Chemotherapy Induces Breast Cancer Stemness in Association with Dysregulated Monocytosis

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Abstract

Purpose: Preoperative or neoadjuvant therapy (NT) is increasingly used in patients with locally advanced or inflammatory breast cancer to allow optimal surgery and aim for pathologic response. However, many breast cancers are resistant or relapse after treatment. Here, we investigated conjunctive chemotherapy-triggered events occurring systemically and locally, potentially promoting a cancer stem–like cell (CSC) phenotype and contributing to tumor relapse.

Experimental Design: We started by comparing the effect of paired pre- and post-NT patient sera on the CSC properties of breast cancer cells. Using cell lines, patient-derived xenograft models, and primary tumors, we investigated the regulation of CSCs and tumor progression by chemotherapy-induced factors.

Results: In human patients and mice, we detected a therapy-induced CSC-stimulatory activity in serum, which was attributed to therapy-associated monocytosis leading to systemic elevation of monocyte chemoattractant proteins (MCP). The post-NT hematopoietic regeneration in the bone marrow highlighted both altered monocyte–macrophage differentiation and biased commitment of stimulated hematopoietic stem cells toward monocytosis. Chemotherapeutic agents also induce monocyte expression of MCPs through a JNK-dependent mechanism. Genetic and pharmacologic inhibitions of the MCP-CCR2 pathway blocked chemotherapy’s adverse effect on CSCs. Levels of nuclear Notch and ALDH1 were significantly elevated in primary breast cancers following NT, whereas higher levels of CCR2 in pre-NT tumors were associated with a poor response to NT.

Conclusions: Our data establish a mechanism of chemotherapy-induced cancer stemness by linking the cellular events in the bone marrow and tumors, and suggest pharmacologic inhibition of CCR2 as a potential cotreatment during conventional chemotherapy in neoadjuvant and adjuvant settings. Clin Cancer Res; 24(10); 2370–82. ©2018 AACR.

Introduction

Cytotoxic chemotherapy is used as conventional treatment for cancer in adjuvant and neoadjuvant settings and as induction therapy in *de novo* stage IV breast cancer. The cancer cell population is influenced directly by the therapeutic agents and indirectly by therapy-associated changes that occur in the tumor microenvironment and possibly also at the systemic level. Compared with adjuvant chemotherapy aiming to target residual cancer cells upon surgical removal of a majority of the tumor mass, neoadjuvant therapy (NT) exerts direct and indirect effects on the entire population of cancer cells that present preoperatively. The promise of NT relies on the potentials to downstage tumors before planning for optima surgery, to eradicate clinically undetectable disseminated cancer cells, and to test the efficacy of therapy. In breast cancer and several major human cancers, NT has been shown to significantly improve clinical parameters and outcomes (1–3).

In the treatment of breast cancer, NT is increasingly administered to candidates for breast preservation and/or present with locally advanced or inflammatory breast cancer. Although only 10% to 30% of treated hormone receptor-positive (HR+) breast cancer patients exhibit pathologic complete response (pCR) in breast and in regional lymph nodes, the pCR rate of untreated breast cancers characterized as triple-negative (TN) or HER2+ breast cancers is over 50% (4, 5). Evidence is emerging about the association between pCR and long-term progression-free and overall survival, particularly in HR+ breast cancers. However, after NT, some patients with pCR and more with non-pCR relapse, or progress (in the case of non-pCR) with stage IV metastatic breast cancer, which is ultimately fatal (6). Therefore, understanding both *de novo* and acquired resistance to NT or induction therapy is of utmost importance. Mechanisms of breast cancer resistance are partly intrinsic to cancer cells, including altered drug metabolism.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

X. Ren, G. Somlo, and S.E. Wang are co senior authors of this article.

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Translational Relevance
Preoperative or neoadjuvant therapy is increasingly administered to patients who are candidates for breast preservation and/or present with locally advanced or inflammatory breast cancer. However, some patients respond poorly to the treatment or relapse after neoadjuvant therapy. Our study using patient blood samples and experimental cell and mouse models shows that cytotoxic chemotherapy induces circulating monocyte-derived chemokines, which stimulate cancer stem-like cells (CSC) to potentially promote tumor relapse. Therefore, effective anticancer therapies need to block the concurrent CSC-stimulating effect to achieve better short-term and long-term outcomes. Using preclinical tumor models, we show that pharmacologic inhibition of CCR2 can serve as a potential cotreatment during conventional chemotherapy by targeting therapy-induced cancer stemness. In primary breast tumors, higher levels of CCR2 in tumor cells are associated with lack of a pathologic complete response to neoadjuvant therapy.

Materials and Methods

Clinical specimens
Human specimens were obtained from voluntarily consenting breast cancer patients at the City of Hope National Medical Center (Duarte, CA; for Figs. 1, 5A, C–E; Table 1: Supplementary Fig. S1) or at the Tianjin Medical University Cancer Institute and Hospital (Tianjin, China; for Fig. 5B) under Institutional Review Board–approved protocols. Written informed consents were obtained from all patients. The studies were conducted in accordance with recognized ethical guidelines. Patients from the City of Hope were participants of clinical trials NCT01525966, NCT01730833, or NCT00295893 (ClinicalTrials.gov Identifier). Clinical information, including age, tumor stage and pathology, as well as NT starting time, regimen, and response, is summarized in Supplementary Table S1.

