CCL2/CCR2-Dependent Recruitment of Functional Antigen-Presenting Cells into Tumors upon Chemotherapy

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Abstract

The therapeutic efficacy of anthracyclines relies, at least partially, on the induction of a T-cell– and T-lymphocyte–dependent anticaner immune response. Here, we show that anthracycline-based chemotherapy promotes the recruitment of functional CD11b+CD11c+Ly6ChighLy6G−MHCIIdendritic cell–like antigen-presenting cells (APC) into the tumor bed, but not into lymphoid organs. Accordingly, draining lymph nodes turned out to be dispensable for the proliferation of tumor antigen–specific T cells within neoplastic lesions as induced by anthracyclines. In addition, we found that tumors treated with anthracyclines manifest increased expression levels of the chemokine Ccl2. Such a response is important as neoplasms growing in Ccl2−/− mice failed to accumulate dendritic cell–like APCs in response to chemotherapy. Moreover, cancers developing in mice lacking Ccl2 or its receptor (Ccr2) exhibited suboptimal therapeutic responses to anthracycline-based chemotherapy. Altogether, our results underscore the importance of the CCL2/CCR2 signaling axis for therapeutic anticaner immune responses as elicited by immunogenic chemotherapy. Cancer Res; 74(2); 1–10. © 2013 AACR.

Introduction

Accumulating evidence indicates that the antineoplastic effects of anthracyclines largely depend on the induction of a T-cell–mediated tumor-specific immune response. In patients with breast cancer subjected to one cycle of anthracycline-based chemotherapy, a high ratio of CD8+ cytotoxic versus FOXP3+ regulatory T cells within the tumor predicts the advent of a complete pathological response after 6 cycles of neoadjuvant treatment (1, 2). In mice, the therapeutic efficacy of anthracyclines is abolished when the extravasation of CD11b+ myeloid cells is blocked (3), when several distinct T-cell subsets (i.e., α/β or γ/δ T cells, CD8+ cells) are eliminated (4, 5) as well as when essential cytokines such as interferon (IFN)–γ, interleukin (IL)-1β, and IL-17 are neutralized (5–7). Thus, the therapeutic efficacy of anthracyclines requires specific cytokines to orchestrate a complex immune response that involves antigen-presenting cells (APC) exhibiting a dendritic cell–like (i.e., CD11b+CD11c+Ly6ChighLy6G−MHCIIdendritic cell–like) phenotype and several T-cell subsets (3, 6). The actual source of such intratumor APCs has not yet been elucidated.

One of the most critical processes for the initiation of an immune response against cancer cells succumbing to anthracyclines is the secretion of ATP (8). Extracellular ATP indeed operates not only as a potent chemotactic signal (by binding to purinergic P2Y2 receptors; ref. 9), but also as a proinflammatory agent, stimulating the activation of the NLRP3 inflammasome (by binding to purinergic P2RX7 receptors) and hence the release of IL-1β (10). In line with this notion, ATP is essential for the accumulation of mononuclear precursors within the tumor bed as well as for their local differentiation into dendritic cell–like APCs (3). Thus, shortly (24–72 hours) after the administration of immunogenic chemotherapy (11–13), ATP levels increase in the tumor interstitium as a consequence of the apoptotic demise of cancer cells. Of note, autophagy is required for such an immunogenic cell death–associated release of ATP to be optimal (8). Myeloid and lymphoid cells indeed are not recruited by anthracycline-treated autophagy-deficient tumors, correlating with the absence of anticaner

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immune responses and hence with therapeutic failure (8). Along similar lines, cancers can be rendered virtually insensitive to the antineoplastic effects of anthranclyenes by the overexpression of CD39, a cell surface ATP-degrading enzyme (8). Besides ATP, other chemotactic factors including multiple chemokines are known to participate into the recruitment of innate and cognate immune effectors to inflammation sites (14). However, the precise contribution of chemokines to anticaner immune responses elicited by immunogenic chemotherapeutics has not yet been established.

