Chemokines are small chemotactic cytokines that are structurally divided into C, CC, CXC, and CX3C subgroups according to the positioning of conserved cysteine residues (1). This classification corresponds only in part with a biological division of chemokines in groups that selectively attract specific subtypes of leukocytes. For example, CC chemokines are capable to activate and chemoattract multiple leukocyte cell types. Alternative attempts were made to classify chemokines as inflammatory chemokines (i.e. inducible proteins that attract leukocytes to sites of inflammation) or constitutively released homeostatic chemokines that regulate leukocyte homing in the lymphoid system (2). Besides their function in leukocyte migration, chemokines are involved in other normal or pathological processes, like hematopoiesis, angiogenesis, cancer growth, and metastasis (3, 4). This indicates that our understanding of the exact role of a number of chemokines remains limited. As a consequence, a systematic chemokine and chemokine receptor nomenclature based on protein structure rather than on function has been introduced recently (3), despite the fact that not all chemokines nor all their receptors have been identified. Indeed, for some recently cloned chemokines such as CCL18/pulmonary and activation-regulated chemokine (PARC)1 (5), the regulated production and function of the natural protein has not yet been investigated in detail, and its agonistic receptor remains unknown.

Chemokines are produced by a variety of cell types including leukocytes and cancer cells (6, 7). Tumor-derived chemokines are important for the characteristic recruitment of leukocytes, such as tumor-associated macrophages (TAM) and lymphocytes, to the tumor environment and hence contribute to the promotion of a specific host anti-tumor immune response. However, this natural cytotoxicity against tumor cells provoked by the leukocytic infiltrate is counteracted by several tumor-favoring processes mediated by chemokines. In fact, some chemotactic cytokines derived from cancer cells or tumor-associated leukocytes act as growth factors for tumor cells (6). Other chemokines promote tumor growth and metastasis by attracting endothelial cells and by causing angiogenesis or by affecting the motility or the migration of cancer cells (4, 8). Metastasis is also indirectly and in a countercurrent way, enhanced by the chemokine-induced release of matrix-degrading enzymes by the infiltrating leukocytes such as TAM (9). In this manner, the "macrophage balance" hypothesis (10) elegantly depicts how TAM possess the dual potential to promote or inhibit malignant cell growth and tumor progression.

Ovarian carcinoma is an intriguing model for detailed inves-
tigation of the role of chemokines and tumor-associated leukocytes in neoplasia. Epithelial ovarian cancer is an important cause of death for women, since, in most of the cases, the tumor has already asymptptomatically disseminated throughout the peritoneal cavity by the time that patients are diagnosed with the disease (11). The presence of TAM, lymphocytes, and dendritic cells has been demonstrated in the lymphoreticular infiltrate in ovarian carcinoma (12, 13). In addition, mRNA for the chemokines CCL2/monocyte chemotactic protein-1 (MCP-1), CXCL8/IL-8, and CCL3/macrophage inflammatory protein-1α (MIP-1α) and, to a lesser extent, CCL4/MIP-1β and CCL5/RANTES were detected in sections of the malignant ovarian tissue and in ovarian carcinoma cell lines (12, 14, 15). Ovarian carcinoma is often accompanied by extensive ascites accumulation, and a number of cytokines have been detected in this peritoneal fluid (16–18). As far as chemokines are concerned, until the present only CCL2/MCP-1, CXCL8/IL-8, and CXCL12/stromal cell-derived factor-1α (15, 17, 19) could be demonstrated by ELISA, and the CCL2/MCP-1 and CXCL8/IL-8 levels were reported to be significantly enhanced in ascites from patients with ovarian cancer compared with nonovarian carcinoma patients (15, 17). Human ascitic fluid is a rich natural source allowing us to identify molecules that may have important functions in the tumor environment and to study their molecular diversity and biological activity at the protein level. This study reports for the first time the isolation of biologically active, postranslationally modified chemokine isoforms from the ascitic fluid from patients with ovarian carcinoma. Furthermore, we found unusually high levels of the chemokine CCL18/PARC (5), alternatively designated dendritic cell-derived CC chemokine-1 (20), alternative macrophage activation-associated CC chemokine-1 (21), and MIP-4 (22). Although CCL18/PARC was not produced by the tumor cells, its levels in ascitic fluids were significantly higher in patients with ovarian carcinoma compared with nonovarian carcinoma.

