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 dendritic cell (DC)- and T lymphocyte-dependent anticancer immune response. Here, we show that anthracycline-based chemotherapy promotes the recruitment of functional CD11b⁺CD11c⁺Ly6ChighLy6G⁻MHCII⁺ DC-like antigen-presenting cells (APCs) 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 DC-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 anticancer immune responses as elicited by immunogenic chemotherapy.
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 breast cancer patients 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 six 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., \(\alpha/\beta\) or \(\gamma/\delta\) T cells, CD8\(^+\) cells) are eliminated (4, 5) as well as when essential cytokines such as interferon \(\gamma\) (IFN\(\gamma\)), interleukin (IL)-1\(\beta\) 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 (APCs) exhibiting a dendritic cell (DC)-like (i.e., CD11b\(^-\)CD11c\(^+\)Ly6C\(^{hi}\)Ly6G\(^{low}\)MHCII\(^+\)) phenotype and several T-cell subsets (3, 6). The actual source of such intratumoral 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) (9), but also as a pro-inflammatory agent, stimulating the activation of the NLRP3 inflammasome (by binding to purinergic P2RX7 receptors) and hence the release of IL-1\(\beta\) (10). In line with this notion, ATP is essential for the accumulation of monocytic precursors within the tumor bed as well as for their local differentiation into DC-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 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 anticancer immune responses and hence with therapeutic failure (8). Along similar lines, cancers can be rendered virtually insensitive to the antineoplastic effects of anthracyclines 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 anticancer immune responses elicited by immunogenic chemotherapeutics has not yet been established.

Driven by these premises and incognita, we investigated whether the APCs that accumulate within neoplastic lesions in response to anthracycline-based chemotherapy derive from local precursors or are recruited from external sources and to which extent chemokines regulate their biogenesis. We found that DC-like APCs are freshly recruited into the tumor bed in response to immunogenic chemotherapy 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 fibrosarcoma MCA205 cells (H2b), their ovalbumin (OVA)-expressing derivatives (a generous gift from Dr Andrew D. Weinberg, Portland Providence Medical Centre, Portland, OR, USA) and AT3 (H2b) mammary carcinoma cells were cultured in GlutaMAX™-I-containing RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM HEPES buffer, 1 mM 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 ATCC (Manassas, VA, USA), 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 (Carlsbad, CA, USA).

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 (FELASA) guidelines and were approved either by the IGR Ethics Committee (CEEA IRCIV/IGR n°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 (H2b) mice (aged between 6 and 8 weeks) were purchased from Janvier (Le Genest St. Isle, France), OVA257-264-specific TCR transgenic OT1 mice (H2b) and congenic mice harboring an alternative CD45/Ly-5 alloantigen (Ly-5.1) were obtained from Charles River (L'Arbresles, France), while 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 hrs lights ON, 12 hrs 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 (DLNs) 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 two 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 *s.c.*. Thereafter, tumor surface (longest diameter multiplied by its perpendicular diameter) was routinely monitored by means of a common caliper, and when it reached 25-45 mm², mice received either 2.9 mg/kg DX *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 *s.c.*, 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™-I-containing RPMI 1640 medium supplemented with 0.4 Wünsch units/mL Liberase TL, from Roche, Mannheim, Germany, and 200 U/mL DNase I, from Calbiochem, San Diego, CA, USA), and then transferred to 24-well plates for enzymatic dissociation (30 min, 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 (BM) cells were recovered by repeatedly flushing the femoral or tibial BM cavities with PBS using 26G needles. 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, San Diego, CA, USA), following the manufacturer’s instructions.

**Flow cytometry and sorting.** For cell-surface immunostaining, primary antibodies targeting the following proteins were employed (at 2 μg/mL unless indicated, staining at 4 °C for 20 min): CD45.1 (A20), CD45.2 (104), TCR Vβ 5.1/5.2 (MR9-4) and CD69 (H1.2F3), all from eBioscience (San Diego, CA, USA); CD11b (M1/70), CD11c (N418) and Ly6C (AL-21), all from BD Pharmingen (San José, CA, USA); 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 (Kidlington, UK), and CCR2 (MC-21), a generous gift from Dr. Matthias Mack (University of Regensburg, Regensburg, Germany). Cytofluorometric assessments were performed on a BD™ LSR II Flow Cytometer (BD Biosciences, San José, CA, USA) and data were analyzed by means of the FlowJo software package (Tree Star, Inc., Ashland, OR, USA). Tumor cell suspensions...
were sorted with a MoFlo™ XDP (Beckman-Coulter, Miami, FL, USA) 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 CFSE (Molecular Probes-Life Technologies, Eugene, CA, USA) for 10 min at 37 °C, followed by quenching of the extra dye with the equal volume of 20% FBS in PBS. These cells were then inoculated *i.v.* into OVA-expressing MCA205 fibrosarcoma-bearing mice (7×10⁶ 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 *i.v.* into CD45.1 congenic mice (1×10⁶ cells/mouse) one day before the subcutaneous inoculation of OVA-expressing MCA205 cells. DX-based chemotherapy or PBS was administered *i.t.* 7 days later. Tumors, DLNs, contralateral lymph nodes and spleens were harvested 60 hrs after chemotherapy to analyze CD69 expression on OT1 cells.