Driven by these premises and incognitae, we investigated whether the APCs that accumulate within neoplastic lesions in response to anthraclyne-based chemotherapy derive from local precursors or are recruited from external sources and to which extent chemokines regulate their biogenesis. We found that dendritic cell-like APCs are freshly recruited into the tumor bed in response to immunogenic chemotheraphy and that the host-derived chemokine Ccl2 plays a major, ATP-independent role in this process. In line with this notion, Ccl2 and its major receptor, Ccr2 (also known as CD192), turned out to be fundamental for the antineoplastic effects of anthracycline-based chemotherapy in mice.

Materials and Methods

Cell lines

Mouse firososarcoma MCA205 cells (H2fl), their ovalumin (OVA)-expressing derivatives (a generous gift from Dr. A.D. Weinberg, Portland, Providence Medical Centre, Portland, OR), and AT3 mammary carcinoma cells (H2fl) were cultured in GlutaMAX–containing RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1 mmol/L pyruvate, 10 mmol/L HEPES buffer, 1 mmol/L nonessential amino acids, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. Unless otherwise indicated, authenticated wild-type cells were obtained from American Type Culture Collection, immediately amplified to constitute liquid nitrogen stocks and (upon thawing) never passaged for more than 1 month before use in experimental determinations. Cell culture products were from Gibco-Life Technologies.

Animal experiments

Mice were housed in specific pathogen-free conditions at either the Institut Gustave Roussy (IGR, Villejuif, France), the Peter MacCallum Cancer Center (East Melbourne, Australia), or the QIMR Berghofer Medical Research Institute (Herston, Australia). All animal experiments were performed in accordance with the Federation of European Laboratory Animal Science Association guidelines and were approved either by the IGR Ethics Committee (CEEA IRCIV/IGR No. 26, registered with the French Ministry of Research) or by the Animal Experimentation Ethics Committee of Peter MacCallum Cancer Center and QIMR Berghofer Medical Research Institute. Female C57BL/6 (H2fl) mice (aged between 6 and 8 weeks) were purchased from Janvier, OVA257–264-specific T-cell receptor (TCR) transgenic OT1 mice (H2fl) and congenic mice harboring an alternative CD45/Ly-5 allotaigan (Ly-5.1) were obtained from Charles River, whereas Ccl2−/− and Ccr2−/− mice were bred at the Peter MacCallum Cancer Center or QIMR Berghofer Medical Research Institute. Mice were maintained under controlled light cycle (12 hours lights ON, 12 hours lights OFF), allowed food and water ad libitum, and were invariably used for experiments between the age of 7 and 16 weeks.

Lymphadenectomy

Mice were subjected to skin decontamination with 10% povidone-iodine (commercial Betadine solution) and anesthesia was maintained by continuous mask inhalation of isoflurane flow during surgery. A narrow incision (3 mm) was performed on the thigh, tumor-draining lymph nodes (DLN) were removed using a dissecting forceps, and the incision was eventually closed with sterile skin closure clips. Sham-operated mice were treated with a similar procedure (but were not subjected to lymphadenectomy), and the duration of surgery was standardized between the 2 groups. After surgery, mice were placed on a heater plate set at 37°C until recovery.

Tumor chemotherapy models

For establishing syngeneic solid tumors, wild-type, Ccl2−/−, or Ccr2−/− CB57BL/6 mice were inoculated with 5 × 105 MCA205, OVA-expressing MCA205, or AT3 cells subcutaneously. Thereafter, tumor surface (longest diameter multiplied by its perpendicular diameter) was routinely monitored by means of a common caliper, and when it reached 25 to 45 mm2, mice received either 2.9 mg/kg doxorubicin intratumorally (i.t.; in 50 μL PBS) or an equivalent volume of solvent.

Transplantation of established tumors

CD45.2 CB57BL/6 mice were anesthetized under continuous isoflurane flow and a small incision was made on the skin near the thigh. MCA205 fibrosarcomas (surface ≈35–50 mm2) freshly harvested from CD45.1 mice were then implanted as a whole subcutaneously, with the help of grooved probes and forceps. Eventually, the incision was closed with sterile skin closure clips.