EXPERIMENTAL PROCEDURES

Characteristics of Patients—Ascitic fluids and surgical specimens were collected from ovarian carcinoma and nonovarian carcinoma patients from the Department of Obstetrics and Gynecology, (San Gerardo Hospital, Monza, Italy), the Departments of Pediatric Hematology/Oncology and Laboratory Medicine (University Hospital, Gent, Belgium), and from the Department of Gynecology (Regional Hospital Heilig Hart, Leuven, Belgium). Collected ascitic fluids were centrifuged and subjected to immunocytochemistry. Twelve patients were comprised of three patients with a benign gynecological disorder (ovarian cyst, fibroma, and mucinous adenoma) and nine women with nonovarian carcinoma.

1. Detection of Chemotactic Activity—Ascitic fluids from two different patients with ovarian carcinoma (FIGO stage IIIc and stage Ic, respectively) were used for immunohistochemistry.

Biochemical Characterization of Natural Chemokines by SDS-PAGE, Amino Acid Sequence Analysis, and Mass Spectrometry—C, RP-HPLC-purified chemokines were analyzed for their purity and M, by SDS-PAGE on Tris/Triicine gels under reducing conditions (25). Proteins were stained with silver, and the M, markers used were aprotinin (M, 6,400), PI (14,400; Bio-Rad), and lysozyme (M, 14,400; Bio-Rad). The NH2-terminal sequence of purified chemokines was determined on a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, CA). The average M, was measured by electrospray ion trap mass spectrometry (Esquire; Bruker Daltonik, Bremen, Germany). Purified proteins were diluted in 50% acetonitrile, 50% water, 0.1% acetic acid at a final concentration of at least 0.2 μg/ml, and 30 μl was injected in the mass spectrometer at a flow rate of 5 μl/min. The average M, was calculated from the summation of 100 spectra, which resulted in an accuracy of ±2.0 mass units.

Induction of Chemokines in Cultured Cells—The human ovarian carcinoma cell line IGROV was grown in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% fetal calf serum (Invitrogen, Groningen, The Netherlands). The human cervix carcinoma HeLa line (American Type Culture Collection, Manassas, Virginia) and the L-cell line HeP-2 were grown in Eagle’s minimum essential medium with Earle’s salts (Invitrogen) supplemented with 10% fetal calf serum. For induction experiments, confluent monolayers of IGROV and HeLa cells (grown in multwell dishes; 1 ml/well, 24 × 1 cm2) and of HEp-2 cells (grown in 25-cm2 flasks; 5 ml/flask; Techno Plastic Products AG) were induced in RPMI 1640 (IGROV) or Eagle’s minimum essential medium with Earle’s salts (HeLa and HEp-2) containing low concentrations (0.5–2%) of fetal calf serum. The stimulating agents used were measles virus (At-tenuvax strain, 106.5 50% tissue culture infectious doses/ml), PMA (26). These inducers did not cross-react in matrix metalloproteinase (MMP)-2 nor MMP-9 activity assays.

Chemokine Isoforms in Ovarian Carcinoma Ascitic Fluid

24585

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sisted of more than 80% CD3+ lymphocytes. Purified granulocytes were obtained after the removal of remaining erythrocytes from the granulocyte pellet by lysis in double-distilled water for 30 s. Human mononuclear THP-1 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum.

CCL18/PARC from ascitic fluid was tested for its chemotactic potential on freshly purified CD3+ lymphocytes (105 cells/ml) in the Boyden microchamber (Neuro Probe Inc., Cabin John, MD) using a 5-μm pore size, polyvinyl pyrrolidone-free polycarbonate membrane (Nuclepore; Corning Separations Division, Acton, MA) that was coated for 24 h at 4 °C with fibronectin (20 μg/ml; Invitrogen). Analogously, the chemotactic activity of ascitic CCL2/MCP-1 and CCL18/PARC was tested on THP-1 cells (1.0 × 106 cells/ml, 2 days after subcultivation) and of CXCL8/IL-8 (106 cells/ml) on neutrophilic granulocytes was analyzed using 5-μm pore size polycarbonate membranes with or without prior polyvinyl pyrrolidone treatment, respectively. Samples (lower compartment of the chamber) and cells (upper compartment of the chamber) were diluted in Hank’s balanced salt solution (Invitrogen) supplemented with human serum albumin (1 mg/ml; Red Cross, Leuven, Belgium). After the incubation of CD3+ lymphocytes, THP-1 cells or granulocytes for 4 h, 2 h, or 45 min, respectively, cells were fixed and stained with Hemacolor solutions (Merck, Darmstadt, Germany). The cells that migrated through the membranes were counted microscopically in 10 oil immersion fields at ×500 magnification. The chemotactic index of a sample (trapped cells in each chamber) was calculated as the number of cells that migrated in response to the test sample divided by the number of cells that migrated in response to the control sample (dilution buffer). Synthetic CCL7/MCP-3 (24) and pure natural IL-8 derived from stimulated monocytes (27) were used as positive controls.

