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Chemotherapy-induced neutropenia elicits metastasis formation in mice by promoting proliferation of disseminated tumor cells

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ABSTRACT

Chemotherapy is the standard of care for most malignancies. Its tumor debulking effect in adjuvant or neoadjuvant settings is unquestionable, although secondary effects have been reported that paradoxically promote metastasis. Chemotherapy affects the hematopoietic precursors leading to myelosuppression, with neutropenia being the main hematological toxicity induced by cytotoxic therapy. We used renal and lung murine tumor models metastatic to the lung to study chemotherapy-induced neutropenia (CIN) in the metastatic process. Cyclophosphamide and doxorubicin, two myelosuppressive drugs, but not cisplatin, increased the burden of artificial metastases to the lung, by reducing neutrophils. This effect was recapitulated by treatment with anti-Ly6G, the selective antibody-mediated neutrophil depletion that unleashed the formation of lung metastases in both artificial and spontaneous metastasis settings. The increased cancer dissemination was reversed by granulocyte-colony stimulating factor-mediated boosting of neutrophils in combination with chemotherapy. CIN affected the early metastatic colonization of the lung, quite likely promoting the proliferation of tumor cells extravasated into the lung at 24–72 hours. CIN did not affect the late events of the metastatic process, with established metastasis to the lung, nor was there any effect on the release of cancer cells from the primary, whose growth was, in fact, somewhat inhibited. This work suggests a role of neutrophils associated to a common cancer treatment side effect and claims a deep dive into the relationship between chemotherapy-induced neutropenia and metastasis.

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Introduction

Metastasis, the process of cancer spreading to different organs from the site of tumor onset, is the primary cause of cancer-related deaths, with estimates reporting that 90% of cancer patients die from metastases within five years of their diagnosis.[1,2] The formation of metastases is complex, requiring different steps: cancer cells invade the stroma surrounding the primary tumor to make their way toward the vasculature. Entering the vessels, circulating tumor cells (CTCs) circulate until they stop in a target organ, exit the vessels, and seed in the new "soil" (disseminated tumor cells, DTCs) to colonize its parenchyma. Despite lethal, metastasis formation is highly inefficient, and most cancer cells cannot adapt to the new environments.[3,4]

Although the formation of metastasis is the final manifestation of the neoplastic progression, the dissemination of cancer cells is likely to occur early during tumor progression, and it might take several years before its clinical appearance.[5] Pre-operative chemotherapy-induced tumor shrinkage facilitates surgical resection and prevents the possible development of micro-metastatic disease.[6] In contrast, the Early Breast Cancer Trialists' Collaborative Group reported that the rate of distant metastasis did not differ between patients who underwent neo or adjuvant cytotoxic therapy, nor did neoadjuvant chemotherapy extend overall survival.[7]

These findings might also hold in other cancers. Several lines of evidence have pointed to the hidden pro-metastatic effects of conventional chemotherapy, which, under certain circumstances, may worsen the disease progression and reduce the patient's life expectancy.[8] For example, paclitaxel enhanced the density of perivascular macrophages in the tumor, facilitating cancer cell intravasation.[9] Chemotherapy also induced tumors to release small extracellular vesicles (sEV) carrying

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signals priming the pre-metastatic niche once the sEV land at the distant sites.[10,11]

Chemotherapy also directly conditioned the organ microenvironments, making them suitable for metastatic colonization. For example, taxanes promoted metastasis by action on the extracellular matrix composition in a $CD8^+$ T cell-dependent manner.[12] Similarly, cyclophosphamide made the lung vasculature more adhesive for CTCs in a metalloproteinase-2-dependent manner, which remodeled the vasculature basal membrane.[13]

The immune system is pivotal in chemotherapy's effects.[8,14] Besides altering immune cell functions, chemotherapy affects the white blood cells (WBCs) by depleting hematopoietic precursors with a rapid proliferation rate, in turn inducing immunosuppression.

The most severe hematological toxicity induced in cancer patients undergoing chemotherapy is neutropenia, which is a long-lasting drop in the Absolute Neutrophil Count (ANC $<2 \times 10^9$/L).[15] About 80% of patients in the first chemotherapy cycle usually develop neutropenic complications, and have to discontinue treatment or reduce drug doses.[16]

The role of neutrophils in tumor progression is controversial. Neutrophils have been described as possessing both metastasis-promoting[17–21] and suppressing capacities[22–25]. This prompted us to address the effect of chemotherapy-induced neutropenia (CIN) on metastasis formation properly.

Here we show that drugs inducing neutropenia [ie, cyclophosphamide (CPA); doxorubicin (DXR)] can promote cancer dissemination and metastasis formation. Mainly, neutropenia enhanced the early colonization phase and the proliferation of DTCs in the target organ during the metastatic cascade. These events were fully reversed when mice recovered their neutrophil levels or after receiving the granulocyte-colony stimulating factor (G-CSF).

This study provides information on chemotherapy's detrimental effects in cancer, emphasizing the role of neutrophils to control chemotherapy-promoted metastasis.

Materials and methods

Animals

Nine-to-twelve-week-old female Balb/C and C57BL/6N mice were obtained from Charles River Laboratories; female athymic Nude-*Foxn1*^{nu} were obtained from Envigo Laboratories. Mice were maintained under specific pathogen-free conditions.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that comply with the Italian Governing Law (D.lgs 26/2014; authorization n.19/2008-A issued March 6, 2008, by Ministry of Health), with the Mario Negri Institutional regulations and policies that provide internal authorization for people conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2015 – Reg. No. 6121) and with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Italian Ministry of Health (authorization no. 602/2019- PR) approved this study. All efforts were made to minimize the number of animals used and their suffering.

Tumor lines

Renal adenocarcinoma cell line Renca was purchased from the American Type Culture Collection (ATCC). Tumor cells were maintained in RPMI-1640 (Microgem), supplemented with 10% fetal bovine serum (FBS; Microgem). The Renca-Luc variant was obtained by infecting Renca cells with a lentiviral vector carrying the coding sequence of synthetic firefly luciferase gene *luc2* and using blasticidin (5 μg mL^{-1}; Gibco) as a selection antibiotic. The Renca-iRFP670 is the fluorescent Renca variant stably expressing the iRFP670 fluorescent protein. The UV-2237 fibrosarcoma cell line[26] was maintained in DMEM (EuroClone) supplemented with 10% FBS. Stocks of the cell lines were stored frozen in liquid nitrogen and used within four weeks after thawing. Cells were routinely tested by PCR for Mycoplasma contamination.

The Lewis Lung carcinoma cell line (LLC) was obtained from the Developmental Therapeutics Program (DTP) – Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository, NCI, US. LLC was stocked frozen as in vivo-derived tumor fragments and maintained subcutaneously in C57/BL6 mice before testing.

Renca metastatic tumor model

Artificial metastases were obtained by injecting Renca cells ($2{,}5\times10^4$) into the tail vein. Mice were euthanized 18–21 days later and lungs excised or metastasis evaluation.