Cells and constructs
Breast cancer cell lines MDA-MB-231, BT474, and 4T1 as well as the monocytic cell line THP-1 were obtained from the American Type Culture Collection and cultured in DMEM (for MDA-MB-231 or RPMI-1640 (for BT474, 4T1, and THP-1) base medium supplemented with 10% FBS. The patient-derived PDX265922 cells originating from a TN breast tumor and propagated in NSG mice as well as the primary cancer-associated fibroblasts (CAF) from the same human tumor are described previously (17). All cells used herein were tested to be free of mycoplasma contamination and authenticated by using the short tandem repeat profiling method at the beginning and end of the study. Aliquots of frozen cell stocks were prepared immediately and used to replace cells in culture every 2 months. Lentiviral constructs expressing shRNAs against CCR1-3 as well as a scrambled control were purchased from GeneCopoeia to generate MDA-MB-231 cells with stable gene knockdown (CCR1: #HSH002198-LVRU6MP; CCR2: #HSH002200-LVRU6MP; CCR3: #HSH002207-LVRU6MP; control: #CShCTR001-LVRU6MP). For CCR1-3, each set contains 4 shRNA expression constructs coded as #1–4. Production of viruses, infection, and selection of transduced cells were carried out as previously described (17). The two shRNA constructs showing greatest gene knockdown efficiency were shown in Supplementary Fig. S3A–S3C. Recombinant human CCL2, CCL7, and CCL8, as well as the neutralizing antibodies against human CCL2/7/8 and the control goat IgG were purchased from R&D Systems. The CCR2 inhibitor MK-0812 was purchased from Cayman Chemical. Doxoruubicin, docetaxel, and SP600125 were purchased from Sigma-Aldrich. RO4929097 was purchased from Selleckchem.

Cytokine array and ELISA
Paired pre- and post-NT human sera were analyzed for changes in cytokine levels by using RayBio C-series human cytokine antibody array C3 (RayBiotech) following the manufacturer’s protocol. Levels of MCPs in human and mouse sera were measured by the corresponding ELISA kits. The human CCL2/7/8 and the mouse CCL2/8 DuoSet ELISA kits were purchased from R&D Systems. The CCL7 mouse ELISA Kit was purchased from Cusabio.

and enhanced damage repair and survival capacities (7). More recently, the complex role of the tumor microenvironment has become clearer; cytokines and other secreted factors produced by recently, the complex role of the tumor microenvironment has become clearer; cytokines and other secreted factors produced by stromal fibroblasts, endothelial cells, and certain tumor-infiltrating immune cells have been shown to impede the tumor response to conventional and targeted therapies (8, 9). In addition, accumulating evidence indicates the critical role of a cancer stem–like cell (CSC) phenotype in resistance to therapy.

CSCs are defined as a subset of cancer cells that can proliferate/maintain/differentiate into a new phenotypically heterogeneous tumor, resembling normal stem cells in the self-renewing and pluripotent capacities and undifferentiated gene expression patterns (10, 11). CSCs are implicated in tumor initiation [as the tumor-initiating cells (TIC)], sustained tumor growth (by undergoing self-renewal and generating cells with diverging phenotypes), therapy refractoriness (by expressing drug transporters and remaining dormant in the tumor), and metastasis (as seeds for distant colonization; ref. 12). A variety of cell surface markers and phenotypic markers have been used to enrich CSCs from bulk tumor cells; for human breast cancer, these include CD44+/CD24−/low expression or activity of aldehyde dehydrogenase 1 (ALDH1), and the ability to escape anoikis and grow into spheres in anchorage-independent conditions (10, 13, 14). The diverse and dynamic nature of CSCs has been recognized: the gene expression markers of CSCs may vary between tumors, and multiple CSC pools may exist within individual tumors; CSCs may undergo genetic evolution during cancer recurrence and metastasis; and nonstem cancer cells may reversibly switch to CSCs (12). Therefore, effective therapies against cancer stemness need to target all CSC subsets existing in the tumor and meanwhile block new CSC emergence, such as those potentially induced by therapy.

Several studies have reported that post-NT breast tumors exhibit a higher CSC frequency (15) and stemness–associated gene expression (16). However, it remains unclear if the CSC population, in addition to escaping the cytotoxic effect of NT, also undergoes therapy-induced expansion. We therefore set out to determine if and how NT affects the CSC traits, to shed light on selecting patients who may benefit from potential combination therapy targeting NT-induced cancer stemness.
Coculture assay

Monocytes used in coculture assays were isolated by an EasySep mouse monocyte isolation kit (Stemcell Technologies) from the peripheral blood of C57BL/6 mice after 4 weekly treatments with doxorubicin (4 mg/kg), docetaxel (25 mg/kg), or PBS. THP-1 cells were pretreated with doxorubicin (125 nmol/L), docetaxel (4 nmol/L), or PBS for 48 hours. The coculture was set up in RPMI-1640 medium supplemented with 10% FBS by seeding breast cancer cells in the lower chamber and mouse or human monocytes in the upper chamber of a 0.4-μm transwell insert (Corning). After 48 hours, cancer cells were harvested for analyses.

Flow cytometry and cell sorting

Single-cell suspensions prepared from cell culture or tissue were analyzed by an ALDEFLUOR assay kit (#01700; Stemcell Technologies) following the manufacturer’s protocol. Flow cytometry was performed using a CyAn ADP flow cytometer (Dako) and analyzed by FlowJo software (TreeStar). Antibodies used for stemness analysis are APC anti-human CD326 (EpCAM/ESA; #324208; BioLegend); FITC anti-human CD44 (#555478; BD Biosciences); and PE anti-human CD24 (#555428; BD Biosciences). For the flow cytometry of bone marrow (BM) hematopoietic cells, femora and tibiae were collected from C57BL/6 mice at the indicated time points, and BM cells were flushed with MACS buffer and analyzed as described (18). To characterize the hematopoietic progenitors, cells were negatively selected for Lin (Ter119-APC-eFluor 780, #47-5921-82; Gr1-APC-eFluor, 780, #47-5931-82; B220-APC-eFluor, 780, #47-0452-82; CD3e-APC-eFluor, 780, #47-0031-82; CD11b-APC-eFluor, 780, #47-1032-82).

Figure 1.