Immunohistochemistry—Tissue sections (6-μm thick) of formalin-fixed, paraffin-embedded human ovarian cancer surgical specimens were deparaffinized in toluol and rehydrated in alcohol. Sections were used for routine analysis (hematoxylin and eosin staining) and for immunohistochemistry. For the latter, the endogenous peroxidase activity was blocked by incubating the sections for 30 min in methanol with 0.3% hydrogen peroxide. The slides were first permeabilized for 30 min with Tween 20 in PBS and then incubated overnight with antibodies against human CCL18/PARC mAb (dilution 1:20; R & D Systems). After incubation with peroxidase-labeled goat anti-mouse Ab (EnVision; DAKO), the reaction product was visualized by 3,3′-diaminobenzidine tetrahydrochloride (Sigma), resulting in brown immunoreactive sites, which eluted at different but expected positions in the salt gradient (0.6–1 M NaCl) after the main protein peak (0.1–0.6 M NaCl) (Fig. 1A). The chemokine-containing fractions were subjected to cation exchange chromatography, allowing us to separate the CCL2/MCP-1 and CXCL8/IL-8 immunoreactivities, which eluted at different but expected positions in the NaCl gradient (Fig. 1B). The CCL2/MCP-1 immunoreactivity was detected at about 0.3 M NaCl, whereas CXCL8/IL-8 eluted later in the salt gradient at 0.7–0.8 M NaCl.

Final purification of the ascitic fluid-derived CXCL8/IL-8 immunoreactivity was achieved by RP-HPLC. SDS-PAGE of CXCL8/IL-8 eluting at about 30.5% acetonitrile allowed us to visualize a 7.0-kDa protein (in Fig. 2A), which corresponded to CXCL8/IL-8 (610 and 550 ng/ml in fractions 56 and 57, respectively, as measured by CXCL8/IL-8 ELISA; Fig. 2B). Taking into account some inevitable loss of chemokine protein during the various purification steps, this amount of purified CXCL8/IL-8 protein matched the original concentration of CXCL8/IL-8 detected in the crude ascites. Capillary Edman degradation and mass spectrometry (detected average Mr of 8538.0 and 8382.0) confirmed the presence of two isoforms of CXCL8/IL-8 with an intact COOH terminus that differ in NH2-terminal truncation (i.e. CXCL8/IL-8-(5–77) and CXCL8/IL-8-(5–67) with a theoretical average Mr of 8538.0 and 8381.0, respectively. The molecule CXCL8/IL-8-(6–77) (SAKELRCQC . . . ) has already been described as the dominant CXCL8/IL-8 form that is produced by mononuclear leukocytes (27). In addition, a similar amount of the previously unreported CXCL8/IL-8-(5–

**RESULTS**

**Molecular Heterogeneity of CXCL8/IL-8, CCL2/MCP-1, and CCL3/MIP-1α Proteins Isolated from Ascitic Fluid of Patients with Ovarian Carcinoma—Immunoreactivity levels of the proinflammatory chemokines CCL2/MCP-1 and CCL3/MIP-1α were reported to be significantly enhanced in the ascitic fluid from patients with ovarian carcinoma (15, 17). However, no data are available on the biochemical characteristics of these chemokines in this pathological fluid. In view of the extensive posttranslational modifications of chemokines and the importance of these alterations for their biological activity, CCL2/MCP-1 and CXCL8/IL-8 proteins were isolated from 850 ml of ovarian carcinoma ascitic fluid through a standard procedure.
(RSAKELRCQC . . . ) isoform was detected, lacking four instead of five amino acids at the NH₂ terminus. In general, NH₂-terminally truncated forms of CXCL8/IL-8 have been shown to be more active than the full-length form, provided that the ELR motif remains intact (28). In the microchamber migration assay, the natural mixture of these two CXCL8/IL-8 isoforms was clearly chemotactic for neutrophilic granulocytes at about 4 ng/ml (Fig. 2B) and even kept its chemotactic potency at about 1 ng/ml (diluted 500-fold, data not shown). This corresponds with the reported biological activity of a natural mixture of leukocyte-derived CXCL8/IL-8 consisting of CXCL8/IL-8-(6–77) and CXCL8/IL-8-(8–77) isoforms (29).

