fig. 3C), an adhesion molecule which had been reported to be expressed at the site of adhesions (21). Furthermore, we confirmed the function of CCL1-induced CCR8 in PMφ using a chemotaxis assay. Pretreatment with CCL1 at the concentration of 50 ng/ml caused specific chemotactic activity for PMφ to CCL1. In contrast, the responses by BMφ or untreated PMφ to CCL1 were poor, although untreated BMφ and PMφ responded to MCP1 (Fig. 3D). CCL1-induced chemotaxis was inhibited by anti-CCL1 mAb as well as by PTX, which confirmed involvement of a G protein-coupled 7 transmembrane receptor such as CCR8 (Fig. 3D). Thus, under conditions where intestinal or peritoneal injury and inflammation occurs, there is a strong and specific positive feedback system to induce the CCL1/CCR8 chemokine system for the recruitment of PMφ.

**CIMA assay: an in vitro model for PMφ aggregate formation and peritoneal adhesions**

Our next experiments were directed to determine whether we could reconstitute aggregate formation of PMφ associated with adhesions in vitro. When QD-labeled PMφ were placed on a monolayer of mouse PMCs, PMφ adhered to PMCs loosely and retained a rounded shape. Addition of CCL1 to this mixed culture led to formation of QD-positive cell aggregates with diameters of >100 μm by 3 h and at later time points (Fig. 4A). Importantly, the aggregates involved PMCs. The PMCs became detached from the culture plates and moved into the Mφ cell aggregates to form a larger mass as shown by the presence of cells, which were QD-labeled. This result suggests that organs and tissues could be pulled onto Mφ aggregates via mesothelial cells to eventually form adhesions. In the absence of PMφ, addition of CCL1...
to the PMC layer did not induce detachment or morphological changes (data not shown). The PMϕ also formed aggregates in the presence of bacterial components including LPS, PGN, and CpG, and proinflammatory cytokines, TNF-α and IL-1β (Fig. 4C). The optimal concentration of CCL1 for forming aggregates was ~5–10 ng/ml (Fig. 4C). This indicated that the concentration gradient of CCL1 made through this CCL1/CCR8 autocrine system of PMϕ was required for cell migration to form aggregates (Fig. 4B). At the high concentration in this one-chamber culture system, the concentration gradient around the cells would not be formed, even if cells produce CCL1. In contrast, BMϕ failed to form CCL1-induced aggregates on PMCs, although they responded to LPS and TNF-α to some extent (Fig. 4C). This CCL1-induced aggregate formation was significantly blocked by addition of anti-CCL1-neutralizing mAb or PTX (Fig. 4D). Because involvement of PMCs in the PMϕ aggregates was now established, we next investigated the responses of PMCs to CCL1. Although mRNA for CCL1 in unstimulated PMCs was hardly detected, LPS, PGN, TNF-α, IL-1β, and CCL1 induced dramatic up-regulation of CCL1 (Fig. 4E). CCR8 was constantly expressed on PMCs and expression level did not change with these stimuli (Fig. 4E). Thus, our in vitro model reproduced the initial steps in aggregate formation of PMϕ-enforming PMCs and demonstrated that PMCs also facilitated the CCL1/CR8-positive feedback system in PMϕ.

Furthermore, many studies have shown that early fibrinolytic events in the peritoneum play a central role in adhesion formation (1). To investigate possible involvement of CCL1 in the fibrinolytic pathway, mRNA levels for tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor 1 (PAI-1) in the PMϕ and PMCs were assessed. Considerable levels of mRNA for tPA and PAI-1 were detected in unstimulated PMCs and PMϕ (data not shown). In PMϕ, expression of tPA was down-regulated, while that of PAI-1 was up-regulated 2 h after stimulation with CCL1 (Fig. 4F). In PMCs, significant up-regulation of PAI-1 was seen 2 h after starting treatment with CCL1 (Fig. 4F). Moderate up-regulation of tPA in PMCs became statistically significant when they were treated with CCL1 for 4 h. This CCL1-induced down-regulation of tPA in PMϕ and early up-regulation of PAI-1 in PMϕ and PMCs may also participate in the promotion of cell aggregation and adhesion formation.