Tissue recovery and processing for flow cytometry

Freshly recovered tumors or spleens were cut into small pieces (using surgical scissors) in digestion buffer (serum-free GlutaMAX–containing RPMI 1640 medium supplemented with 0.4 Winsch units/mL Liberase TL, from Roche, and 200 U/mL DNase I, from Calbiochem), and then transferred to 24-well plates for enzymatic dissociation (30 minutes, 37°C). Upon washing with PBS, single-cell suspensions were obtained by filtering samples through a 70-μm cell strainer. Lymph nodes were gently squeezed with dissecting forceps in digestion buffer to obtain single-cell suspensions. Bone marrow cells were recovered by repeatedly flushing the femoral or tibial bone marrow cavities with PBS using 26G needles. The peripheral blood was harvested from the inner canthus of the eye with heparin-coated Pasteur pipettes. Red blood cells were removed with the RBC Lysis Buffer (BioLegend), following the manufacturer’s instructions.

Flow cytometry and sorting

For cell surface immunostaining, primary antibodies targeting the following proteins were used (at 2 μg/mL unless
indicated, staining at 4°C for 20 minutes): CD45.1 (A20), CD45.2 (104), TCR VB 5.1/5.2 (MR9-4), and CD69 (H1.2F3), all from eBioscience; CD11b (M1/70), CD11c (N418), and Ly6C (AL-21), all from BD Pharmingen; B220 (RA3-6B2), I-A/I-E (M5/114.15.2; used at 0.2 μg/mL), Ly6G (1A8), and CD103 (2E7), all from BioLegend; Ly-6B.2 (7/4), from AbD Serotec; and CCR2 (MC-21), a generous gift from Dr. Matthias Mack (University of Regensburg, Regensburg, Germany). Cytotoxicity assays were performed on a BD LSR II Flow Cytometer (BD Biosciences) and data were analyzed by means of the FlowJo software package (Tree Star, Inc.). Tumor cell suspensions were sorted with a MoFlo XDP (Beckman-Coulter) or a FACSAria (BD Biosciences) cell sorter.

In situ T-cell activation assays
For testing antigen-specific T-cell proliferation in situ, splenic OT1 cells were stained with 5 μM/L CFSE (Molecular Probes-Life Technologies) for 10 minutes at 37°C, followed by quenching of the extra dye with an equal volume of 20% FBS in PBS. These cells were then inoculated intravenously into OVA-expressing MCA205 fibrosarcoma-bearing mice (7 x 10^6 cells/mouse), 4 days after tumor cell inoculation (day-5). Chemotherapy was administered on day 0 and mice were subjected to lymphadenectomy or sham surgery on day 1. OT1 cell proliferation in tumors and DLNs was assessed by CFSE dilution on day 3.

Alternatively, CD45.2^+ OT1 cells were inoculated intravenously into CD45.1 congenic mice (1 x 10^6 cells/mouse) 1 day before the subcutaneous inoculation of OVA-expressing MCA205 cells. Doxorubicin-based chemotherapy or PBS was administered i.t. 7 days later. Tumors, DLNs, contralateral lymph nodes, and spleens were harvested 60 hours after chemotherapy to analyze CD69 expression on OT1 cells.

Microarray studies
Total RNA was isolated from established MCA205 fibrosarcomas 2 and 7 days after the i.t. administration of doxorubicin or PBS. Biotin-labeled cRNA was synthesized using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems). cRNA was hybridized to Mouse WG-6 V2 Expression BeadChips (Illumina) and scanned with a BeadStation 500x (Illumina). Raw data collection from Illumina BeadChips was performed using the BeadStudio software v. 3.1.1.0 (Illumina). Data were further analyzed by an R-based informatics platform by means of packages from the Bioconductor project (15). Upon quantile normalization, genes were defined as differentially expressed when associated with absolute changes in expression levels (absolute fold change) >1.7. This transcriptomic dataset has been deposited in the Gene Expression Omnibus database (accession number GSE46275; ref. 16).