Postchemotherapy sera from breast cancer patients and mice stimulate a CSC phenotype through elevated MCP levels. A, Twenty pairs of pre- and post-NT sera from TNBC (n = 8; black) or HER2+ breast cancer (n = 12; blue) patients were analyzed for the activity to stimulate the ALDEFLUORbright population of breast cancer cells. MDA-MB-231 cells were cultured for 48 hours in base medium supplemented with 10% human serum before ALDEFLUOR assays by flow cytometry. Wilcoxon test was performed. B, ELISA to determine the levels of human CCL2/7/8 in the 20 pairs of sera. Wilcoxon tests were performed. C, ALDEFLUOR assays using 6 pairs of sera (3 cases for each breast cancer subtype) were performed as in A except that CCL2/7/8-neutralizing antibodies (NAb; 30 ng/mL; alone or all 3 combined) or control IgG were added during serum treatment. D, ALDEFLUOR assays of MDA-MB-231 cells treated with patient sera in the presence of a CCR2 inhibitor (MK-0812; 600 nmol/L) vehicle. E, NSG mice with or without MDA-MB-231 xenograft tumors and tumor-free BALB/c mice received 3 weekly injections with doxorubicin (DOXO; 4 mg/kg) or docetaxel (DTX; 25 mg/kg; n = 3). Six days later, serum was collected to treat MDA-MB-231 cells for ALDEFLUOR assays as in A. F, ELISA to determine the serum levels of mouse CCL2/7/8. G, ALDEFLUOR assays of MDA-MB-231 cells treated with mouse sera in the presence of the CCR2 inhibitor MK-0812 or vehicle. H, Sera from chemotherapy-treated patients and mice induce tumorigenicity. MDA-MB-231 cells pretreated with human (case T1) or mouse (tumor-free BALB/c) sera for 48 hours were injected into the mammary fat pad of NSG mice (n = 10) at the indicated numbers. Tumor incidence after 4 weeks is shown. The estimated TIC frequency was estimated by ELDA. *P < 0.05; **P < 0.01; and ***P < 0.001 (compared with the corresponding IgG group in C or as indicated).
MCPs Mediate Chemotherapy-Induced Cancer Stemness

Table 1. Serum CSC-stimulating activity and MCP levels in stratified patients

<table>
<thead>
<tr>
<th>Stratification by response to NT (total n = 20)</th>
<th>pCR (n = 9)</th>
<th>Non-pCR (n = 11)</th>
<th>pCR vs. non-pCR P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDEFLUOR* (% of MDA-MB-231)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-NT</td>
<td>1.02 ± 0.19</td>
<td>1.03 ± 0.23</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Post-NT</td>
<td>3.52 ± 1.36</td>
<td>1.96 ± 0.61</td>
<td>0.004</td>
</tr>
<tr>
<td>Pre vs. Post P value</td>
<td>0.004</td>
<td>0.005</td>
<td></td>
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<tr>
<td>ESA+CD44+CD24−/low (% of BT474)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-NT</td>
<td>0.66 ± 0.20</td>
<td>0.68 ± 0.27</td>
<td>0.55</td>
</tr>
<tr>
<td>Post-NT</td>
<td>2.67 ± 0.68</td>
<td>2.01 ± 0.77</td>
<td>0.05</td>
</tr>
<tr>
<td>Pre vs. Post P value</td>
<td>0.004</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>CCL2/MCP-1 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-NT</td>
<td>109.1 ± 24.6</td>
<td>116.1 ± 34.1</td>
<td>0.52</td>
</tr>
<tr>
<td>Post-NT</td>
<td>222.2 ± 83.8</td>
<td>177.8 ± 54.8</td>
<td>0.23</td>
</tr>
<tr>
<td>Pre vs. Post P value</td>
<td>0.004</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>CCL7/MCP-3 (pg/mL)</td>
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<td></td>
<td></td>
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<tr>
<td>Pre-NT</td>
<td>15.6 ± 8.1</td>
<td>14.0 ± 7.2</td>
<td>0.71</td>
</tr>
<tr>
<td>Post-NT</td>
<td>48.2 ± 18.1</td>
<td>37.9 ± 13.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Pre vs. Post P value</td>
<td>0.004</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>CCL8/MCP-2 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Pre-NT</td>
<td>74.7 ± 29.7</td>
<td>73.5 ± 34.5</td>
<td>0.72</td>
</tr>
<tr>
<td>Post-NT</td>
<td>185.9 ± 59.0</td>
<td>136.8 ± 42.8</td>
<td>0.10</td>
</tr>
<tr>
<td>Pre vs. Post P value</td>
<td>0.004</td>
<td>&lt;0.001</td>
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</table>

Stratification by HER2 status (total n = 20)

| ALDEFLUOR* (% of MDA-MB-231)                  |            |                 |                        |
| Pre-NT                                        | 1.06 ± 0.23| 1.00 ± 0.21     | 0.68                   |
| Post-NT                                       | 3.65 ± 1.42| 2.00 ± 0.57     | 0.005                  |
| Pre vs. Post P value                          | 0.02       | <0.001          |                        |
| ESA+CD44+CD24−/low (% of BT474)               |            |                 |                        |
| Pre-NT                                        | 0.63 ± 0.30| 0.70 ± 0.18     | 0.68                   |
| Post-NT                                       | 2.15 ± 0.89| 2.41 ± 0.74     | 0.68                   |
| Pre vs. Post P value                          | 0.02       | <0.001          |                        |
| CCL2/MCP-1 (pg/mL)                            |            |                 |                        |
| Pre-NT                                        | 128.8 ± 29.2| 102.4 ± 26.0   | 0.05                   |
| Post-NT                                       | 262.3 ± 53.6| 154.9 ± 43.3   | <0.001                 |
| Pre vs. Post P value                          | 0.02       | <0.001          |                        |
| CCL7/MCP-3 (pg/mL)                            |            |                 |                        |
| Pre-NT                                        | 16.4 ± 7.1 | 13.7 ± 7.6      | 0.34                   |
| Post-NT                                       | 37.7 ± 16.3| 45.7 ± 15.6     | 0.25                   |
| Pre vs. Post P value                          | 0.02       | <0.001          |                        |
| CCL8/MCP-2 (pg/mL)                            |            |                 |                        |
| Pre-NT                                        | 98.5 ± 27.3| 57.7 ± 22.8     | 0.002                  |
| Post-NT                                       | 207.5 ± 39.0| 126.5 ± 38.1   | <0.001                 |
| Pre vs. Post P value                          | 0.008      | <0.001          |                        |

*The 20 pairs of pre- and post-NT sera in Fig. 1A and B and Supplementary Fig. S1A were stratified by breast cancer’s response to NT (pCR vs. non-pCR) or HER2 status (TN vs. HER2*) before being summarized for the activity to stimulate CSC populations and for levels of CCL2/7/8 in the format of mean ± SD. The Wilcoxon test was used to calculate the Pre vs. Post P value, whereas the Mann–Whitney test was used for pCR vs. non-pCR P value and TN vs. HER2* P value. P values in boldface are statistically significant.