The cation exchange fractions containing CCL2/MCP-1 immunoreactivity were also subjected to RP-HPLC for final purification. Up to 370 ng/ml of CCL2/MCP-1 immunoreactivity eluted from the RP-HPLC column at the expected position (26–27% acetonitrile), corresponding to a distinctly stained 10-kDa protein band on SDS-PAGE (Fig. 2C, fractions 42 and 43). Immunoblotting of the CCL2/MCP-1-containing fractions clearly demonstrated a 10-kDa protein with CCL2/MCP-1 immunoreactivity (data not shown). The measured average Mr of 8664.0 (by ion trap mass spectrometry) is identical to the theoretical average Mr of unglycosylated CCL2/MCP-1-(1–76). Natural unglycosylated CCL2/MCP-1-(1–76) was previously isolated from leukocytes and was more potent in chemotaxis assays on monocytes than glycosylated intact CCL2/MCP-1 (12 and 13.5 kDa) and NH₂-terminally truncated isoforms of CCL2/MCP-1, the latter being practically devoid of chemotactic activity (30). It can be concluded that both CXCL8/IL-8 and CCL2/MCP-1 occur in their biologically most active form in ascitic fluid from ovarian carcinoma patients.

Furthermore, CCL3/MIP-1α mRNA has been demonstrated in ovarian carcinoma biopsies (12). Here, up to 30 ng/ml CCL3/MIP-1α immunoreactivity was detected in the heparin-Sepharose chromatography fractions, co-eluting with the CXCL8/IL-8 and CCL2/MCP-1 immunoreactivities (Figs. 1A and 3A). Subsequent cation exchange chromatography and RP-HPLC were necessary to separate the CCL3/MIP-1α (up to 180 ng/ml) and CCL2/MCP-1 peaks (Fig. 3B). Sequence analysis demonstrated the presence of truncated CCL3/MIP-1α lacking four residues at the NH₂ terminus (ADTPTACC . . . ), and this ascitic CCL3/MIP-1α form was chemotactically active on monocytic THP-1 cells (50-fold dilution; Fig. 3B).

In a further attempt, the heparin-Sepharose fractions of the purified ascitic fluid were screened for the presence of CCL7/MCP-3, CCL18/PARC, and CCL20/LARC by the use of specific and sensitive ELISAs (Fig. 3A). In most body compartments, these chemokines occur less abundantly than CCL2/MCP-1 and CXCL8/IL-8, but they might be selectively up-regulated in ovarian carcinoma. CCL20/LARC was detected as a minor peak (up to 4 ng/ml) eluting at about the same position as CCL3/MIP-1α, whereas only marginal CCL7/MCP-3 levels (less than
ELISA (data not shown) was further purified by RP-HPLC and detected by MIP-1CCL18/PARC immunoreactivity (330 ng/ml), eluting at 1.0 M exchange chromatography, a single peak of CCL18/PARC immunoreactivity. Due to this high CCL18/PARC concentration, crude ascitic fluid (50 ml) was directly loaded onto a heparin-Sepharose column, without prior concentration by adsorption to silica or any other method.

The heparin-Sepharose fractions purified in Fig. 1A were subjected to immunohistochemical staining analysis (Fig. 8A). Reliable staining with antibodies directed against CCL18/PARC in the carcinoma cells could not be demonstrated in this tumor. The cellular infiltrate in the stromal areas was minimal. However, some of these inflammatory cells stained positive for CCL18/PARC (data not shown). In addition, the ascitic fluid of CCL18/PARC underwent final purification by RP-HPLC, resulting in a single peak of 1500 ng of CCL18/PARC immunoreactivity eluting at 28% acetonitrile (Fig. 4B). On SDS-PAGE (Fig. 4C), primarily RP-HPLC fraction 49 revealed a clearly visible 7.0-kDa protein band corresponding to 2100 ng/ml (400 µl/fraction) of CCL18/PARC immunoreactivity. Mass spectrometrical analysis demonstrated the presence of two CCL18/PARC isoforms in this fraction, namely intact CCL18/PARC with an average Mr of 7850.4 (theoretical average Mr of 7851.2) and, to a lesser extent, a CCL18/PARC isoform with an average Mr of 7778.8 (theoretical average Mr of 7780.4), lacking either the NH₂- or the COOH-terminal alanine (Fig. 5). Amino acid sequence analysis yielded NH₂-terminally intact CCL18/PARC protein only. Therefore, it was concluded that besides intact CCL18/PARC-(1–68) also a truncated CCL18/PARC-(1–68) isoform, lacking the COOH-terminal alanine, was isolated from the ascitic fluid.