Quantitative RT-PCR
Total RNA extraction and genomic DNA removal were performed by means of the RNeasy Micro Kit (Qiagen), following the manufacturer’s instructions. Total RNA (0.5–2 μg per sample) was then reverse transcribed into cDNA with the SuperScript III Reverse Transcriptase (Life Technologies), in the presence of random primers, the RNasin Plus RNase Inhibitor (Promega) and the Deoxynucleoside Triphosphate Set, PCR grade (Roche). The expression of genes coding for inflammatory cytokines, chemokines, M1 or M2 macrophage-related factors, and protein involved in MHC class I or class II antigen presentation was analyzed with TaqMan Gene Expression Assays using the Universal Master Mix II (with UNG) on a StepOnePlus Real-Time PCR System (everything from Applied Biosystems). Quantitative RT-PCR data were invariably normalized to the expression levels of the housekeeping gene peptidylprolyl isomerase A (Ppia).

Immunofluorescence microscopy
Freshly harvested tumors were processed for the immunofluorescence microscopy-assisted quantification of caspase-3 activation and infiltration by CD11c^+ CD86^+ cells as previously reported (3). Thereafter, 5-μm-thick cryosections were stained with the following antibodies or antibody conjugates: CD11c-AlexaFluor 488 (N418, from eBioscience), CD86-AlexaFluor 647 (GL-1, from BioLegend), and anti-cleaved caspase-3 (Asp175, from Cell Signaling Technology), followed by AlexaFluor 568 goat anti-rabbit immunoglobulin G (H+L; from Molecular Probes-Life Technologies). Nuclear counterstaining was achieved by incubating slides in 10 μM/L Hoechst 33342 (Molecular Probes-Life Technologies) for 5 minutes before mounting. Finally, 20 to 40 view-fields were acquired for each slide by means of a confocal SPE microscope (Leica Microsystems GmbH), and 8 to 10 images were randomly picked for statistical analysis.

Statistical analyses
Unless otherwise indicated, results are expressed as means ± SEM or means ± SD, as appropriate, of n = 3 parallel assessments. All experiments were repeated at least twice, yielding similar results. Normal distributions were compared by unpaired, 2-tailed Student t tests; tumor growth curves with Mann–Whitney U tests. Statistical analyses were performed with Prism 5 (GraphPad) or Excel 2007 (Microsoft). P-values <0.05 were considered as statistically significant.

Results and Discussion
Chemotherapy stimulates intratumoral antigen presentation
Shortly (38 hours) after the i.t. administration of doxorubicin, a prototypical anthracycline, MCA205 fibrosarcomas established in immunocompetent C57BL/6 mice contain an elevated percentage of CD11b^+Ly6C^hiLy6G^-CD11c^-MHCI^I^ restricted antigen presentation (Fig. 1B and C), as well as genes coding for multiple cytokines, including IL-1β, TNF-α, IL-10, and IL-12 (Fig. 1D). Besides becoming more abundant with time, this subset of myeloid cells overexpresses genes involved in MHC class I- and II-restricted antigen presentation (Fig. 1B and C), as well as genes coding for multiple cytokines, including IL-1β, TNF-α, IL-10, and IL-12 (Fig. 1D). However, these cells do not manifest an obvious polarization of gene expression toward a pro-inflammatory phenotype (Fig. 1D, E). Besides becoming more abundant with time, this subset of myeloid cells overexpresses genes involved in MHC class I- and II-restricted antigen presentation (Fig. 1B and C), as well as genes coding for multiple cytokines, including IL-1β, TNF-α, IL-10, and IL-12 (Fig. 1D). However, these cells do not manifest an obvious polarization of gene expression toward a profile associated with M1 or M2 macrophages (Supplementary Fig. S2). At this time point, no increase in CD11b^+Ly6C^hiLy6G^-CD11b^-CD11b^+Ly6C^hiLy6G^- and CD11b^+Ly6G^- cells was observed in the DLNs of mice bearing doxorubicin-treated.
MCA205 fibrosarcomas. Conversely, CD11b<sup>+</sup>Ly6Chigh/Ly6G<sup>+</sup> neutrophil-like cells were found to accumulate in the bone marrow and spleen of these animals, respectively (Supplementary Fig. S1). The biological significance of this latter observation remains obscure.