**Disruption of CCL1/CCR8 interaction prevents peritoneal adhesions**

The establishment of a role for CCL1 in an in vitro model of cellular aggregate formation prompted us to investigate the effects of disruption of the CCL1/CCR8 system in vivo. Measurement of the levels of CCL1 in peritoneal lavage fluid revealed that CCL1 was significantly increased in mice with TNBS-induced colitis (Fig. 5A). Then, we found that the anti-CCL1-neutralizing mAb efficiently inhibited the formation of aggregates of QD-labeled PMϕ to the colonic serosa after induction of colitis (Fig. 5, B and C). Four days after induction of TNBS colitis, treatment with anti-CCL1 mAb caused less peritoneal adhesions when compared with mice treated with control rat IgG (Fig. 5, D and E). In our postoperative adhesion model, two ischemic buttons were created on both sides of the parietal peritoneum. Mice in the control group formed membranous thick adhesions to most of the ischemic buttons (Fig. 5F). In contrast, treatment with anti-CCL1 mAb efficiently reduced these adhesions (Fig. 5G). Frequency of adhesion formation in CCR8−/− mice was also decreased to the levels comparable to the group treated with anti-CCL1 mAb (Fig. 5, F and G). Of note, blocking the CCL1-CCR8 interaction did not affect the healing of the initial operative incision.

**Discussion**

Little is known about cell trafficking between the peritoneal cavity and the organs of this locale including the gastrointestinal tract. We describe here for the first time the migration and aggregate formation of PMϕ at the site of injury. We have revealed the mechanism for this aggregate formation; a specific positive feedback system in PMϕ of the chemokine CCL1 and its receptor CCR8 when tissue damage or infection occurs. We have further established here an in vitro model for aggregation of PMϕ and PMCs, which was triggered by this same CCL1/CCR8 system. Finally, we were able to interrupt the migration of PMϕ and development of subsequent peritoneal adhesions by abrogating CCL1/CCR8 interaction. Each of these significant new findings is discussed in detail in the following paragraphs.

Practically no attention has been given to the serosal cell response in inflammatory disease of visceral organs. However, the damage and injury to visceras reaching the peritoneum is often fatal. In the case of murine colitis, we found that PMϕ form specific aggregates at the site of transmural ulcers and do not migrate into the inflamed colon. It is reasonable that these cell aggregates physically cover this tissue defect in the intestine and maintain a barrier to prevent further exposure to the flora, potential pathogens, or intoxicants. Apparently, the localization and function of PMϕ is distinct from other types of Mϕ, which are recruited directly from the bone marrow via the blood circulation and diffusely infiltrate into the mucosal and submucosal layer of the colon. This unique function of PMϕ is largely mediated through the restricted expression of a specific chemokine and its receptor. Naïve PMϕ are responsive to many chemokines; however, PMϕ stimulated with CCL1 specifically up-regulate the expression of CCR8, which then facilitated the development of cell aggregates at this particular site of tissue damage.