eFluor 780, #47-0112-82; CD4-APC-eFluor 780, #47-0041-82; CD8a-APC-eFluor 780, #47-0081-82; eBioscience) and stained with c-Kit/PECy7 (#25-5981-81), CD34-FTC (#11-0341-81), and CD16/CD32-PE (FgR-III/II; #12-0161-81) antibodies (eBioscience) for 30 minutes before being analyzed by a BD FACSCanto II flow cytometer and BD FACSDiva software (BD Biosciences). Macrophage and monocyte characterization did not include the Lin-negative selection, and cells were stained with CD3-Alexa Fluor 700 (#561388), B220-Alexa Fluor 700 (#557957; BD Biosciences), NK1.1-FTC (#11-5941-81), CD115-APC (#17-1152-82), and Fcγ/80-PE (#12-4801-82; eBioscience) antibodies. The cell populations were identified as: HSC, Lin−c-Kit−Sca-1+; CMP, Lin−c-Kit−Sca-1+CD16/CD32lowCD34+; GMP, Lin−c-Kit−Sca-1+CD16/CD32highCD34+; MEP, Lin−c-Kit−Sca-1+CD16/CD32−CD34+; macrophages, CD3−B220−NK1.1−F4/80+CD115−; Low SSC, monocytes, CD3−B220−NK1.1−F4/80+CD115+. Complete blood count was performed using a Sysmex XT-2000i hematology analyzer (Sysmex Corporation).

Sphere formation assay

Mammosphere culture was performed as previously described (17). Cells pretreated with MCPs for 48 hours were seeded in ultralow attachment 6-well plates (Corning). The number of spheres (diameter ≥ 70 μm) was counted on day 10, and sphere-forming efficiency was calculated based on the number of initially seeded cells.

RNA extraction and quantitative reverse transcription PCR

These procedures were performed as described previously (17). Primers used are indicated in Supplementary Table S2. An annealing temperature of 55°C was used for all primers.

Western blot analysis

These procedures were performed as described previously (19). Protein extracts were separated by electrophoresis on a 10% or 12% SDS polyacrylamide gel. Protein detection was performed using the following antibodies: NICD (#4147; Cell Signaling Technology); SOX9 (#AB5353; EMD Millipore); NANOG (#5380; Cell Signaling Technology); and GAPDH (#2118; Cell Signaling Technology).

IHC

IHC staining of formaldehyde-fixed, paraffin-embedded tumor tissues was performed as previously reported (17) using the following antibodies and dilutions: Ki-67 (#11680; Abcam), 1:3, NOS3 (#7952; Cell Signaling Technology); and CD24 (#H0002151MGR, 1:200 dilution). Stained slides were scored according to intensity of staining (−: 0; +: 1; ++: 2; and

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+ + + : 3) and percentage of tumor cells staining positive for each antigen (0%: 0; 1%: 3) and percentage of tumor cells staining positive for each

Animals

All animal experiments were approved by the institutional animal care and use committee at the University of California San Diego or the City of Hope Beckman Research Institute. Six-week-old female NOD/SCID/IL2Rγ-null (NSG), BALB/c, or C57BL/6 mice were used. For the mouse serum analyses in Fig. 1E–G, tumor-free NSG mice and those with MDA-MB-231 xenograft tumors of approximately 200 mm³ in the No. 4 mammary fat pad, as well as tumor-free BALB/c mice received 3 weekly i.p. injections with doxorubicin (4 mg/kg), docetaxel (25 mg/kg), or PBS as control. Serum was collected 6 days after the last injection. For the limiting-dilution transplantation in Fig. 1H, MDA-MB-231 cells pretreated with human serum (case T1) or mouse serum (pooled from 5 tumor-free BALB/c mice) for 48 hours were injected into the mammary fat pads of NSG mice at the indicated numbers. Tumor incidence after 4 weeks was shown. The TIC frequency was estimated by extreme limiting dilution analysis (ELDA; ref. 20). For the monocye depletion experiments in Fig. 2A–C, clodronate liposome or control (Liposoma B.V.; 200 μL for the first treatment and 100 μL thereafter) were injected into BALB/c mice through the tail vein every 2 days starting at 2 days prior to the first chemotherapy treatment. At day 6 after 3 weeks of chemotherapy, blood and BM were collected. For the complete blood count analyses in Fig. 2D and Supplementary Fig. S2, BALB/c and C57BL/6 mice received 4 times of treatment with doxorubicin, docetaxel, or PBS, on days 1, 7, 14, and 21. Blood was collected via the tail vein at each indicated time point. For the bulk cell cancer cell transplantation in Fig. 4A–C, 10⁶ MDA-MB-231 cells with stable knockdown of CCR2 (shCCR2 #1) or those expressing control shRNA (shCTRL) were injected into the #4 mammary fat pad of NSG mice. When tumor size reached approximately 250 mm³, mice were treated weekly with docetaxel for 3 weeks, and then left free of chemotherapy until the end of experiment. One group with MDA-MB-231-shCTRL tumors also received the CCR2 inhibitor MK-0812 (oral 30 mg/kg twice a day) starting with the chemotherapy and continuing for a total of 30 days. For the ALDEFLUORbright cancer cell transplantation in Fig. 4D–I, BALB/c and NSG mice were treated with doxorubicin, docetaxel, or PBS for 3 weeks and then left free of chemotherapy for 1 week, before 10⁴ freshly sorted ALDEFLUORbright 4T1 or PDX265922 cells were injected into the #4 mammary fat pad to assess tumor development. As indicated, some mice received oral MK-0812 or vehicle twice a day at 30 mg/kg starting with the chemotherapy and continuing for 30 days after breast cancer cell engraftment. Tumor volume was determined by caliper measurements.