For spontaneous metastasis studies, a cell suspension (1×10^5) of the Renca-Luc variant was injected orthotopically under the left renal capsule of Balb/C mice as previously described[27] and detailed in Supplemental methods. Primary tumor growth and metastasis were monitored by bioluminescent imaging (BLI) as detailed in Supplemental methods.

Survival endpoints were primarily labored breathing, ≥ 10% weight loss and abdominal distension for tumor burden.

Lewis lung metastatic tumor model

Tumor cells (5×10^4) from enzymatically digested subcutaneous tumors were injected into the right tibia muscle of C57BL/6N mice, and spontaneous metastases to the lungs analyzed.[27] Primary tumor growth was measured with a calliper twice a week, and tumor volume (mm^3) calculated as [length x width^2)]/2. The survival endpoint was based on the primary tumor dimension (1,5–2 cm^3).

Metastasis assessment

Lungs were harvested and fixed in Bouin's solution (Bio-Optica) for examination with a dissecting microscope (Olympus). Macroscopic lung nodules were counted by an investigator blinded to the treatment group. The number of colonies per lung and the diameter were recorded. Metastasis volume was calculated as [(4/3 π radius3 (μm3)], and the lung tumor burden was derived from the sum of the volumes of all

colonies counted. Histological validation of metastasis is detailed in Supplemental methods.

Drug preparation and in vivo dosing

Cyclophosphamide (CPA; Sigma Aldrich), Doxorubicin (DXR; Sigma Aldrich) and Cisplatin (DDP, Sigma Aldrich) were dissolved in 0.9% NaCl (saline), and mice received a single shot of each drug. CPA[28,29] was injected intraperitoneally (i.p.) at 200 mg kg^{-1}, and DXR and DDP were injected intravenously (i.v.) at 10 mg kg^{-1}. G-CSF (PeproThec, #250–05) was dissolved in water, and mice were treated every day for five consecutive days. G-CSF[30–32] was given s.c. at 0.25 mg kg^{-1}. All drugs were administered in a volume of 10 mL kg^{-1}. Drugs were used at the minimal doses required to induce neutropenia without showing evident toxicities.

Neutrophil depletion

For neutrophil depletion mice were i.p. treated with 100 µg of the monoclonal antibody (mAb) Rat anti-Ly6G clone 1A8 (BioXcell). To improve the depleting efficacy, mice were injected i.p. with 100 µg of the mAb mouse anti-rat kappa immunoglobulin light chain clone MAR18.5 (BioXcell) the day after the anti-Ly6G, as recently reported.[33] The mAb rat anti-trinitrophenol clone 2A3 (BioXcell) was used as isotype control. The administration schedule of the antibodies, and the histological validation of neutrophil depletion is detailed in the Supplemental Methods.

Cell proliferation, treatments and viability

To assess the effect of the neutropenic or healthy mouse serum on the growth of Renca and UV2237, 10×10^3 cells/well were seeded in 96-well plates in complete RPMI-1640 or DMEM media, respectively, with 4% healthy mouse serum. After 24 hours (day 1) media were replaced with fresh ones prepared with either 4% healthy or neutropenic mouse serum, and cell proliferation was measured every 48 hours with crystal violet staining. Proliferation was calculated as an increment from day 1. Serum collection is described in the Supplemental Methods.

To assess the effect of the G-CSF (PeproThec, #250–05) on the proliferation of Renca cells, 10×10^4 cells/well were seeded in 24-well plates in complete media with 5% FBS supplemented with 50 ng mL^{-1} of G-CSF, after 24 (day 0) and 72 (day 3) hours after the seeding. Proliferation was quantified every 24 hours using the Coulter Counter (Beckman Coulter). Alternatively, 10×10^3 Renca cells/well were seeded in 96-well plates in complete media with 10% FBS supplemented with 50 or 500 ng mL^{-1} of G-CSF, and proliferation was quantified every 24 hours with crystal violet staining.

Immunofluorescence and confocal microscopy

Mice were deeply anesthetized with Medetomidine, 2 mg kg^{-1}, and Ketamin, 150 mg $kg^{-1,}$ and transcardially perfused with 50 mL of 0.1 M PBS. The lungs were harvested and snap-frozen in liquid nitrogen; 20 µm longitudinal serial lung cryosections were collected on polylysine objective slides (VWR International). Sections were fixed in cold acetone for 10 minutes, then blocked in PBS with 10% normal goat serum and 0.3% Triton X-100, for 1 hour at room temperature. Sections were incubated overnight with primary antibodies: anti-CD11b, rat (1:200; BioRad) or anti-Ki67, rabbit (1:200; Abcam) at 4°C, then stained with the secondary antibody Alexa 488 anti-rat or anti-rabbit. The nuclei were counterstained whit Hoechst (1:1000; Roche). Images were acquired using a sequential scanning mode with an A1 Nikon confocal running NIS Elements at 20X or 40X magnification. Image analyses were done as detailed in Supplemental Methods.

Organ collection, digestion and flow cytometry analysis

Single-cell suspensions of primary tumors, blood and lungs were obtained and stained. Tumors and lungs were disaggregated mechanically and incubated with 0.5 and 0.1 mg mL^{-1} Type IV Collagenase in $PBS^{-/-}$ at 37°C for 40 and 20 minutes, respectively. Cell suspensions were filtered using 70 µm cell strainers. Red blood cells were lysed using red blood cell lysing buffer (Sigma Aldrich) for analysis. The number of viable cells extracted was determined by counting cells mixed with the Trypan blue exclusion dye. Extracellular staining was done using a $PBS^{-/-}$ buffer containing 2% FBS, 2 mM EDTA and 0.05% NaN3. Cells were then stained with ViaKrom 808 nm (Beckman Coulter), and negative cells were considered viable. Fc blocking reagent (Clone 24G2, MiltenyiBiotech) was added to cell suspensions for 10 minutes at 4° C, and then, for the extracellular staining an antibody mix was added on the cell suspensions for 20 minutes at 4°C in the dark. The antibodies used are listed in the Supplemental Methods.

Immune cell quantification is expressed as a percentage of CD45 positive cells (relative) or as absolute count, quantified as [no. of WBCs x (% neutrophils)]/100. Spectral overlap was calculated using live cells, and cells were acquired on a CytoFLEX LX (Beckman Coulter).

Statistical analyses

Statistics were calculated using GraphPad Prism 7.0b (GraphPad, La Jolla, CA), and values were expressed as mean ± SD. Data were analyzed with the following tests (see figure legends for details): Unpaired two-tailed Student's t-test, One-way ANOVA adjusted for multiple comparisons using Tukey's test, two-way ANOVA with Sidak's or Bonferroni's multiple comparisons. A p-value <0.05 ($^*P = 0.05$, $^{**}P = 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$) was considered significant.