In mice carrying OVA-expressing MCA205 fibrosarcomas, doxorubicin-based chemotherapy increased the i.t. proliferation of adoptively transferred CFSE-labeled OT1 cells expressing a transgenic TCR specific for the MHC class I H<sub>2</sub>K<sup>b</sup>-restricted OVA-derived peptide SIINFEKL (Fig. 1E and

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Figure 1. Impact of anthracycline-based chemotherapy on gene expression by tumor-infiltrating CD11b<sup>+</sup>Ly6Chigh cells and activation of antigen-specific T cells. A–D, wild-type (WT) C57BL/6 mice bearing MCA205 fibrosarcomas were treated with doxorubicin i.t. or an equivalent volume of PBS, as a single injection. Thirty-eight hours later, CD11b<sup>+</sup>Ly6Chigh tumor-infiltrating leukocytes were purified by cytofluorometry (A) and the expression of genes involved in MHC class I (B) or class II (C) antigen presentation and cytokine secretion (D) was quantified by RT-PCR. Results are expressed in arbitrary units (AU) upon normalization to the expression levels of Ppia. E, WT C57BL/6 mice were inoculated with OVA-expressing MCA205 cells subcutaneously (on day-9), and then (on day 5) received OVA-specific, CFSE-labeled OT1 cells intravenously (i.v.). On day 0, mice were treated with doxorubicin i.t. or an equivalent volume of PBS, as a single injection, followed (on day 1) by lymphadenectomy or sham surgery (as a control condition). On day 3, tumors were harvested and dissociated to determine the proliferation (CFSE dilution) of OT1 cells. Representative histograms of CFSE staining and quantitative data are depicted. Results are shown as means ± SEM (n = 5 mice/group); *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, nonsignificant (unpaired, 2-tailed Student t test), as compared with PBS-receiving tumors (unless otherwise indicated). DOX, doxorubicin.
Supplementary Fig. S3). Sixty hours after the administration of doxorubicin, the percentage of activated (CD69\(^+\)) OVA-specific OT1 cells was increased in the tumor bed, but not in the spleen, DLNs, and contralateral lymph node (Supplementary Fig. S3). Of note, the chemotherapy-induced proliferation of i.t. OT1 cells was maintained upon the surgical ablation of DLNs (Fig. 1E). This is in line with previous findings from our group demonstrating that anticancer immune responses elicited by doxorubicin-based chemotherapy are insensitive to the administration of a lymphotoxin \(\beta\) decoy that impedes the formation of DLNs (3). Moreover, the proliferation of adoptively transferred CFSE-labeled OT1 cells in DLNs was not affected by chemotherapy (Supplementary Fig. S4). Taken together, these findings indicate that the presentation of tumor antigens elicited by immunogenic chemotherapy occurs within the tumor bed.