Statistical analysis

All quantitative data are presented as mean ± SD. Two-sample two-tailed Student t tests were used for comparison of means of quantitative data between two groups. For multiple independent groups, one-way ANOVA with post hoc Tukey tests were used. Nonparametric Wilcoxon tests were used for comparison of paired pre- and post-NT patient samples. Nonparametric Mann–Whitney U tests were used for comparison between two independent patient groups. Values of P < 0.05 were considered significant. Sample size was generally chosen based on preliminary data indicating the variance within each group and the differences between groups. For animal studies, sample size was predetermined to allow an 80% power to detect a difference of 50%. Animals were randomized before treatments. For experiments in which no quantification is shown, images representative of at least three independent experiments are shown.

Data and materials availability

All materials, data, and protocols described in the article are available from the corresponding author on reasonable request.

Results

Postchemotherapy sera stimulate a CSC phenotype through elevated MCP levels

To determine if systemic factors (e.g., cytokines), altered by anticancer therapies, may regulate the CSC phenotype, we first compared the effects of paired pre- and post-treatment sera from TN and HER2+ breast cancer patients who had received NT. TNBC patients received carboplatin and a taxane and those with HER2+ breast cancers received these agents plus a HER2-targeting therapy. Patient serum was added to MDA-MB-231 TNBC cells or BT474 HER2+ breast cancer cells at 10% to replace the FBS in regular medium. In both breast cancer models, the post-NT serum significantly induced cell populations that are enriched for BCSCs (the ALDEFLUORbright population in MDA-MB-231 and the ESA+CD44+CD24low population in BT474; refs. 10, 14) compared with paired pre-NT serum (Fig. 1A; Supplementary Fig. S1A). Using a semi-quantitative cytokine array, we detected elevated levels of CCL2 (MCP-1), CCL7 (MCP-3), and CCL8 (MCP-2) in post-NT sera (Supplementary Fig. S1B). This was confirmed by ELISA (Fig. 1B). When cases were stratified by breast cancer’s response to NT or by HER2 status, the blood-borne CSC-stimulating activity as well as levels of all three MCPs were significantly induced following NT in both pCR and non-pCR groups, and in both TNBC and HER2+ groups (Table 1), indicating these NT-associated effects occur regardless of tumor response and HER2 status. Antibody-mediated neutralization of MCPs, especially CCL2, or inhibition of the MCP receptor CCR2 that serves as a major receptor for all three MCPs using its antagonist MK-0812 (21), impaired or abolished the ability of post-NT sera to induce CSCs (Fig. 1C and D; Supplementary Fig. S1C). Therefore, MCP signaling through CCR2 plays an essential role in mediating the CSC-inducing activity in post-NT human blood.

We next examined if chemotherapy led to a similar result in mouse serum, and if the presence of a tumor is required for this therapy-induced effect. Female NSG mice with or without MDA-MB-231 orthotopic xenograft tumors, as well as tumor-free BALB/c mice, received 3 weeks of doxorubicin or docetaxel treatment before the serum was collected and added to MDA-MB-231 cells in vitro for evaluation of CSC markers. The results indicate that, similar to humans, mice induce a tumor-independent blood-borne activity after chemotherapy that expands ALDEFLUORbright breast cancer cells, and that MCPs as well as their receptor CCR2 also mediate this effect in both immuno-competent and -compromised mice (Fig. 1E–G). Importantly, when varying numbers of MDA-MB-231 cells pretreated with pre-/post-NT patient sera, or with pre-/post-docetaxel sera from tumor-free NSG mice, were injected into the mammary fat pad of NSG mice in a limiting-dilution transplantation assay, postchemotherapy sera from both human and mice exhibited enhanced ability to stimulate tumorigenicity (Fig. 1H). Therefore, the posttherapy serum activity to
Chemotherapy-induced monocytosis is responsible for the elevation of circulating MCPs. A, Clodronate liposomes or control were injected into BALB/c mice through the tail vein every 2 days starting at 2 days prior to the first chemotherapy treatment ($n = 3$). At day 6 after 3 weeks of chemotherapy, the number and percentage (out of total WBCs) of monocytes were analyzed by a complete blood count (left). The CD11b+CD115+ monocyte population in the BM was analyzed by flow cytometry (right). B, ELISA to determine the serum levels of mouse CCL2/7/8 ($n = 3$). C, ALDEFLUOR assays of MDA-MB-231 cells treated with indicated mouse sera ($n = 3$). D, Chemotherapy induces expansion of monocytes in the rebound phase. BALB/c mice received 4 treatments with DOXO or DTX, or PBS on days 1, 7, 14, and 21 (blue arrowheads). At each indicated time point, complete blood count was conducted to determine the numbers and percentages of various cell populations ($n = 3$). Total BM cell count was also shown. LY, lymphocytes; MO, monocytes; NE, neutrophils; RBC, red blood cells; PLT, platelets. On days 7 and 14, blood was collected before treatment. E, BM cells were collected at the indicated time from C57BL/6 mice that had received 1 to 4 treatments with DOXO or DTX, or PBS (treatments given on days 1, 7, 14, and 21). Flow cytometry was performed to determine the population of indicated cell types ($n = 3$). F, Representative flow cytometry plots on day 28 from all three groups in E. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (compared with the corresponding PBS group in D and E or as indicated).
cells and platelets did not dramatically elevation of circulating MCPs in the blood results from BM reconstitution. This posttherapy rebound was not likely to result from injection-associated inflammation, because injections with PBS did not significantly alter WBC or monocyte numbers, and the two tested drugs injected at the same frequency showed differences in the strength and time course of monocyte regulation.

The postchemotherapy induction of circulating monocytes was also observed in C57Bl/6 mice (Supplementary Fig. S2). Using established cell surface markers (18), we analyzed the hematopoietic regeneration in the BM of these mice following chemotherapy treatment. Compared with the control group receiving PBS, both doxorubicin and docetaxel induced an expansion of HSCs that began immediately and climaxed after the last treatment. Although little effects were observed with the common myeloid progenitors (CMP), concurrently increased granulocyte-monocyte progenitor (GMP) and decreased megakaryocyte-erythrocyte progenitor (MEP) populations were detected after 4 times of chemotherapy treatment (Fig. 2E and F). In addition, we observed a significant shift of the F4/80+CD115+ macrophages to F4/80+CD115+ monocytes starting after the first treatment and accumulating thereafter. These results suggest the involvement of a series of postchemotherapy events in the BM, including the immediately altered monocyte–macrophage differentiation as well as the stimulation of HSC expansion and their biased lineage commitment to generate more GMPs versus MEPs in a relatively later phase for sustained monocytosis.