Results

Chemotherapy-induced neutropenia is associated with increased metastasis

Healthy Balb/c mice were given CPA, and total circulating leukocytes and absolute neutrophil counts (ANC), two parameters predictive of a myelosuppressive effect, were longitudinally monitored by flow cytometry. CPA treated-mice had reductions in WBCs from two to six days after but had recovered by eight days (Supplemental Figure S1a). The ANC

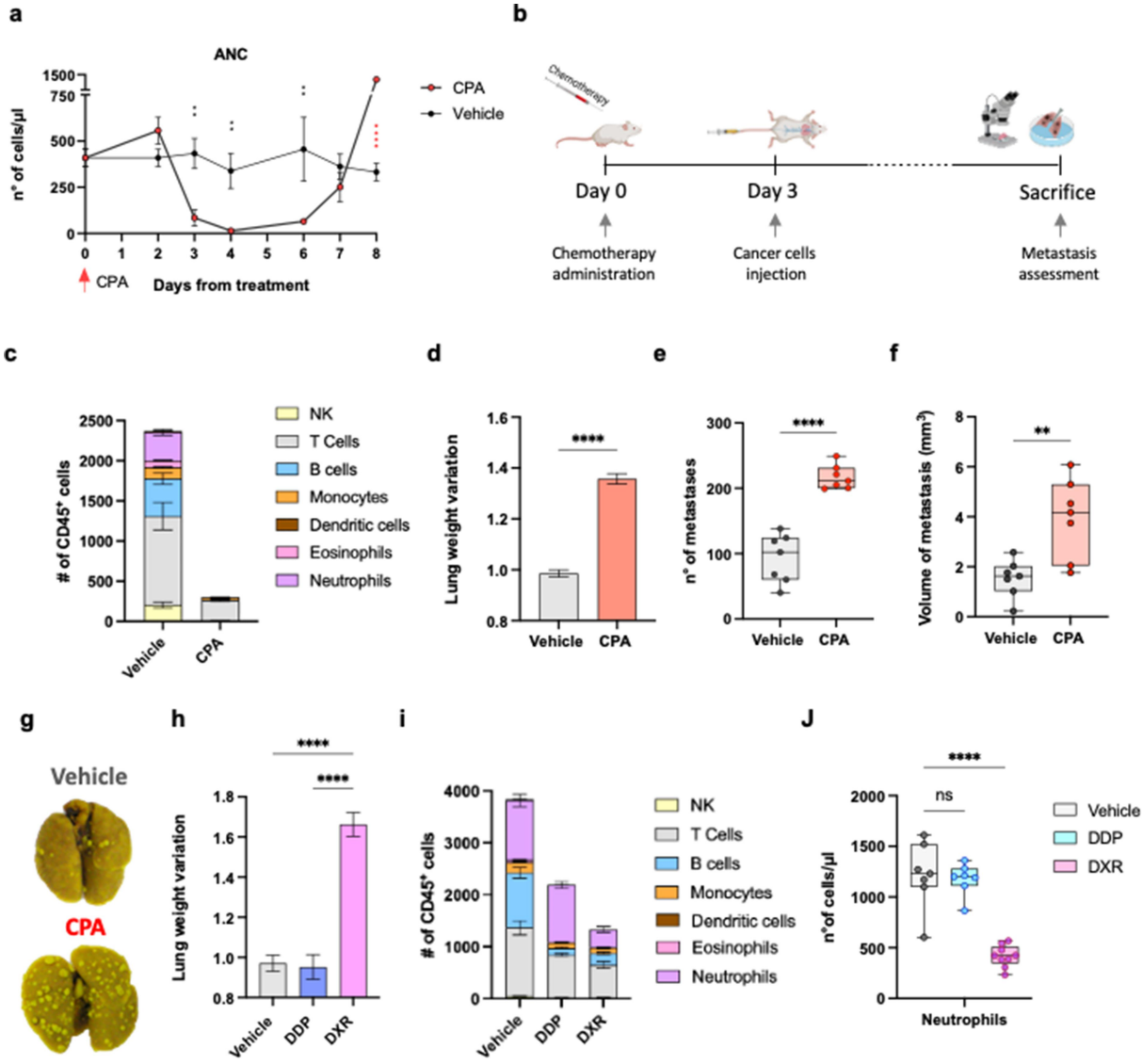

Figure 1. Effect of chemotherapy-induced myelosuppression on metastasis formation. (a) Absolute neutrophil counts (ANC) over the period tested, after treatment of nine week healthy Balb/c mice with CPA (day 0, red arrow; 3–6 mice per group). (b) Experimental outline: on day 0 mice were treated with either CPA (200 mg kg^{-1}) or vehicle; on day 3 mice were-tail vein injected with Renca tumor cells; on day 18, mice were euthanized, lungs were harvested, fixed and stained for metastasis evaluation. (c) Total number of viable CD45+ immune cells in mice treated three days earlier with vehicle ($n = 5$), and CPA ($n = 5$). (d) Lung weights of mice pretreated with CPA or vehicle-treated mice and injected with Renca (e) Macroscopic evaluation of metastasis number. (f) Volume of metastatic deposits macroscopically assessed on the surface of the lungs. (g) Metastatic lungs fixed in Bouin's solution from Balb/c mice i.v. injected with Renca cells and culled on day 18 post inoculum (h) Burden of metastasis expressed as lung weight of chemotherapy (DDP, DXR) over vehicle-treated mice (8 per group). (i) Total number of viable CD45+ immune cells in mice treated three days earlier with the vehicle ($n = 7$), DDP (10 mg kg^{-1}; $n = 7$) and DXR ($n = 10$ mg kg^{-1}; 9). (j) Total circulating neutrophils. Data in a, c, d, h and i are presented as mean ± SEM.**$P = 0.01$, ***$P < 0.001$, ****$P < 0.0001$, 2way ANOVA with Šídák multiple comparisons test (a), unpaired two-tailed t-test (d-f) or one-way ANOVA with Tukey multiple comparisons test (h,j).

too decreased three days after treatment, and gradually recovered after five days (Figure 1a; Supplemental Figure S1b). To rule out any direct effect of chemotherapy on cancer cells, we investigated the consequence of cancer treatment-induced myelosuppression on the formation of artificial lung metastasis by Renca tumor cells injected i.v. in CPA pre-treated mice[34] (Figure 1b). The treatment ablated the mouse immune system, as assessed by flow cytometry at the day of cancer cell injection (Figure 1c; Supplemental Figure S1d-j; Supplemental Figure S2). These mice had an increase in lung weight (Figure 1d) resulting from the larger metastatic burden, quantified as the number (Figure 1e,g) and volume (Figure 1f,g) of metastases, compared to the vehicle-treated group.

To strengthen the CPA findings, two other chemotherapeutics, DXR and DDP[35] were studied. Mice were injected i.v. with Renca tumor cells three days after DXR and DDP pretreatment (Figure 1b; Supplemental Figure S1c). While DDP pretreated mice showed no difference from the vehicle group, DXR-treated mouse lungs had a significantly higher burden of metastases, assessed as the increment of lung weight (Figure 1h).