**Intratumoral recruitment of APCs upon immunogenic chemotherapy**

Theoretically, the APCs that accumulate within anthracycline-treated neoplastic lesions might either originate from preexisting precursors that differentiate locally or be recruited de novo from external sources. To discriminate between these possibilities, we established MCA205 fibrosarcomas in CD45.1 mice, treated them with vehicle or doxorubicin, and (1 day later) transplanted them into isogenic CD45.2 C57Bl/6 recipients. This allowed us to quantify the intratumoral abundance of sessile (CD45.1\(^+\)) versus freshly recruited (CD45.2\(^+\)) leukocytes, more specifically CD11c\(^+\)MHCII\(^+\) cells. In this setting, we observed that most tumor-sessile leukocytes are replaced by their recipient-derived counterparts within the first 4 days after transplantation, irrespective of the administration of vehicle or doxorubicin (Fig. 2A–C). Globally, the ratio of recipient-derived (CD45.2\(^+\)) versus donor-derived (CD45.1\(^+\)) cells was not altered by chemotherapy (Fig. 2E). However, in doxorubicin-treated tumors, the ratio of CD45.2\(^+\) versus CD45.1\(^+\) CD11c\(^+\)MHCII\(^+\) dendritic cell–like cells was higher than in PBS-receiving lesions (Fig. 2E), supporting the notion that anthracyclines trigger the recruitment of dendritic cell precursors rather than the mere differentiation of preexisting myeloid cells in situ. Accordingly, CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells accumulating within neoplastic lesions in response to anthracyclines phenotypically resembled a cell subset present in the bone marrow (both were also CD11c\(^+\)F4/80\(^int\)/Ly6C\(^{-}\)/CD103\(^+\); Supplementary Fig. S1). Moreover, the frequency of several distinct circulating CD11b\(^+\) cell subsets fluctuated in response to anthracyclines (Supplementary Fig. S5), in concert with the possibility that at least a fraction of CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells found in the tumor bed upon immunogenic chemotherapy is blood-borne.

**CE12 is required for the intratumoral recruitment of APCs**

ATP is known to operate as a chemotactic and danger signal in the context of immunogenic chemotherapy (3, 8). In line with this notion, MCA205 fibrosarcoma cells that had been engineered to overexpress the ecto-ATPase CD39 on their surface generated lesions in immunocompetent mice that were unable to recruit myeloid, especially CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells in response to doxorubicin (Fig. 3A). To identify additional factors that are involved in the chemotherapy-induced recruitment of antigen-presenting leukocytes into the tumor bed, we performed microarray-based transcriptomic analyses. In this setting, several chemokine-encoding genes were found to be upregulated (at the mRNA level) 2 days after the i.t. administration of doxorubicin (Fig. 3B). In particular, anthracycline-based chemotherapy turned out to promote the expression of Cc12 and Ccl7 (two chemokines involved in myeloid cell chemotaxis; refs. 17 and 18) by intratumoral CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells. This phenomenon was not influenced by CD39 and hence must be independent from the immunogenic cell death–associated secretion of ATP (Fig. 3C). The levels of Cc3c11 in CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells were not influenced by anthracycline-based chemotherapy, yet were a priori more elevated in the context of CD39 overexpression (Supplementary Fig. S6). Conversely, extracellular ATP seemed to have a positive impact on the expression levels of Cxcl1, Cxcl9, Cxcl10, Cxcl11, and Ccl5 (Fig. S6). Of note, both CD45\(^+\) leukocytes and CD45\(^-\) tumor cells responded to doxorubicin by producing Cc12 and Ccl7, yet only the former did so in a statistically significant fashion (Fig. 3D). Moreover, tumors growing in Cc12\(^-\) mice failed to recruit CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells in response to doxorubicin (Fig. 3E). Interestingly, several CD11b\(^+\) cell subsets including CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) leukocytes were found to express the Cc12 receptor Ccr2 (which also binds Ccl7), irrespective of their previous exposure to doxorubicin. In this context, the highest levels of Ccr2 were expressed by CD11b\(^+\) cells isolated from neoplastic lesions (as opposed to the circulation, spleen, bone marrow and DLNs; Fig. 3F). Conversely, we failed to detect significant amounts of Ccr2 on the surface of malignant cells, be them recovered from PBS- or doxorubicin–treated tumors (Supplementary Fig. S7). Taken together, these observations suggest that Cc12 produced by host leukocytes plays a prominent role in recruitment of CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) cells into the tumor bed upon anthracycline–based chemotherapy.