Chemotherapy induces monocyte expression of MCPs, which promote the stemness-associated properties by inducing Notch

We next cocultured breast cancer cells with mouse monocytes isolated before and after chemotherapy or with THP-1 human monocytic cells pretreated with doxorubicin or docetaxel. In a dose-dependent manner, the postchemotherapy monocytes exhibited an enhanced ability to stimulate the CSC traits in breast cancer cells, which was abolished by CCR2 inhibition (Fig. 3A) or MCP neutralization (Fig. 3B). We further found that the chemotherapeutic agents induced the secreted levels of MCPs in monocytes but not in CAF (Fig. 3C), and that this induction occurred at the RNA level through a JNK-dependent mechanism (Fig. 3D), consistent with a previous report showing JNK-mediated upregulation of CCL2 through c-Jun–binding sites in the gene promoter (22). When MDA-MB-231 cells were individually treated with recombinant MCPs, all three MCPs, especially CCL7, induced dose-dependent increases in mammosphere formation as well as the population of ALDEFLUORbright cells (Fig. 3E). Similar results were observed with patient-derived PDX265922 TNBC cells (Fig. 3F). In BT474 HER2+ breast cancer cells, which naturally harbor a high percentage of ALDEFLUORbright cells (23), MCPs significantly induced mammosphere formation as well as the subset of ESA−/CD44+/CD24−/low cells that are known to contain enriched CSCs (ref. 10; Fig. 3G). In all breast cancer cells tested, MCPs significantly induced the expression of stemness-related genes, including SOX9 and NANOg, at the mRNA and protein levels, and increased the protein levels of the Notch intracellular domain (NICD), indicating activation of Notch signaling (Fig. 3H and I). In support of our previously reported mechanism of CCL2 to activate Notch (17), we found that the ability of all three MCPs to induce stemness genes was completely abolished by a γ secretase inhibitor RO4929097 (Fig. 3J). These results collectively indicate that chemotherapy induces monocyte expression of MCPs, which promote CSC properties by inducing Notch signaling.

Chemotherapy promotes CSC properties in vivo through MCP-CCR2 signaling

Based on the in vitro data, we hypothesized that the MCP-CCR signaling mediates the post-NT CSC induction and tumor progression. To this end, we generated MDA-MB-231 cells with individual knockdown of CCR1-3 (shCCR1-3) or those expressing control shRNA (shCTRL). Stable knockdown of CCR2 but not the other two CCRs efficiently blocked the effects of all three MCPs on inducing stemness-related gene expression and mammosphere formation (Supplementary Fig. S3A–S3C). Pharmacologic inhibition of CCR2 with MK-0812 also abolished MCP-mediated induction of NICD and stemness-related genes (Supplementary Fig. S3D). We therefore focused on CCR2 and injected 10⁶ of MDA-MB-231 cells with stable expression of shCCR2 or shCTRL into the #4 mammary fat pad of NSG mice. When tumor size reached approximately 250 mm³, mice were treated with docetaxel for 3 weeks and then left free of chemotherapy until the end of experiment (Fig. 4A). One group with MDA-MB-231-shCTRL tumors also received the CCR2 inhibitor MK-0812 starting with the chemotherapy and continuing for a total of 30 days. Tumors with CCR2 knockdown grew slower than the control tumors from the beginning and exhibited significantly suppressed regrowth after the completion of chemotherapy. Treatment with the CCR2 inhibitor also significantly reduced posttherapy tumor regrowth (Fig. 4B). The posttherapy tumors with CCR2 knockdown or treated with CCR2 inhibitor also contained a lower content of ALDEFLUORbright cells, compared with the control tumors (Fig. 4C).

We next examined if chemotherapy prior to breast cancer cell engraftment altered the host environment to enhance tumor
Figure 3.
Chemotherapy induces monocyte expression of MCPs, which promote the stemness-associated properties by inducing Notch.

A and B, Cocultures were set up using MDA-MB-231 or BT474 breast cancer cells with monocytes isolated from C57BL/6 mice treated with DOXO, DTX, or PBS, or with DOXO/DTX/PBS-pretreated THP-1 cells, in the presence or absence of the CCR2 inhibitor MK-0812 (A) or CCL2/7/8 NAb (B) as described in Materials and Methods. Breast cancer cells and monocytes were seeded at a ratio of 1:1 or 1:3. ALDEFLUOR assays (for MDA-MB-231) and ESA+CD44+CD24low flow cytometry (for BT474) were performed using breast cancer cells harvested after 48 hours of coculture.

C, MCP secretion by THP-1 and CAF was determined by ELISA of the conditioned media (CM) of 10^5 THP-1 cells or cancer-activated CAF (pretreated with the CM from PDX265922 cancer cells) that had been treated with DOXO (125 nmol/L), DTX (4 nmol/L), or PBS for 48 hours.

D, THP-1 cells under DOXO/DTX/PBS treatment and mouse monocytes isolated as in A were cultured in the presence or absence of a JNK inhibitor SP600125 (1 μmol/L) for 48 hours and analyzed by quantitative RT-PCR using GAPDH/Gapdh for normalization.

E, MDA-MB-231 cells were treated with CCL2/7/8 at the indicated concentrations for 48 hours and analyzed by sphere formation assay or ALDEFLUOR assay.

F, PDX265922 cells derived from a primary TNBC were treated with CCL2/7/8 (1 ng/mL) or PBS for 48 hours and analyzed by sphere formation assay or ALDEFLUOR assay.

G, CCL2/7/8-treated BT474 cells were analyzed by sphere formation assay or flow cytometry for the ESA+CD44+CD24low population.

H, Indicated breast cancer cells were treated with CCL2/7/8 (1 ng/mL) or PBS for 24 hours and analyzed by quantitative RT-PCR for indicated stemness-related genes. Data are normalized to GAPDH and compared with the PBS group.

I, Western blot analysis showing stemness-associated gene expression in breast cancer cells treated with CCL2/7/8 or PBS for 24 hours.