Since the two CCL18/PARC isoforms could not be separated by column chromatography, the chemotactic potency of the mixture was tested in a microchamber migration assay using CD3⁺ lymphocytes, freshly purified from peripheral blood. The ascitic CCL18/PARC isoforms significantly chemotactically lymphocytes at about 1 ng/ml (Fig. 6).

Lack of CCL18/PARC Production by Carcinoma Cells—To identify the possible cell types responsible for the production of CCL18/PARC in the ascitic fluid from patients with ovarian carcinoma, the conditioned media of different human tumor cell types were analyzed in the CCL18/PARC ELISA. Heparin-Sepharose fractions of a semipurified conditioned medium of nonstimulated ovarian carcinoma SW626 cells were found to lack any detectable CCL18/PARC immunoreactivity (data not shown). To find out whether tumor cells required the administration of specific stimulating agents for CCL18/PARC protein production, induction experiments were carried out on IGROV ovarian carcinoma cells, HeLa cervix carcinoma cells and Hep₂ larynx carcinoma cells (Fig. 7). IGROV ovarian carcinoma cells spontaneously released 0.6 ng/ml CXCL8/IL-8 during 48 h of incubation. This constitutive secretion was significantly increased after stimulation with the tumor-promoting agent PMA (up to 17 ng/ml) and to a lesser degree after induction with the double-stranded RNA poly(rI-rC) and the cytokine IL-1β (up to 1.7 ng/ml). However, none of these stimulators could significantly induce CCL18/PARC protein above the detection limit (<0.2 ng/ml), nor did measles virus or IFN-γ affect the production of either of the two chemokines. In HeLa cells, the CCL2/MCP-1 production was significantly up-regulated from 2 ng/ml in unstimulated cells up to 15 ng/ml after 48 h of incubation with measles virus, poly(rI-rC), or the cytokine IFN-γ or IL-1β, but not with PMA, which induced CXCL8/IL-8 in the IGROV cells. None of these stimulators could significantly induce CCL18/PARC protein above the control level (0.1 ng/ml). In addition, although measles virus and IL-1β induced CCL2/MCP-1 in HeP₂ carcinoma cells, these inducers as well as PMA, poly(rI-rC), and IFN-γ failed to significantly stimulate the CCL18/PARC production above the detection limit (0.1 ng/ml). It can be concluded that the carcinoma cells are probably not the source of the ascitic CCL18/PARC.

Tumor-infiltrating Monocyte/Macrophages Produce CCL18/PARC—In search of the CCL18/PARC-producing cells in ovarian carcinoma, a section of a mucinous cystadenocarcinoma was subjected to immunohistochemical staining analysis (Fig. 8A). Reliable staining with antibodies directed against CCL18/PARC in the carcinoma cells could not be demonstrated in this tumor. The cellular infiltrate in the stromal areas was minimal. However, some of these inflammatory cells stained positive for CCL18/PARC (data not shown). In addition, the ascitic fluid...
fluid from a nonovarian carcinoma patient with Budd-Chiari syndrome, containing 11 ng/ml CCL18/PARC, showed cells that stained positive for both CCL18/PARC (red) and the monocyte/macrophage marker CD68 (black) (Fig. 8B). Furthermore, cytospins derived from the ascitic fluid (170 ng/ml CCL18/PARC) of the mucinous cystadenocarcinoma patient contained CCL18/PARC-negative, nonmalignant mesothelial cells but also cells with monocyte/macrophage morphology staining positive for CCL18/PARC (brown) (Fig. 8, C and D). Therefore, TAM are assumed to be a cellular source of the CCL18/PARC.
Elevated Levels of CCL18/PARC in the Ascitic Fluid from Ovarian Carcinoma Patients—The CCL18/PARC levels in ascitic fluids from ovarian carcinoma patients were compared with the CCL18/PARC concentrations in ascitic fluids originating from diseases other than ovarian carcinoma. Ascitic fluids from women without ovarian carcinoma (n = 12) contained on average 44 ± 7 ng/ml CCL18/PARC protein (Fig. 9). However, in patients diagnosed with ovarian carcinoma (n = 12), the ascitic CCL18/PARC levels were on average 3-fold higher (124 ± 15 ng/ml on average, p = 0.0002), the individual levels ranging between 58 and 200 ng/ml. The reported increase of CXCL8/IL-8 in ascitic fluid from patients with ovarian carcinoma compared with nonovarian carcinoma patients (17) was confirmed to be statistically significant (2.0 ± 0.6 versus 0.7 ± 0.2 ng/ml, p = 0.01). In apparent contrast with previous findings (15), the observed CCL2/MCP-1 concentrations in the ascitic fluids did not significantly differ between patients with or without ovarian carcinoma (3.2 ± 0.4 versus 3.3 ± 1.0 ng/ml, p = 0.26). However, it should be noticed that in this previous study only nonneoplastic effusions were used as controls. In addition, CCL3/MIP-1α, CCL7/MCP-3, and CCL20/LARC levels did not rise above the detection limit (2.0 ng/ml, 0.5 ng/ml and 0.4 ng/ml, respectively) in the two groups of patients (data not shown).