The hypothesis that different myelosuppressive effects of DXR and DDP might explain the difference in metastasis burden prompted us to characterize the immune phenotype of WBCs on the day of tumor injection, during chemotherapy-induced alterations (Figure 1i; Supplemental Figure S2). DXR and DDP both affected the immune cell fingerprint,

significantly reducing NK cells, T-cells, B-cells (Figure 1i; Supplemental Figure S1k-m), monocytes, eosinophils, and dendritic cells (Figure 1i; Supplemental Figure S1n-p) compared to vehicle. However, only DXR significantly reduced neutrophils (Figure 1j), arguing that they might play protective role against metastasis when the other WBCs were depleted by chemotherapy.

These results suggested a possible association between chemotherapy-induced neutropenia and the risk of metastasis.

Neutrophils play a role in controlling metastasis formation

Upon neutrophil recovery (day 8 post-treatment), the CPA treated-mice had an ANC beyond the physiological levels (Figure 1a; Supplemental Figure S1b). Given that mice were killed three weeks after the injection of tumor cells, the increase in metastases might be due to the neutrophil rebound, rather than their initial depletion. To test whether higher levels of neutrophils correlated with reduced metastasis, we injected Renca cells i.v. in mice that had recovered from neutropenia (chemotherapy 8 days before; CPA-8) or when the ANC was at its nadir (CPA 3 days before; CPA-3), and compared the metastasis to untreated mice (Figure 2a–c). All mice (11/11) inoculated during neutropenia (CPA-3) had macroscopic metastasis (100% incidence). In comparison, only two out of 11 mice (18% incidence) developed metastasis when tumor cells were injected at recovery (CPA-8), lower than vehicle-treated mice (50% incidence). This finding suggested that low neutrophil levels correlated positively with metastasis formation, and that increased number of circulating neutrophils is associated with reduced metastatization.

To further demonstrate the involvement of neutropenia in chemotherapy-promoting metastasis, mice were treated with the granulocyte-colony stimulating factor (G-CSF), used as a vehicle to boost neutrophil levels.[36] Mice were given CPA or CPA and G-CSF, 6 hours before CPA (day 0) and up to day 3, when Renca cells were injected i.v. (Figure 2d), and WBCs and ANC monitored. As expected, mice challenged with CPA had fewer WBCs and neutropenia (Figure 2e; Supplemental Figure S3a-b). In contrast, in mice treated with CPA + G-CSF the ANC was comparable to healthy controls (Figure 2e; Supplemental Figure S3a-b). After CPA or CPA + G-CSF, neutrophils did not show any different activation, as indicated by the similar expression of CD11b, a surrogate activation marker[37], compared to healthy controls (Figure 2f). The monocytes and lymphoid cells remained significantly reduced compared to healthy mice (Supplemental Figure S3 g-i). The metastatic burden was significantly lower in mice receiving CPA with G-CSF than mice with CPA alone (Figure 2g–j). The finding shows that a high ANC correlates with reduced metastasis formation, and implies that preventing the chemotherapy-induced neutropenia counteracted its pro-metastatic effects. G-CSF treatment did not significantly affect Renca cell proliferation or their ability to form colonies *in vitro* compared to cells not exposed to the growth factor (Supplemental Figure S3c-f).

Since chemotherapy affects the whole immune system, the involvement of other leukocytes in metastasis cannot be excluded. To mirror neutropenia, we selectively targeted neutrophils in Balb/C mice using the depleting antibody anti-Ly6G in combination with the anti-rat Kappa Immunoglobulin Light Chain (Figure 2k). This depletion strategy has proved superior to the more commonly used single anti-ly6G dose.[33] As expected, neutrophil depletion did not alter the number of circulating leukocytes except for neutrophils that were virtually absent over the period tested (Supplemental Fig S4a-c). Neutrophil-depleted mice, injected with Renca cells i.v., had more metastases than control mice (Figure 2l,m; Supplemental Figure S4d). This confirmed that the absence of neutrophils was associated with increased metastasis. To exclude T-cell involvement, FOX1 nude mice lacking T cells were used (Figure Supplemental 4e). Similarly, neutrophil-depleted nude mice had more metastases (Figure Supplemental S4f-g), confirming that neutrophils are required for metastasis formation. Nude and syngeneic mice, neutropenic or not, did not have different T cell frequencies after treatment with the depleting cocktail (Supplemental Figure S4h).

Chemotherapy-induced neutropenia affects the early colonization of the lungs but not established metastases

To assess the effect of neutropenia on already implanted tumor lesions, neutrophil depleting antibodies were administered (day 12) once metastatic lung deposits by bioluminescent Renca cells (Renca-Luc) were established (Figure 3a), and secondary tumor progression was monitored by bioluminescence (BLI) longitudinally (Figure 3b,c). Depleting antibodies reduced circulating neutrophils, as shown 24 hours after the last antibody dose (Supplemental Figure S4i). However, as the disease progressed, treated mice presented the same lung metastatic load as untreated mice (Figure 3c; Supplemental S4j). This supported the notion that neutrophils play a role in the early formation of metastases.

To assess the effect of chemotherapy or neutropenia on early metastasis formation, we measured BLI 24 hours and 6 days after Renca-Luc injection. At 24 h, CPA pretreated mice had higher BLI counts than the vehicle group, indicating more cancer cells retained in the lungs (Figure 3d). Vehicle or DDP pretreated mice had comparable BLI counts, while DXR pretreatment reduced the tumor cell load in the lungs at 24 hours (Figure 3e). Neutrophil depletion reduced the arrest of cancer cells, alike DXR, as indicated by a BLI count lower than in the vehicle group (Figure 3f). Despite these somewhat contrasting data, six days later, the signal of mice treated with the neutropenia-inducing agents – CPA, DXR (Figure 3g,h) and the depleting antibodies (Figure 3i) – increased significantly. This suggests that the colonization of the target organ, rather that the arrival of cancer cells, is the step in the metastatic cascade mainly affected by neutropenia. We cannot exclude that CPA might facilitate metastasis by other mechanisms favoring the early arrest of cancer cells into the lungs.