**The efficacy of anthracycline-based chemotherapy in mice depends on Cc12/Ccr2**

The aforementioned findings indicate that Cc12 is required for the doxorubicin-stimulated intratumoral accumulation of CD11b\(^+\)Ly6C\(^{high}\)Ly6G\(^-\) dendritic cell–like cells, including the APCs that are essential for the optimal efficacy of anthracycline-based chemotherapy (3). MCA205 fibrosarcomas developing in Cc12\(^-\) and Ccr2\(^-\) mice exhibited a similar apoptotic response to doxorubicin than tumors growing in wild-type hosts (Fig. 4A and B). However, the absence of Cc12 or Ccr2 in the host dampened the accumulation of CD11c\(^+\)CD86\(^+\) dendritic cells in tumor areas enriched of dead cells (Fig. 4A and C). In line with this notion, Cc12\(^-\) and Ccr2\(^-\) mice bearing MCA205 fibrosarcomas were less sensitive to doxorubicin–based chemotherapy than their wild-type counterparts (Fig. 4D). Similarly, AT3 breast carcinomas only responded to the i.t. administration of doxorubicin in
mice expressing both Ccl2 and Ccr2. Conversely, the growth of AT3 cancers in Ccl2−/− or Ccr2−/− hosts was poorly affected by anthracycline-based chemotherapy (Fig. 4E). Altogether, these data indicate that the CCL2/CCR2 signaling axis is essential for optimal therapeutic responses to anthracyclines, at least in mice.

**Concluding remarks**

Taken together, the results presented here indicate that anthracycline-based chemotherapy promotes the intratumoral accumulation of (presumably blood-borne) myeloid cells, including cells with a dendritic cell-like phenotype that mediate antigen presentation. Immunogenic chemotherapy
seems to cause major shifts in the composition of myeloid cell subsets that affect the tumor microenvironment but not lymphoid organs. In addition, available evidence suggests that neoplastic lesions contain all the cellular and humoral elements that are required to mount a local anticancer immune response even in the absence of DLNs, hence constituting a sort

Figure 3. Contribution of ATP and chemokines to the chemotherapy-induced accumulation of myeloid tumor-infiltrating leukocytes. A–D, wild-type (WT) C57BL/6 mice bearing WT or CD39-expressing (CD39) MCA205 fibrosarcomas were treated with doxorubicin i.t. or an equivalent volume of PBS, as a single injection. Tumors were then harvested and processed either for the cytofluorometric quantification of tumor-infiltrating leukocytes of the indicated surface phenotype at the indicated time points (A), either for microarray-based transcriptomic studies, 48 hours after chemotherapy (B), or for the RT-PCR-assisted quantification of Ccl2 and Ccl7 expression levels among cells of the indicated surface phenotype (isolated by cytofluorometry in C or magnetic bead separation in D), 20 hours after chemotherapy (C and D). In B, chemokine-encoding genes are indicated by asterisks. In C, results are expressed in arbitrary units (AU) upon normalization to the expression levels of Ppia. E, WT C57BL/6 or Ccl2−/− mice harboring MCA205 fibrosarcomas were treated with doxorubicin or PBS, as a single i.t. injection. Thirty-eight hours later, tumors were collected and processed for the cytofluorometric quantification of tumor-infiltrating leukocytes of the indicated surface phenotype. F, the bone marrow (BM), blood, spleen, neoplastic lesions, and DLNs of WT C57BL/6 mice harboring MCA205 fibrosarcomas were harvested and processed for the cytofluorometric quantification of Ccr2 expression among cells of the indicated surface phenotype. Representative expression profiles are shown (Iso BM, BM cells stained with an isotype-matched control antibody). Quantitative results are reported as means ± SEM (n = 5 mice/group). **, P < 0.05; ***, P < 0.01; ns, nonsignificant (unpaired, 2-tailed Student t test), as compared with PBS-receiving tumors (unless otherwise indicated). DOX, doxorubicin.
Figure 4. Contribution of the CCL2/CCR2-signaling axis to the antineoplastic effects of anthracyclines. A–E, wild-type (WT), Ccl2−/−, or Ccr2−/− mice were inoculated with MCA205 fibrosarcoma (A–D) or AT3 mammary cancer cells (E), and were treated with doxorubicin i.t. or an equivalent volume of PBS, as a single injection 7 days later.