**FIG. 7. Lack of CCL18/PARC production by carcinoma cells.** Confluent monolayers of IGROV ovarian carcinoma cells (upper panel), HeLa cervix carcinoma cells (middle panel), and HEP-2 larynx carcinoma cells (lower panel) were stimulated for 48 h with different chemokine inducers (i.e. measles virus (MV; 10^5–5% tissue culture infectious doses/ml), PMA (10 ng/ml), recombinant IFN-γ (200 ng/ml), the double-stranded RNA poly(rI-rC) (PIC; 100 µg/ml), and IL-1β (10 units/ml). Supernatants from unstimulated cells were used as negative controls (Co). The production of CXCL8/IL-8 (open bars), CCL2/MCP-1 (hatched bars), and CCL18/PARC (black bars) was analyzed by specific ELISAs. Results represent the mean ± S.E. of at least three independent experiments. Statistically significant increases in chemokine production above the control value are indicated (*, p < 0.05; **, p < 0.01).

**DISCUSSION**

The appearance of ascitic fluid due to epithelial ovarian cancer has allowed researchers to study the role of cytokines in this disease (16–18). In view of the accumulation of leukocytes in this peritoneal fluid, the presence of chemokines has also been investigated. Enhanced immunoreactivity for the prototypic CXC chemokine CXCL8/IL-8 and CC chemokine CCL2/MCP-1 has been found in ascitic fluid from ovarian carcinoma patients (15, 17). The substantial volumes of ascites in the tumor environment allowed us to study for the first time the molecular heterogeneity of multiple chemokines present in this pathological fluid. We purified CCL2/MCP-1 and CXCL8/IL-8 isoforms from 850 ml of ascitic fluid from a patient with ovarian carcinoma through a standard four-step purification procedure using specific ELISAs as a screening method. Mass spectrometry, NH2-terminal sequence analysis, SDS-PAGE, and immunoblotting revealed the presence of unglycosylated intact CCL2/MCP-1 (1–76). This natural CCL2/MCP-1 was clearly chemotactically active on monocyteic THP-1 cells and could therefore contribute to the general recruitment and retention of TAM and lymphocytes in the malignant ovary tissue and the ascitic fluid. However, in a group of 24 patients, we were not able to demonstrate a statistically significant increase of the CCL2/MCP-1 concentrations in ovarian carcinoma ascitic fluid compared with nonovarian carcinoma ascites. Once infiltrated in the ovarian carcinoma environment, TAM selectively displayed defective mRNA and surface expression of CC chemokine receptor-2, the unique receptor for CCL2/MCP-1 (31). As a consequence, TAM did not migrate anymore in response to CCL2/MCP-1. Very recently, CC chemokine receptor-1 was found to be the only CC chemokine receptor consistently expressed in the solid ovarian tumor by the infiltrating CD68+ macrophages and CD8+ lymphocytes (32).