A neutropenia-conditioned environment provides cancer cells with a proliferative advantage responsible for increased metastasis

To further study the metastatic cascade after the extravasation of cancer cells into the lungs (from 24 h to 72 h), we used the

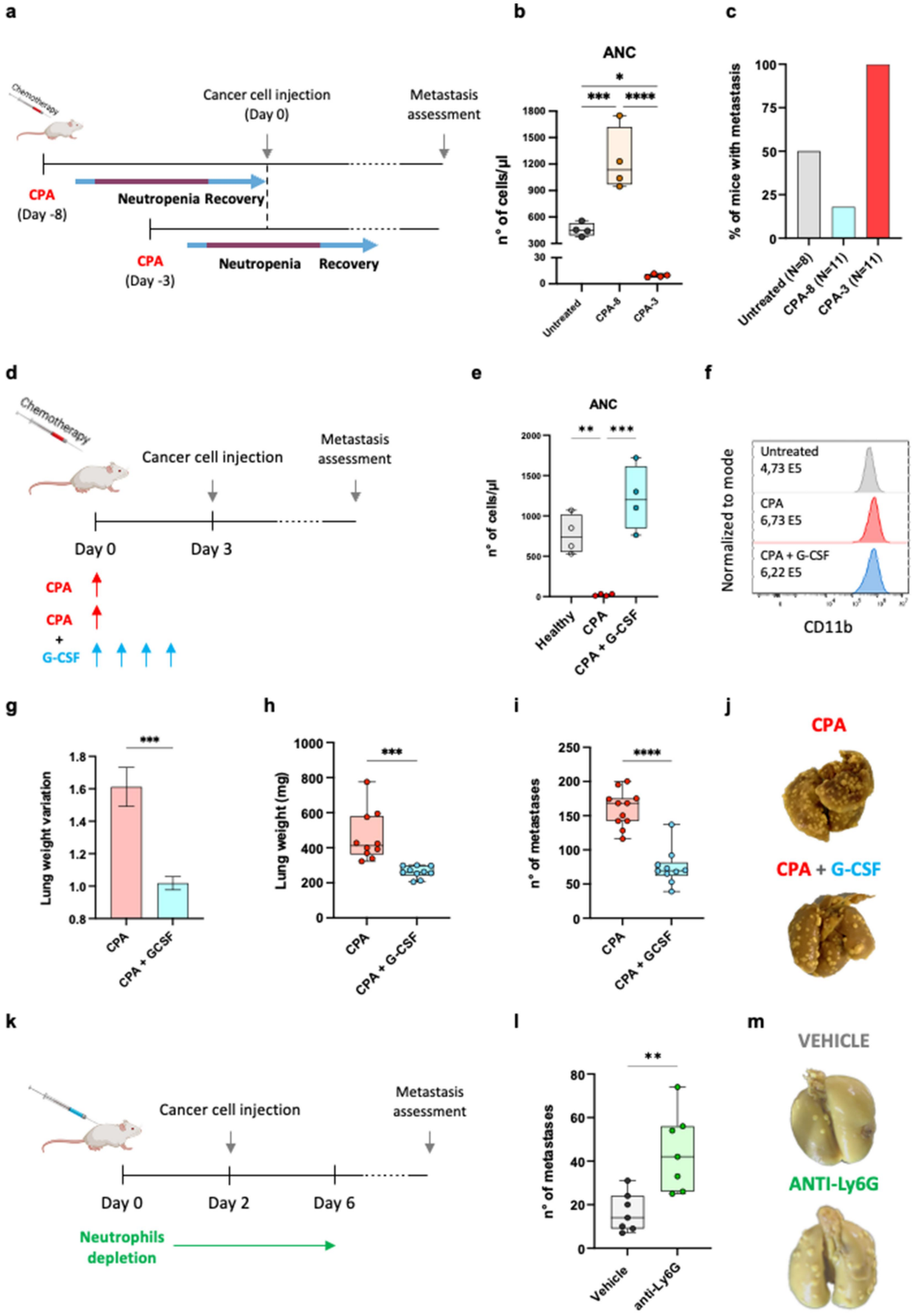

Figure 2. Neutrophils are required to control the formation of metastasis. (a) Experimental outline: nine week healthy Balb/C mice were divided into two treatment groups and pre-conditioned with CPA eight (CPA-8; 200 mg kg^{-1}, $n = 11$) or three (CPA-3; 200 mg kg^{-1}, $n = 11$) days before i.v. injection of Renca cells. (b) ANCs of pre-conditioned mice compared to untreated mice. (c) Mice with metastases at death. (d) Experimental outline: four days before the i.v. injection of Renca tumor (day 0), 12 week Balb/C mice were treated with either CPA (200 mg kg^{-1}) or CPA and G-CSF for five doses (0.25 mg kg^{-1}). During the test, mice were bled for recording leukopenia and neutropenia. (e) ANC at the tumor injection, day 3. (f) Geometric mean fluorescent intensity histograms showing the expression of CD11b in neutrophils of untreated, CPA-treated or CPA followed with G-CSF on the day of tumor injection (3 per group). (g) Variation of the weight of the lungs of CPA over CPA + G-CSF treated mice. Data is represented as mean ± SEM. (h) Weights of the lungs with metastases. (i) Number of macro-metastases. (j) Representative pictures of metastatic lungs. (k) Experimental outline: nine week Balb/C mice were pretreated with the anti-Ly6G and the anti-Rat antibody two days before the i.v. injection of Renca tumor cells. (l) Number of macro-metastases counted on the lungs. (m) Representative pictures of metastatic lungs. *$P = 0.05$, **$P = 0.01$, ***$P < 0.001$, ****$P < 0.0001$ as determined by ordinary one-way ANOVA with Tukey's multiple comparisons test (b and e) or two-tailed, unpaired Student's *t*-test (g-i and l).