**Microarray studies.** Total RNA was isolated from established MCA205 fibrosarcomas 2 and 7 after the intratumoral administration of DX or PBS. Biotin-labeled cRNA was synthesized using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Foster City, CA, USA). cRNA was hybridized to Mouse WG-6 V.2 Expression BeadChips (Illumina, San Diego, CA, USA) 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 then 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 (GEO) database (16) (accession number GSE46275).

**Quantitative RT-PCR.** Total RNA extraction and genomic DNA removal were performed by means of the RNeasy Micro Kit (Qiagen, Hilden, Germany), 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, Carlsbad, CA, USA), in the presence of random primers, RNasin® Plus RNase Inhibitor (Promega, Madison, WI, USA) 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 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, five μm-thick cryosections were stained with the following antibodies: CD11c-AlexaFluor® 488 (N418, from eBioscience), CD86-AlexaFluor® 647 (GL-1, from BioLegend) and anti-cleaved Caspase-3 (Asp175, from Cell Signaling Technology, Danvers, MA, USA), followed by AlexaFluor® 568 goat anti-rabbit IgG (H+L) (Molecular Probes-Life Technologies). Nuclear counterstaining was achieved by incubating slides in 10 μM Hoechst 33342 (Molecular Probes-Life Technologies) for 5 min before mounting. Finally, 20-40 view-fields were acquired for each slide by means of a confocal
SPE microscope (Leica Microsystems GmbH, Wetzlar, Germany), and 8-10 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, two-tailed Student’s *t* tests; tumor growth curves with Mann-Whitney *U* tests. Statistical analyses were performed with Prism 5 (GraphPad, San Diego, CA, USA) or Excel 2007 (Microsoft, Rockville, MD, USA). *p* values < 0.05 were considered as statistically significant.
Results and Discussion

Chemotherapy stimulates intratumoral antigen presentation. Shortly (38 hours) after the intratumoral administration of doxorubicin (DX), a prototypical anthracycline, MCA205 fibrosarcomas established in immunocompetent C57BL/6 mice contain an elevated percentage of CD11b$^+$Ly6C$^{high}$Ly6G$^-$ and CD11c$^+$MHCII$^+$ cells, as compared to vehicle-injected lesions (3) (Figure 1A and Figure S1). Besides becoming more abundant with time, this subset of myeloid cells overexpresses genes involved in MHC Class I and II-restricted antigen presentation (Figure 1B,C), as well as genes coding for multiple cytokines, including Il-1β, tumor necrosis factor α (Tnf), Il-10 and Il-12 (Figure 1D). However, these cells do not manifest an obvious polarization of gene expression towards a profile associated with M1 or M2 macrophages (Figure S2). At this time point, no increase in CD11b$^+$Ly6C$^{high}$Ly6G$^-$, CD11b$^+$, CD11b$^+$Ly6C$^{high}$Ly6G$^-$ and CD11b$^+$Ly6G$^+$ cells was observed in the DLNs of mice bearing DX-treated MCA205 fibrosarcomas. Conversely, CD11b$^+$Ly6C$^{high}$Ly6G$^-$ and CD11b$^+$Ly6G$^+$ neutrophil-like cells were found to accumulate in the BM and spleen of these animals, respectively. (Figure S1). The biological significance of this latter observation remains obscure.