The recruitment of PMϕ to the inflamed colonic serosa was dramatic. This was most probably explained by a specific and positive feedback system of CCL1/CCR8 in the PMϕ that we found here. In the case of transmural damage in the colon where normal flora reside, each stimulant positively induced up-regulation of the CCL1/CCR8 system in PMϕ. Because TNBS colitis induced in C57BL/6 mice was ameliorated by the administration of anti-CCL1 mAb (our unpublished data), there may be various inflammatory pathways downstream of CCL1 up-regulation. Previous reports showed that CCL1 functions as a migration inducer of Th2-type cells in both humans and mice (22, 23) as well as neutrophil Mϕ (24). Recent studies demonstrated that CCR8 was expressed in CD4⁺CD25⁺ T cells with IL-10 production (25) or FOXP3 expression (26). In addition, CCL1 was produced by a type of M2 (alternatively activated, M2b) Mϕ (27). Furthermore, rhadinovirus-transformed human T cells produced CCL1 with expression of CCR8, which supported cell growth and cytokine production (28). It is of interest that monocye-derived dendritic cells use CCR8 in their migration to lymph nodes (29) and Langerhans-type dendritic cells in the skin also produced CCL1 when stimulated with various bacterial components (30). In humans, CCR8 is also expressed in vascular smooth muscle cells and mediates their chemotaxis (31). CCR8 was shown to play a significant role after bacterial challenge in the abdominal cavity in mice (32). However, autoinduction of the receptor CCR8, shown in our results, has not been clearly described to occur in any of these past studies. This vigorous autoactivation system is unique for PMϕ and may explain the rapid and massive recruitment of PMϕ into the injured visera. This characteristic formation of aggregates by PMϕ certainly plays a key role in the immune system of the peritoneal cavity.
The reaction of PMφ described here is a very effective defense system; however, in the case of surgical stress or chronic inflammation, we assumed that the reaction of PMφ to serosal injury might represent a harmful mechanism that ultimately results in severe peritoneal adhesions. To address this notion, we first succeeded in the reconstruction of adhesions between PMφ and PMCs in vitro. To our surprise, addition of only CCL1 to cocultures of PMCs and PMφ induced the formation of large cell aggregates. Of interest, we found that mesothelial cells also showed striking up-regulation of CCL1 after various inflammatory conditions including incubation with CCL1 itself. When the intestinal damage reaches the serosal layer, mesothelial cells are exposed to bacterial components or inflammatory cytokines. At this point, CCL1 is first produced locally by mesothelial cells, where it initiates the recruitment and brisk activation of the CCLI/CCR8 system in PMφ to support their migration and formation of cell aggregates. Furthermore, the enhanced expression of integrin molecules during the aggregate formation of PMφ as well as up-regulation of PAI in PMCs and down-regulation of tPA in PMφ via the CCLI/CCR8 system supports the significance of this chemokine system for promotion of further cell aggregation and adhesions, and finally for induction of firm fibrous adhesion tissue. Previous study described the role of T cells in the formation of adhesions (6, 7). Our experiment using T cell-deficient mice also indicated partial involvement of T cells in adhesion formation, however, CCLI-exposed PMφ did not show enhanced production of TNF-α, IL-6, IL-4, or IL-10 (our unpublished data). The mechanism of the T cell activation along with the CCLI-driven PMφ recruitment requires further investigation.

In the postoperative model, blockade of the CCLI/CCR8 interaction either with anti-CCLI mAb or disruption of the CCR8 gene decreased peritoneal adhesions, but did not affect the healing of the initial midline incision. This suggests a specific effect by blocking the CCLI/CCR8 system in PMφ to prevent postoperative adhesions without affecting wound healing. Currently, many clinical trials and experimental studies for prevention of peritoneal adhesions have been based upon the idea of modification of the fibrino-lysic pathway (33) or the placement of chemical (34) or physical barriers (35, 36). Physical barrier placement was effective in preventing adhesions between viscosa and the peritoneal wall; however, it failed to prevent adhesions between viscosa. Antiadhesion treatments also includes antibiotics (37), the neurokinin 1 receptor antagonist (17), or cyclooxygenase-2 inhibitors (38, 39) which are mostly nonspecific anti-inflammatory regimen. In contrast, specific blocking of CCLI/CCR8 inhibited the aggregation of PMφ but would not block the diffuse infiltration of BMφ into the inflamed site, for the lack of CCR8. This feature suggests the advantage of targeting CCLI/CCR8 for prevention of adhesions, a procedure which would not affect mucosal or systemic defense systems or the wound healing process. Furthermore, we newly developed the CIMA assay in this study. It is now possible to select suitable molecules for use in prevention of peritoneal adhesions in combination of our CIMA assay and high throughput screening of CCR8 antagonists. We now provide a novel target to prevent excess inflammatory responses in the peritoneal cavity and also to point the possibility of prevention of postoperative peritoneal adhesions by blocking CCLI/CCR8 using Abs or antagonists.

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Disclosures
The authors have no financial conflict of interest.

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identifies CD4 memory T cells enriched for FOXP3