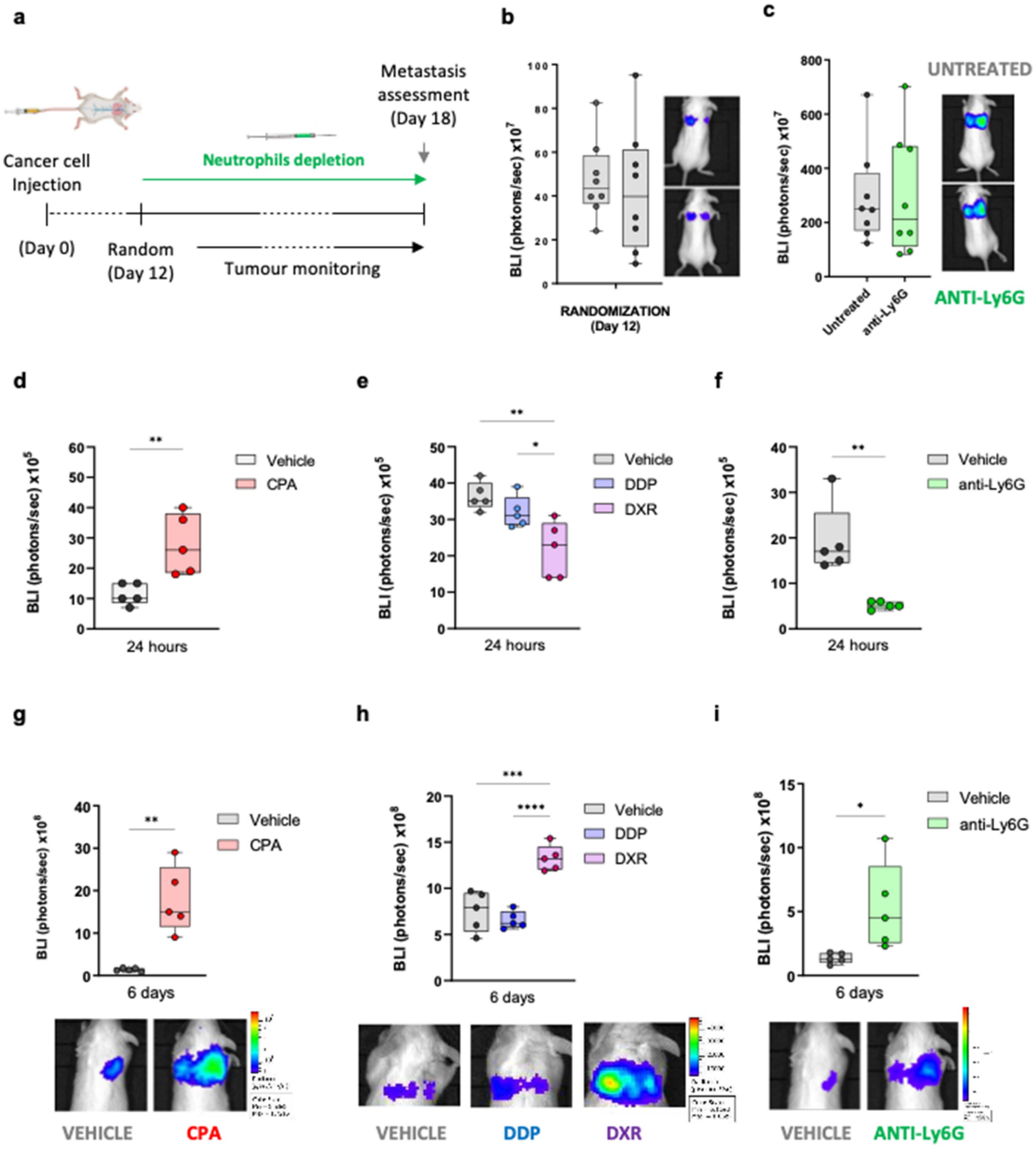

Figure 3. Chemotherapy-induced neutropenia affects the early colonization of the lung by circulating tumor cells. (a) Experimental outline: nine week Balb/C mice were injected i.v. with Renca-Luc cells and scanned with the IVIS imaging system to verify the presence of the lesions. After the establishment of metastases, mice were treated with anti-Ly6G and anti-Rat antibodies. During the depletion, mice were scanned with the IVIS for metastasis evaluation. At the end of the treatment, mice were bled to assess neutrophil depletion. (b) Bioluminescent analyses, presented as photons/second, to randomize mice to the treatment groups. (c) Bioluminescent analysis of neutropenic and untreated mice at the end of the treatment. (d-i) Bioluminescent counts of cancer cells at two time points after their i.v. injection in mice pretreated with CPA (24 hours d; day 6 g), DDP and DXR (24 hours e; day 6 h) or the neutrophil depleting antibody cocktail (24 hours f; day 6 i). Representative pictures refer to mice scanned with the IVIS imaging system on day 6. $^{*}P = 0.05$, $^{**}P = 0.01$, $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparisons test (e, h) or two-tailed unpaired Student's *t*-test (d, f, g, i).

fluorescent variant Renca-iRFP670, which permit a direct *ex vivo* cancer cells visualization by confocal microscopy.

We validated the BLI findings, with fewer Renca-iRFP670 cells arrested in the lungs of neutropenic mice 24 h after the i.v. injection, compared to vehicle-treated mice (Figure 4a). In siblings inoculated with the same cancer cell suspension but killed after 72 h, vehicle-treated mice had a similar number of cancer cells as at 24 h (vehicle range 24 hours 24–36; 72 hours 26–48; Figure 4a). Conversely, there were more Renca-iRFP670 cells in the lungs of neutrophil depleted mice at 72 h than 24 h (neutropenic mice range 24 hours 10–12; 72 hours 24–29; Figure 4a). This suggested that Renca-iRFP670 cells in the neutropenic environment acquired a proliferative advantage. Following this, Renca-iRFP670 cells retained at 24 hours in the lungs of neutropenic mice proliferated significantly more than cells in the lungs of the vehicle mice, as assessed by Ki-67 immunoreactivity (Figure 4b).