In mice carrying OVA-expressing MCA205 fibrosarcomas, DX-based chemotherapy increased the intratumoral proliferation of adoptively transferred CFSE-labeled OT1 cells expressing a transgenic T-cell receptor (TCR) specific for the MHC Class I H2K$^b$-restricted OVA-derived peptide SIINFEKL (Figure 1E and Figure S3). Sixty hours after the administration of DX, 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 (Figure S3). Of note, the chemotherapy-induced proliferation of intratumoral OT1 cells was maintained upon the surgical ablation of DLNs (Figure 1E). This is in line with previous findings from our group demonstrating that
anticancer immune responses elicited by DX-based chemotherapy are insensitive to the administration of a lymphotoxin β 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 (Figure 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 accumulated 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 DX and (one 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 DX (Figure 2A-C). Globally, the ratio of recipient-derived (CD45.2⁺) _versus_ donor-derived (CD45.1⁺) cells was not altered by chemotherapy (Figure 2E). However, in DX-treated tumors the ratio of CD45.2⁺ _versus_ CD45.1⁺ CD11c⁺MHCII⁺ DC-like cells was higher than in PBS-receiving lesions (Figure 2E), supporting the notion that anthracyclines trigger the recruitment of DC 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 BM (both were also CD11c⁺F4/80^{int}7/4⁻CD103⁺) (Figure S1).
Moreover, the frequency of several distinct circulating CD11b⁺ cell subsets fluctuated in response to anthracyclines (Figure S5), in concert with the possibility that at least a fraction of CD11b⁺Ly6C<sup>high</sup>Ly6G⁻ cells found in the tumor bed upon immunogenic chemotherapy is blood-born.

**Ccl2 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<sup>high</sup>Ly6G⁻ cells in response to DX (Figure 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 intratumoral administration of DX (Figure 3B). In particular, anthracycline-based chemotherapy turned out to promote the expression of Ccl2 and Ccl7 (two chemokines involved in myeloid cell chemotaxis) (17, 18) by intratumoral CD11b⁺Ly6C<sup>high</sup>Ly6G⁻ cells. This phenomenon was not influenced by CD39 and hence must be independent from the immunogenic cell death-associated secretion of ATP (Figure 3C). The levels of Cx3cl1 in CD11b⁺Ly6C<sup>high</sup>Ly6G⁻ cells were not influenced by anthracycline-based chemotherapy, yet were a priori more elevated in the context of CD39 overexpression (Figure S6). Conversely, extracellular ATP seemed to have a positive impact on the expression levels of Cxcl1, Cxcl9, Cxcl10, Cxcl11 and Ccl5 (Figure S6). Of note, both CD45⁺ leukocytes and CD45⁻ tumor cells responded to DX by producing Ccl2 and Ccl7, yet only the former did so in a
statistically significant fashion (Figure 3D). Moreover, tumors growing in Ccl2−/− mice failed to recruit CD11b+Ly6C^{high}Ly6G− cells in response to DX (Figure 3E). Interestingly, several CD11b+ cell subsets including CD11b+Ly6C^{high}Ly6G− leukocytes were found to express the Ccl2 receptor Ccr2 (which also binds Ccl7), irrespective of their previous exposure to DX. In this context, the highest levels of Ccr2 were expressed by CD11b+ cells isolated from neoplastic lesions (as opposed to the circulation, spleen, BM and DLNs) (Figure 3F). Conversely, we failed to detect significant amounts of Ccr2 on the surface of malignant cells, be them recovered from PBS- or DX-treated tumors (Figure S7). Taken together, these observations suggest that Ccl2 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 Ccl2/Ccr2. The aforementioned findings indicate that Ccl2 is required for the DX-stimulated intratumoral accumulation of CD11b+Ly6C^{high}Ly6G− DC-like cells, including the APCs that are essential for the optimal efficacy of anthracycline-based chemotherapy (3). MCA205 fibrosarcomas developing in Ccl2−/− and Ccr2−/− mice exhibited a similar apoptotic response to DX than tumors growing in WT hosts (Figure 4A,B). However, the absence of Ccl2 or Ccr2 in the host dampened the accumulation of CD11c+CD86+ DCs in tumor areas enriched of dead cells (Figure 4A,C). In line with this notion, Ccl2−/− and Ccr2−/− mice bearing MCA205 fibrosarcomas were less sensitive to DX-based chemotherapy than their wild-type counterparts (Figure 4D). Similarly, AT3 breast carcinomas only responded to the intratumoral administration of DX 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 (Figure 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 DC-like phenotype that mediate antigen presentation. Immunogenic chemotherapy appears 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 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 two Ccr2 ligands Ccl2 and Ccl7. Tumors established in Ccl2−/− hosts failed to recruit DC-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-sufficient mice. These findings support the importance of host-derived Ccl2 acting in host Ccr2 for the anticancer immune response elicited by immunogenic cell death.

The CCL2/CCR2 system has been previously been implicated in the accumulation of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSC) within neoplastic lesions, hence supporting tumor growth via pro-inflammatory and immunosuppressive
mechanisms (19). In addition, an autocrine CCL2-dependent signaling pathway has been suggested to promote the survival and mobility of tumor cells (20), while 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 cancer patients (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 (Figure 4), implying that none of the aforementioned pro-tumorigenic 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 DX. In this experimental setting, the negative impact of CCL2/CCR2 ablation on the therapeutic activity of DX correlated with a reduced intratumoral recruitment of DC-like APCs. It should be noted that the CCL2/CCR2 system mediates the accumulation of myeloid-derived DCs 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 cancer patients 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 DC-like APCs into the tumor bed constitutes a limiting step for the elicitation of therapeutic anticancer immune responses.