Further purification and NH2-terminal sequence analysis demonstrated the presence of two CXCL8/IL-8 isoforms in the purified ascitic fluid (i.e. CXCL8/IL-8-(6–77), the isoform that is predominantly produced by PBMC (27), and CXCL8/IL-8-(5–77), an as yet undescribed isoform lacking four residues at its NH2 terminus). A mixture of these two natural isoforms did still chemoattract neutrophilic granulocytes at about 1 ng/ml. This is in agreement with the fact that NH2-terminally truncated forms of CXCL8/IL-8 are more active than the intact form and with the reported chemotactic potency of a natural mixture of leukocyte-derived CXCL8/IL-8-(6–77) and CXCL8/IL-8-(5–77) isoforms (28, 29). In addition, we found 3-fold higher CXCL8/IL-8 levels (p = 0.01) in ovarian carcinoma compared with nonovarian carcinoma ascites, in support of existing reports on significant CXCL8/IL-8 increases in the ascitic or cyst fluid of epithelial ovarian cancer patients (17, 33). With an average CXCL8/IL-8 concentration of 2 ng/ml measured in the crude ascitic fluids, the natural mixture of CXCL8/IL-8 isoforms is in theory able to chemoattract neutrophilic granulocytes to the ovarian carcinoma environment. However, the cellular infiltrate in ovarian carcinoma appears to contain only few neutrophils, mainly in the lumina of blood vessels (12). Therefore, CXCL8/IL-8 is implicated in ovarian carcinoma, it is probably not predominant in the chemoattraction of neutrophils. Instead, CXCL8/IL-8 could promote angiogenesis or influence the motility of the ovarian epithelial tumor cells, expressing the CXCL8/IL-8 receptor CXCR1 (34, 35). As a contrast, out of 14 investigated chemokine receptors including CXCR1, only CXCR4 was expressed on ovarian cancer cells (19). Until the present, it has remained unclear whether the expression of CXCL8/IL-8 by ovarian cancer cells is beneficial or detrimental for the tumor progression.

In addition, we isolated a biologically active isoform of CCL3/MIP-1α missing four NH2-terminal amino acids from the same ovarian carcinoma ascitic fluid, extending the reported presence of CCL3/MIP-1α mRNA in ovarian cancer biopsies (12).
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Whether CCL3/MIP-1α could play a major role in ovarian cancer biology remains to be seen, since the CCL3/MIP-1α levels were lower than 2 ng/ml in the ascitic fluids from both ovarian carcinoma and nonovarian carcinoma patients. Two other CC chemokines not previously reported in relation to ovarian carcinoma (i.e. CCL7/MCP-3 and CCL20/LARC) were recovered, albeit in minor quantities, from ovarian carcinoma ascitic fluid. CCL7/MCP-3 was originally isolated from osteosarcoma cells (36). CCL20/LARC mRNA was previously detected in pancreatic adenocarcinoma cells and in epithelial cells of the appendix and of inflamed tonsils (37–39). In addition, CCL20/LARC protein was inducible in epidermal carcinoma cells (23). However, we found that the CCL7/MCP-3 and CCL20/LARC concentrations in ascitic fluid accompanying epithelial ovarian cancer remained below 0.5 and 0.4 ng/ml, respectively.

In contrast to CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL7/MCP-3, and CCL20/LARC, extremely high levels of CCL18/PARC were detected in the ascitic fluid from ovarian carcinoma patients (124 ng/ml). Furthermore, these levels were significantly more elevated than in nonovarian carcinoma patients (44 ng/ml; p = 0.0002). CCL18/PARC was only recently described as a T- and B-cell chemotactant, structurally most homologous to CCL3/MIP-1α (5, 20–22, 40). Although CCL18/PARC has been reported to exhibit CC chemokine receptor-3 antagonistic activity (41), so far no agonistic receptor has been identified for this chemokine. Moreover, no homologue for human CCL18/PARC has been found yet in any other species. Human CCL18/PARC mRNA expression was observed in monocyteic and dendritic cells (5, 20–22), in normal lung, in hypersensitivity pneumonitis-affected lungs, in atherosclerotic plaques, in inflamed liver, and in the dermis of contact hypersensitivity patients (5, 20, 42–45). However, little is known yet about the CCL18/PARC protein levels in vitro or in vivo. Only recently, dendritic cells and macrophages have been demonstrated to produce CCL18/PARC (40, 42, 46, 47). Furthermore, the protein is inducible in monocyte/macrophages by IL-4 and staphylococcal enterotoxins and is significantly enhanced in septic and rheumatoid arthritis (47). The presence of authentic CCL18/PARC protein in human ovarian carcinoma ascitic fluid was confirmed here by the isolation of substantial amounts of chemokine (1.5 μg from 50 ml), which occurred in two isoforms: intact CCL18/PARC-(1-69) and, to a lesser extent, truncated CCL18/PARC-(1-68) lacking the COOH-terminal alanine. These CCL18/PARC isoforms showed significant chemotactic activity on lymphocytes at about 1 ng/ml. The presence of significantly enhanced levels of biologically active CCL18/PARC in the ovarian carcinoma ascitic fluid could point to an important role for CCL18/PARC in the recruitment of lymphocytes to the ovarian carcinoma environment. In addition, CCL18/PARC might be involved in other processes involving tumor progression (e.g. as growth factor for the tumor cells or as a factor directly or indirectly influencing angiogenesis or metastasis). Indeed, the recent reports on the specific chemokine receptor expression in the ovarian carcinoma environment (19, 32) could not take into account the yet unknown PARC receptor. The identification of a functional CCL18/PARC receptor and of a CCL18/PARC homologue in a laboratory animal would facilitate the search for other possible target cells and relevant functions of CCL18/PARC in ovarian carcinoma.