A–C, neoplastic lesions were harvested 48 hours after treatment and processed for the immunofluorescence microscopy–assisted quantification of cells bearing active caspase-3 (Casp-3a) and tumor infiltration by CD11c+CD86+ cells. Representative microphotographs are depicted in A (scale bar, 25 μm). B and C report quantitative data in the form of means ± SEM (n = 5 mice/group, 8–10 randomly picked view-fields out of 20–40 acquired per sample). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, nonsignificant (unpaired, 2-tailed Student’s t test), as compared with PBS-receiving tumors (unless otherwise indicated). D and E, alternatively, tumor growth was routinely assessed throughout the experiment. Results are shown as means ± SEM (n = 5 mice/group).

This experiment has been performed twice, yielding comparable results. *, P < 0.05 (Mann–Whitney U test). Please note that the results depicted in D have been split over two plots (entailing the duplication of WT mice-related curves) to allow for the visualization of otherwise nearly overlapping Ccl2−/− and Ccr2−/− mice–related data. DOX, doxorubicin.
of minimal lymphoid organ. Independently from the accumulation of ATP in the tumor microenvironment, anthracyclines stimulated distinct types of tumor-associated cells, but mainly CD45+ myeloid cells, to produce a series of chemokines including the 2 Ccr2 ligands Ccl2 and Ccl7. Tumors established in Ccl2−/− hosts failed to recruit dendritic cell-like APCs and exhibited a suboptimal therapeutic response to upon anthracycline-based chemotherapy. Along similar lines, tumor growing in Ccr2−/− did not respond to anthracyclines as efficiently as tumors implanted in Ccl2- and Ccr2-proficient mice. These findings support the importance of host-derived Ccl2 acting on host Ccr2 for the anticancer immune response elicited by immunogenic cell death.

The CCL2/CCR2 system has been previously implicated in the accumulation of tumor-associated macrophages and myeloid-derived suppressor cells within neoplastic lesions, hence supporting tumor growth via proinflammatory and immunosuppressive mechanisms (19). In addition, an autocrine CCL2-dependent signaling pathway has been suggested to promote the survival and mobility of cancer cells (20), whereas tumor cell–derived CCL2 has been shown to stimulate metastasis (21) and angiogenesis (22). In line with these observations, CCL2- or CCR2-targeting agents have been shown to improve the antineoplastic activity of distinct immunotherapeutic regimens (23, 24) as well as the efficacy of gemcitabine- and cisplatin-based chemotherapy (25, 26). Based on these observations, several clinical trials have recently been launched to evaluate the safety and therapeutic potential of CCL2- or CCR2-blocking antibodies, either as standalone therapeutic interventions or combined with conventional chemotherapy, in patients with cancer (source http://clinicaltrials.gov).

Here, we investigated the impact of the CCL2/CCR2 system in a rather peculiar context. First, the growth of the tumor models that we chose was unaffected by the absence or presence of Ccl2 or Ccr2 in the host (Fig. 4), implying that none of the aforementioned protumorigenic functions of CCL2/CCR2 operate in this scenario. Second, we focused our attention on the therapeutic immune response elicited by the immunogenic cell death inducer doxorubicin. In this experimental setting, the negative impact of CCL2/CCR2 ablation on the therapeutic activity of doxorubicin correlated with a reduced intratumoral recruitment of dendritic cell–like APCs. It should be noted that the CCL2/CCR2 system mediates the accumulation of myeloid-derived dendritic cells during allergic (27) and autoimmune inflammation (28), as well as at sites of parasitic (29), fungal (30), and mycobacterial infection (31). Thus, the inhibition of the CCL2/CCR2 signaling axis may blunt a wide range of inflammatory and immune reactions, including those required for the control of infectious pathogens and (pre)malignant cells.

Based on these considerations, the use of CCL2- or CCR2-blocking agents in patients with cancer must be carefully considered on a personalized basis. This may indeed constitute a therapeutic option to subvert CCL2-dependent metastatic spread, angiogenesis, and immunosuppression. However, blocking the CCL2/CCR2 system should be avoided in conditions in which the recruitment of dendritic cell–like APCs into the tumor bed constitutes a limiting step for the elicitation of therapeutic anticancer immune responses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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