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Supplemental Material is linked to the online version of the paper.
References


Figure Legends

Figure 1. Impact of anthracycline-based chemotherapy on gene expression by tumor-infiltrating CD11b<sup>+</sup>Ly6C<sup>high</sup> cells and activation of antigen-specific T cells. (A-D) Wild-type (WT) C57BL/6 mice bearing MCA205 fibrosarcomas were treated with doxorubicin (DX) i.t. or an equivalent volume of PBS, as a single injection. Thirty-eight hrs later, CD11b<sup>+</sup>Ly6C<sup>high</sup>tumor-infiltrating leukocytes were purified by cytofluorometry (A) and the expression of genes involved in MHC Class I (B) or 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 ovalbumin (OVA)-expressing MCA205 cells s.c. (on day -9), and then (on day -5) received OVA-specific, CFSE-labeled OT1 cells i.v.. On day 0, mice were treated with DX 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 = non significant (unpaired, two-tailed Student’s t test), as compared to PBS-receiving tumors (unless otherwise indicated)

Figure 2. Origin of tumor-infiltrating dendritic cell-like antigen-presenting cells. (A-E) MCA205 fibrosarcomas were established in histocompatible CD45.1 C57BL/6 mice, allowed to reach an area of 25-40 mm<sup>2</sup>, and then treated with doxorubicin (DX) i.t. or an equivalent volume of PBS, as a single injection. Twenty-four hrs later, tumors were transplanted s.c. to isogenic CD45.2 C57BL/6 recipients. The frequency of tumor-sessile (CD45.1<sup>+</sup>) and freshly recruited (CD45.2<sup>+</sup>) leukocytes, and more specifically that of CD11c<sup>+</sup>MHCII<sup>+</sup> cells (among live cells) was
determined by cytofluorometry at the indicated days post-transplantation. Representative cytofluorometric results are shown in A and quantitative data (means ± SEM, n = 3 mice/group) in B-E. *p<0.05, ns = non significant (unpaired, two-tailed Student’s t test), as compared to PBS-treated tumors.

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 (DX) 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 (TILs) of the indicated surface phenotype, at the indicated time points (A), either for microarray-based transcriptomic studies, 48 hrs post-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 hrs post-chemotherapy (C,D). In B, chemokine-encoding genes are indicated by asterisks. In C, results are expressed in arbitrary units (AUs) upon normalization to the expression levels of Ppiα. (E) WT C57BL/6 or Ccl2−/− mice harboring MCA205 fibrosarcomas were treated with DX or PBS, as a single intratumoral injection. Thirty-eight hrs later, tumors were collected and processed for the cytofluorometric quantification of TILs of the indicated surface phenotype. (E) The bone marrow (BM), blood, spleen, neoplastic lesions, and tumor-draining lymph nodes (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 = non significant (unpaired, two-tailed Student’s t test), as compared to PBS-receiving tumors (unless otherwise indicated).

**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 (DX) i.t. or an equivalent volume of PBS, as a single injection 7 days later. A-C. Neoplastic lesions were harvested 48 hrs 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). Panels 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 = non significant (unpaired, two-tailed Student’s t test), as compared to PBS-receiving tumors (unless otherwise indicated). D,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 panel 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.
Figure 2

A

Day 0

PBS

0.0% 36.8%

CD45.2

0.0% 43.1%

CD45.1

Day 1

8.8% 19.9%

Day 2

5.7% 10.7%

Day 4

7.7% 1.9%

Day 8

14.0% 1.0%

10.1% 4.5%

13.1% 1.8%

28.3% 1.4%

B

PBS-treated transplant

CD45.1

CD45.2

CD11c^MHCII^ cells (% of live)

Time (days)

0 1 2 4 8

Cells (%) 0 10 20 30 40 50

C

DX-treated transplant

CD45.1

CD45.2

CD11c^MHCII^ cells (% of live)

Time (days)

0 1 2 4 8

Cells (%) 0 10 20 30 40 50

D

Total cells

PBS

DX

CD45.1

CD45.2

Time (days)

0 1 2 4 8

E

CD11c^MHCII^ cells

PBS

DX

CD45.2/CD45.1^* cells

Time (days)

0 1 2 4 8

ns

*
CCL2/CCR2-dependent recruitment of functional antigen-presenting cells into tumors upon chemotherapy

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