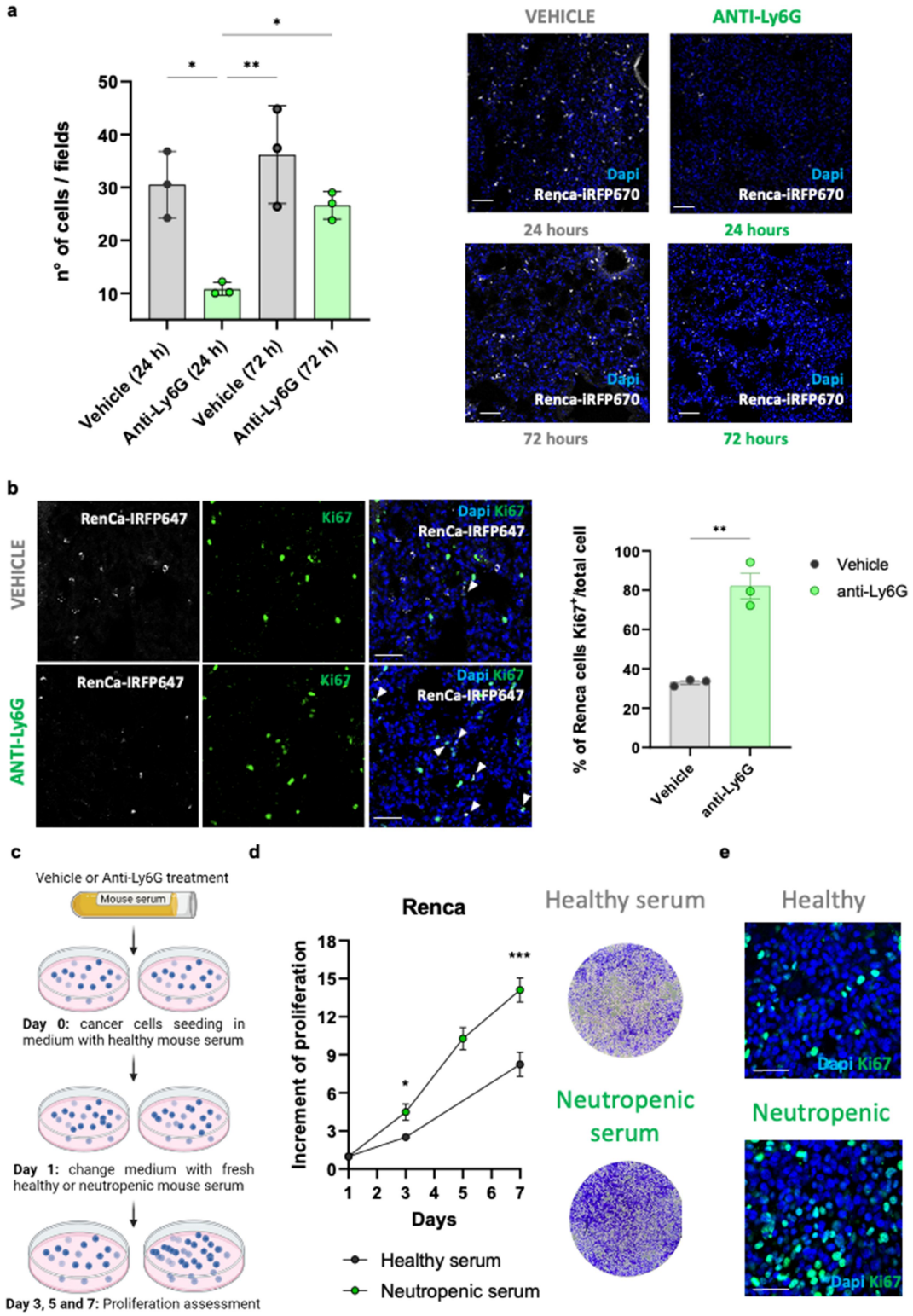

Figure 4. The neutropenic environment promotes cancer cell fitness, giving a proliferative advantage. (a) Renca-iRPF670 cells counted in the lungs of neutropenic or control mice 24 and 72 hours after their injection (3 mice per group/per time point). Scale bar 100 µm. (b) Proliferation of Renca-iRFP670 cells with representative confocal images. The proliferation was assessed by counting the Ki-67 (green) and Renca-iRFP670 (white) juxtapositions in the lungs of neutropenic or healthy mice injected i.V. 24 hours earlier with the Renca-iRFP670 (3 mice per group). Scale bar 50 µm. c-e Experimental design (c) for the proliferation assessment of Renca cells (d) cultivated in media supplemented with 4% sera of neutropenic or healthy mice with representative images of crystal violet staining (on day 7), and (e) Ki-67 immunoreactivity counterstained with DAPI (blue). Scale bar 50 µm. Mean ± SD of four neutropenic or control mouse sera. $^{*}P = 0.05$, $^{**}P = 0.01$, $^{***}P < 0.001$, two-tailed unpaired Student's *t*-test (b), or one-way ANOVA with Tukey's multiple comparisons test (a-d).

The increase in proliferation occurred when mice were still neutropenic, as confirmed by the reduction in the percentage of $CD11b^{+}$ cells (Supplemental Figure S5a-b).

To test whether a neutropenia-induced systemic alteration accounted for the greater proliferation of cancer cells, we examined whether the serum of neutrophil-depleted mice

promoted the growth of cultured Renca cells (Figure 4c). This serum significantly stimulated the proliferation of Renca cells compared to cells grown with the healthy mouse serum in the medium (Figure 4d,e).

To strengthen these observations, we performed proliferation assays in a different cancer cell line, the murine fibrosarcoma UV2237, which recapitulated these findings (Supplemental Figure S5c-d).

This evidence suggests that, in a neutropenic environment, cancer cells acquired a proliferative advantage that ultimately results in widespread cancer dissemination.

Neutrophil depletion promotes spontaneous metastasis formation from a primary tumor

The artificial metastasis assay is not representative of the whole metastatic process, as it excludes the role of the primary tumor. To study the effect of neutropenia on spontaneous metastasis, Renca-Luc cells were orthotopically transplanted under the renal capsule (intra-kidney; i.k.) of mice. Given the sensitivity of Renca cells to chemotherapy, including CPA, its anti-tumor activity likely would hide the pro-metastatic effect of neutropenia. Orthoptic tumor bearing mice were then treated with the neutrophil-depleting cocktail (anti-Ly6G plus anti-Rat) mirroring the neutropenia induced by chemotherapy (Supplemental Figure S6c). The antibodies were administered when the primary tumor was established but not yet metastatic (assessed by *in vivo* imaging six days post-transplantation, Figure 5a). Histological analyses were conducted to verify the presence of micro-metastases at the end of the treatment with the depleting antibodies (day 12). One mouse out of five in the control group had metastases, while four of the five in the neutropenic group had metastases (Figure 5b). Primary tumor monitoring with BLI showed that the antibody treatment delayed the primary tumor growth compared to control group (Figure 5c). Conversely, neutropenic mice had raising bioluminescent counts in the lungs (Figure 5d), confirmed at autopsy by a significantly higher burden of lung metastasis in mice treated with antibodies, as reported.[38] (Figure 5e; Supplemental Figure S6a-b). Immunohistochemistry (Figure 5f,g) and flow cytometry (Figure Supplemental Figure S6f-g) further confirmed the reduction of neutrophils compared to the controls.

No differences in neutrophil frequencies related to the surgical procedures for tumor transplantation were observed in tumor-bearing or sham-operated mice (Supplemental Figure S6c-e). Interestingly, tumor bearing mice showed an increase of circulating neutrophils associated with primary tumor progression (Supplemetal Figure S6C), surmising the presence of cancer-related inflammatory processes.

To extend these observations, the depleting antibodies were administered to mice bearing another tumor model that spontaneously metastasizes to the lungs, the Lewis Lung Carcinoma (LLC). Two schedules were tested (Figure 5h). The early treatment (schedule-1) involved administration of the depleting antibody cocktail when primary tumors were palpable but metastasis not yet implanted, as confirmed by histology (day 5; Figure 5i). In the late treatment (schedule-2), the depleting cocktail was administered when metastatic lesions were implanted, and mice received the antibodies until euthanasia (day 12; Figure 5h,i). The depleting cocktail reduced circulating neutrophils irrespective of the schedule (Supplemental Figure S6h). Primary tumor growth delay was negligible in mice receiving the depleting antibodies (Figure 5j). Increased metastases were observed in neutropenic mice treated only with schedule-1 (early treatment) compared to mice assigned to the vehicle or irrelevant antibody group (Figure 5k; Supplemental Figure S6i). This did not occur in the lungs of mice receiving antibodies with schedule-2 when metastases were already established (Figure 5k; Supplemental Figure S6i). These data confirmed that neutropenia mainly affects metastasis dissemination/early implantation, and ruled out a direct association between lung metastasis and the primary tumor growth.

Discussion

This study shows that myelosuppressive anticancer drugs such as CPA and DXR facilitate lung metastases in preclinical mouse models, with the induction of neutropenia. We suggest that neutropenia promotes the proliferation of DTCs during the early phases of lung colonization and that this effect depends on the host-tumor interaction, not on the direct effect of chemotherapy on tumor cells.