J, Western blot analysis of MDA-MB-231 cells treated with CCL2/7/8 or PBS in the presence or absence of a γ-secretase inhibitor (GSI) RO4929097 (10 μmol/L). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 (compared with the control group in the first column of each group or as indicated).
Figure 4.
Chemotherapy promotes CSC properties in vivo through MCP-CCR2 signaling. A, Schema of the mouse model used to examine if CCR2 intervention suppresses postchemotherapy tumor progression. One million MDA-MB-231 cells with stable knockdown of CCR2 (shCCR2) or those expressing control shRNA (shCTRL) were injected into the #4 mammary fat pad of NSG mice. When tumor size reached approximately 250 mm$^3$, mice were treated with DTX for 3 weeks, and then left free of chemotherapy until the end of the experiment. One group with MDA-MB-231-shCTRL tumors also received the CCR2 inhibitor MK-0812 starting with the chemotherapy and continuing for a total of 30 days, whereas the other two groups received the vehicle. B, Tumor onset and volume (n = 8). The two groups with MDA-MB-231-shCTRL tumors received 3 treatments with DTX, on days 24, 31, and 38 (blue arrowheads); the group with MDA-MB-231-shCCR2 tumors received DTX on days 32, 39, and 46 (blue arrows). C, ALDEFLUOR assay of dissociated MDA-MB-231 tumor cells. D, Schema of the mouse models used to examine if chemotherapy prior to breast cancer cell engraftment enhances tumor formation. BALB/c and NSG mice were treated with DOXO, DTX, or PBS for 3 weeks and then left free of chemotherapy for 1 week, before 1,000 FACS-isolated ALDEFLUOR bright 4T1 or PDX265922 cells were injected into the #4 mammary fat pad to assess tumor development. E, Tumor onset and volume for the 4T1 model in BALB/c mice (n = 8). Inset shows numbers of mice with palpable tumors on day 14. F, ALDEFLUOR assay of dissociated 4T1 tumor cells. G, Relative RNA levels of indicated genes (normalized to Gapdh) in 4T1 tumor tissue determined by quantitative RT-PCR assay. H, Tumor onset and volume for the PDX265922 model in NSG mice (n = 8). CCR2 inhibitor MK-0812 or vehicle was orally administered at 30 mg/kg twice a day starting with the chemotherapy and continuing for 30 days after breast cancer cell engraftment. Inset shows numbers of mice with palpable tumors on day 18. I, ALDEFLUOR assay of dissociated PDX265922 tumor cells. J, Western analysis showing indicated protein levels in PDX265922 tumor tissue. * P < 0.05; ** P < 0.01; and *** P < 0.001 (compared with the corresponding PBS group in E-G or as indicated).
Figure 5.
Chemotherapy-induced monocytosis and tumor expression of NOTCH1, ALDH1, and CCR2 in breast cancer patients. A, Complete blood count showing changes of various cell populations upon NT in a total of 13 patients, including 8 cases of TNBC (black) and 5 cases of HER2+ breast cancer (blue) that were treated at the City of Hope National Medical Center. The green boxes in the background indicate normal ranges. Wilcoxon tests were performed. B, Complete blood count showing the dynamics of various cell populations during 4 cycles of NT in 19 breast cancer patients treated at the Tianjin Medical University Cancer Institute and Hospital. The start of each cycle is indicated by a blue arrowhead. The green boxes in the background indicate normal ranges. C, Eighteen pairs of pre- and post-NT breast tumors from the City of Hope patients were analyzed by IHC to show the percentages of tumor cells positive for Ki-67 or ALDH1, as well as the staining scores for nuclear NOTCH1 in tumor cells. Wilcoxon tests were performed. D, Representative IHC images from two cases. E, Pre-NT breast tumors were analyzed by IHC for the expression of CCR2 in tumor cells. Tumors with pCR (n = 15) were compared with those with non-pCR (n = 20). The Mann-Whitney test was performed. Representative IHC images are shown.
form from prospectively enriched CSCs. One thousand of ALDEFLUOR<sup>high</sup> 4T1 or PD265922 cells were injected into the mammary fat pad of BALB/c or NSG mice, respectively, which had previously received three weekly injections of doxorubicin, docetaxel, or PBS (Fig. 4D). Mice pretreated with chemotherapy developed mammary tumors at an earlier time, and presented tumors with larger size, higher content of ALDEFLUOR<sup>high</sup> cells, and increased expression of stemness-related genes (Fig. 4E–J). These effects were suppressed by cotreatment with the CCR2 inhibitor MK-0812 (Fig. 4H–J). Thus, data from mouse tumor models collectively suggest that chemotherapy promotes CSC-mediated tumor growth through MCP-CCR2 signaling, which may be targeted by pharmacologic inhibition of CCR2.

Chemotherapy induces monocytosis as well as markers of NOTCH activation and CSCs in breast cancer patients, whereas tumor expression of CCR2 is associated with NT response

Our data thus far suggest that the BCSC population may undergo a dramatic expansion in response to NT through a mechanism involving therapy-induced monocytosis and MCP-CCR2 signaling in cancer cells. To seek additional clinical evidence, we performed complete blood count in two independent cohorts of breast cancer patients to determine changes of various cell populations upon NT. Among patients treated at City of Hope, although the WBC, RBC, and platelet counts were significantly decreased after 6 to 12 weeks of NT, a significant induction of monocyte content was detected (Fig. 5A). Among patients treated at the Tianjin Medical University Cancer Hospital, for which blood was examined weekly, we observed significant induction of monocytes during the rebound phase after each time of chemotherapy (Fig. 5B). These results, together with the previously described MCP elevation in post-NT blood (Fig. 1B), indicate an upstream role of chemotherapy-induced monocytosis in the herein identified CSC regulatory mechanism.