With regard to the potential producer cells of CCL18/PARC, unstimulated ovarian carcinoma SW626 cells as well
as stimulated ovarian and other carcinoma cell lines failed to secrete CCL18/PARC protein, although they were good cellular sources of CCL2/MCP-1, CXCL8/IL-8, and CCL20/LARC (Fig. 7; 23; data not shown). Thus, ovarian carcinoma cells can account for the presence of the relatively low levels of the inflammatory chemokines CXCL8/IL-8 (Fig. 7; 34) and CCL2/MCP-1 (12) but not for the unexpectedly high concentrations of CCL18/PARC, formerly classified as a homoeostatic chemokine. The lack of CCL18/PARC expression by ovarian carcinoma cells was confirmed by immunohistochemical staining of malignant ovarian tissue and of cells isolated from the ascitic fluid. The inflammatory mononuclear cells infiltrating the tumor environment were demonstrated to be the CCL18/PARC-producing cells. It is plausible that subsets of the ovarian carcinoma-infiltrating TAM and dendritic cells are a main source of ascitic CCL18/PARC. Indeed, CCL18/ PARC is expressed by macrophages, mainly by alveolar macrophages in vivo and after activation of monocyte/macrophages with alternative macrophage mediators such as IL-4 and IL-10 in vitro (5, 21, 47). These alternatively activated macrophages are presumed to be immunosuppressive, since they down-modulate Th1-mediated immunity and exert Th2-associated effector functions (48). In this context, IL-10-producing immunosuppressive TAM have been detected in the ascites from ovarian carcinoma patients (49). Therefore, the elevated CCL18/PARC levels in ovarian carcinoma ascites may point to the presence of alternatively activated TAM in this tumor environment. CCL18/PARC could thus be involved in the immunosuppression of a host anti-tumor response by attracting T-lymphocytes to suppressive TAM. Furthermore, dendritic cells in ascites from patients with peritoneal carcinomatosis are reported to be rather immature and inadequate in antigen presentation (13), and immature dendritic cells are shown to express CCL18/PARC mRNA (20, 21, 46, 50). It is still controversial whether maturation of dendritic cells down-regulates rather than further increases the CCL18/PARC production (20, 21, 46, 50). In addition to differences in macrophage activation and dendritic cell maturation in an ovarian carcinoma versus nonovarian carcinoma environment, CCL18/PARC production might be induced in tumor-associated dendritic cells and TAM by a factor produced by other specific cells in the ovarian carcinoma microenvironment. Alternatively, a chemotactic factor could be secreted that attracts more constitutively CCL18/ PARC-expressing dendritic cells and TAM to the tumor environment.

In conclusion, we were able to identify biologically active isoforms of CXCL8/IL-8, CCL2/MCP-1, CCL3/MIP-1α, and CCL18/PARC in human ascitic fluid. In addition, protein levels of CXCL8/IL-8 and CCL18/PARC were found to be significantly increased in ovarian carcinoma ascitic fluid in contrast to CCL2/MCP-1, CCL3/MIP-1α, CCL7/MCP-3, and CCL20/LARC. This could point to a more relevant association between CXCL8/IL-8 and CCL18/PARC and epithelial ovarian cancer. Whereas CXCL8/IL-8 was secreted by the ovarian carcinoma cell line IGROV, the tumor-infiltrating mononuclear leukocytes were indicated as the cellular source of CCL18/PARC. Further investigations are required to reveal the specific role of these two chemokines in ovarian carcinoma tumor biology.

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Identification of Biologically Active Chemokine Isoforms from Ascitic Fluid and Elevated Levels of CCL18/Pulmonary and Activation-regulated Chemokine in Ovarian Carcinoma
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