We further analyzed paired pre- and post-NT breast tumors from the City of Hope patients by immunohistochemistry and detected significantly increased levels of nuclear Notch 1 in the tumor, together with increased percentage of tumor cells expressing the BCSC marker ALDH1, upon NT treatment (Fig. 5C and D). Unlike in pre-NT tumors where ALDH1 showed a sparse and scattered staining pattern, ALDH1<sup>+</sup> cells in post-NT tumors notably existed in patches, possibly suggesting a clonal origin (Fig. 5D). When pre-NT tumors were compared for tumor cell expression of CCR2, those exhibiting PCR during the subsequent NT had significantly lower CCR2 level compared with tumors with non-pCR (Fig. 5E), suggesting that CCR2 expression level which sets tumor responsiveness to MCPs could be a factor influencing tumor response to NT.

Discussion

Although conventional chemotherapy has been known to induce cycles of myelosuppression and restoration (24), how the process of therapy-induced myeloid cell homeostasis may influence tumor relapse has not been fully studied. We show that chemotherapy induces monocytosis and the consequent systemic elevation of MCP chemokines, which occurs regardless of tumor response to therapy, the expression status of HER2, or even the presence of a tumor. Compared with the HER2<sup>+</sup> cases, the TNBC cases exhibited higher pre- and post-NT levels of CCL2 and CCL8 and a higher post-NT ALDEFLUOR-stimulating activity in the blood (Table 1). This could suggest the involvement of TNBC-derived factors that also influence MCPs. Our data suggest that HSCs display enhanced commitment to the granulocyte-mono
cyte lineage following chemotherapy (Fig. 2). During hematopoietic development and homeostasis, cytokines derived from hematopoietic and stromal cells play critical roles in the fate determination of HSCs. Lineage-specific cytokines, such as granulocyte-colony stimulating factor and granulocyte/macrophage-colony stimulating factor secreted by BM mesenchymal cells, can be induced by TNFα and IL1α produced at sites of inflammation, leading to stimulation of multiple stages of granulopoiesis (25). Secretion of inflammatory cytokines has been shown in fibroblasts in response to persistent DNA damage (26) and in peripheral blood mononuclear cells in response to apoptosis induction (27). Therefore, cytotoxic therapeutic agents, by damaging hematopoietic and stromal cells in the BM and/or inducing hematopoietic stresses, may alter the local cytokine network to affect the complex cell dynamics in the BM observed in Fig. 2E and F.

In addition to enhanced monocytosis, chemotherapy also increases the production of MCPs by monocytes but not CAF, which also secrete MCPs but at lower levels (Fig. 3C). This effect is dependent on JNK, a stress-activated protein kinase that acts through the c-Jun transcriptional factor to regulate gene expression during stress response and apoptosis (28). In turn, MCPs may recruit monocytes/macrophages to the tumor, and CCL2 has been shown to induce angiogenesis (29). These events may further regulate the cellular composition and cytokine environment of the tumor, exerting additional effects on CSCs (30). Thus, local productions of MCPs by monocytes/macrophages and nonmo
cytic cells (e.g., CAF) in the tumor would likely contribute to the overall effects of MCPs before and after therapy, and be blocked by CCR2 inhibition. Although CAF does not increase MCP production upon chemotherapy treatment, previous reports show that chemotherapy increases the frequency of CAF in primary tumors and stimulates CAF to produce other cytokines that promote CSCs and chemoresistance, including IL1βA, IL11, and IL6 (31–33).

Notch signaling promotes self-renewal of adult stem cells (including human mammary stem cells; ref. 34) and lineage-specific proliferation of multipotent progenitor cells (e.g., expansion of luminal progenitor cells in the mammary epithelial hierarchy; ref. 35). We and others have reported that Notch signaling promotes CSC phenotypes and contributes to a higher degree of tumor malignancy (17, 36, 37). The post-NT activation of Notch and acquisition of CSC properties could potentially promote tumor progression and metastasis through the regulation of epithelial-to-mesenchymal transition, angiogenesis, and genes involved in invasiveness (38, 39). Importantly, MCP-directed regulation of Notch may also influence hematopoietic stem cells/progenitor cells (HSC/HPSC), as Notch activation has been shown to induce expansion of hematopoietic stem/progenitor cells (40, 41). If true, this may contribute to a feed-forward loop in which MCPs produced by initial monocytes induce an expansion of HSCs and/or monocyte progenitors to produce more monocytes as sustained sources for MCPs.

Our study is based on clinical specimens from NT trials as well as experimental tumor models to simulate NT administered at an early tumor stage and to assess CSC-mediated tumor formation in an immediately posttherapy host environment. The stage of cancer and therapy simulated by these models represents the
phase when CCR2 inhibition may have a beneficial effect by antagonizing the therapy-induced, monocytosis-associated cancer stemness. It is worth noting that the induction of CSC traits during NT may also affect a subsequent metastatic event. However, metastasis is ultimately determined by multiple factors influencing cancer dormancy, molecular evolution, reprogramming upon arrival to a new site, as well as adaptation of the metastatic niche, and can occur years after the therapy-induced monocytosis through independent mechanisms (42).

CCR2 in monocytes and HSCs/HPCs mediates the mobilization of these cells from BM to inflammatory sites (43). Therefore, CCR2 inhibitors in phase I/II clinical trials for noncancer diseases (21, 44) may act on both cancer and hematopoietic cells to efficiently block chemotherapy-induced breast cancer stemness. Although higher levels of CCL2 in primary breast cancers are associated with poorer prognosis (45) and its neutralization in mice has been shown to inhibit metastasis (46), a recent study reports that cessation of CCL2 inhibition promotes breast cancer metastasis suggesting the complication of targeting CCL2 as a monotherapy (47). Indeed, a human monoclonal antibody against CCL2 has not shown antitumor activity as a single agent (12, 48), a recent study (21, 44) may act on both cancer and hematopoietic cells to influence these cells from BM to inflammatory sites (43).

Metastatic disease may also affect a subsequent metastatic event. However, metastasis is ultimately determined by multiple factors influencing cancer dormancy, molecular evolution, reprogramming upon arrival to a new site, as well as adaptation of the metastatic niche, and can occur years after the therapy-induced monocytosis through independent mechanisms (42).

Disclosure of Potential Conflicts of Interest
G. Somlo reports receiving commercial research grants from Celgene, other commercial research support from Roche, and is a consultant/advisory board member for Pfizer and Roche. No potential conflicts of interest were disclosed by the other authors.

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