Cancer patients frequently receive neoadjuvant chemotherapy, the primary treatment with anticancer drugs for resettable solid tumors. Despite substantially improved responses, patients with residual disease often suffer worse distant recurrence-free survival.[14] Accumulating evidence indicates that cytotoxic treatments may promote metastasis by inducing primary tumor alterations.[10,39] Here we exploit artificial metastasis assays to uncouple the effect of neutropenia on the release of cancer cells from a primary tumor (intravasation), from the circulating tumor cell arrival to the target organ. We show that an effect on the primary tumor is not necessarily associated with an increase of metastases. Our data indicate that neutropenia influences the lungs, promoting metastatic outgrowth without a primary tumor.

A number of reports show that the immunological alteration induced by chemotherapy is responsible for worsening cancer.[8] Here we show that CPA, DXR and DDP reduced the number of circulating leukocytes, but only CPA and DXR elicited neutropenia, inducing hospitable “soil” for lung metastasis growth. Chemotherapy was administered 3–4 days before tumor cell inoculation[30,35,40,41] to specifically study alterations in the host avoiding anti-tumor effects on cancer cells. In fact, secondary lesions develop because metastatic cancer cells are usually insensitive or resistant to chemotherapy.

Preclinical studies have shown a causal relationship between neutrophils and metastasis. Because of their context-dependency and plasticity, neutrophils can exert both pro- and anti-metastatic activities.[42,43] To study the importance of neutrophils in metastasis, we challenged our models with an antibody-mediated strategy (the anti-Ly6G antibody with the anti-rat Kappa Immunoglobulin Light Chain), recently proposed as inducing controlled mouse neutrophil depletion.[33] The finding that their depletion increased metastasis suggests

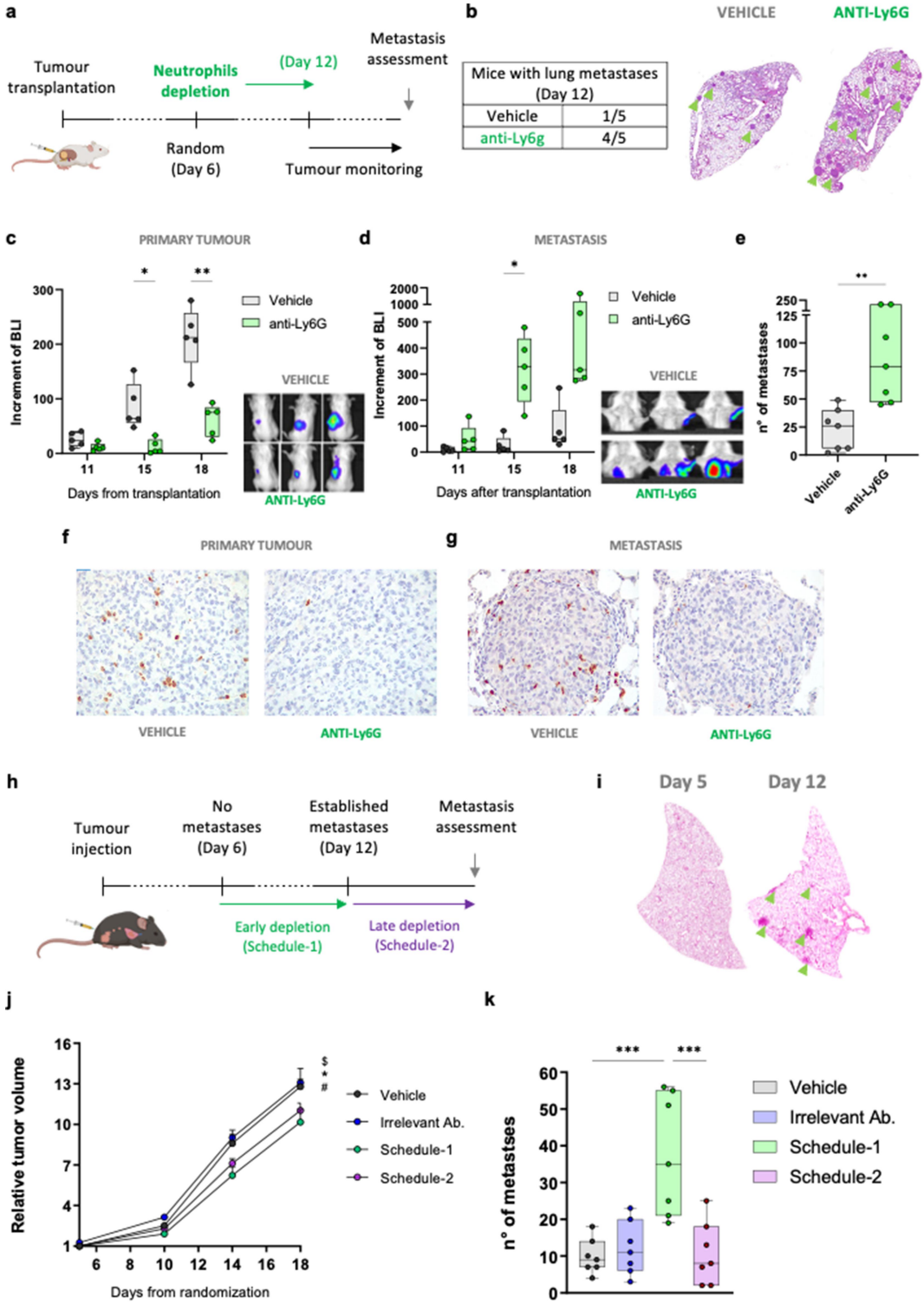

Figure 5. Effect of neutrophil depletion in mice bearing metastatic tumors. (a) Experimental outline: Renca-Luc cells were injected i.k. in ten week Balb/C mice, the tumor take was assessed by IVIS on day 6 and mice randomized by BLI to treatment with the anti-Ly6G and the anti-Rat or vehicle (13 per group), once a day for three times. On days 6 (randomization day) and 12 (end of the treatment), four and six mice per group, respectively, were euthanized to assess neutrophil depletion in the tumor and lungs. Primary tumors and half-lungs were used to measure neutrophils by flow-cytometry. The remaining half of the lung was fixed and stained for metastasis evaluation. (b) Number of mice with metastases at the end of the treatment with the depleting antibodies (day 12), assessed by histology with representative pictures. Green arrows point to metastases. c-d Increment of the bioluminescent signal during the test and representative images of the primary (c) and secondary (d) tumors. (e) Metastatic deposits macroscopically counted on the surface of the lungs. f-g Representative immunohistochemistry images of primary tumors (f) and metastasis (g) of vehicle and neutrophil-depleted mice. (h) Experimental outline: LLC tumor cells were injected i.m. into nine week C57Bl/6N mice and, on day 6 after transplantation, mice were randomized by primary tumor volume to the treatment groups (7 per group). Schedule-1: treatments from day 6 to 12; Schedule-2: treatments from day 12 to 18; the irrelevant antibody was administered with the same timing as schedule-1. (i) Representative images of lungs with metastases at 5 and 12 days after tumor transplantation, assessed by histology. Green arrows point to metastases. (j) Relative primary tumor growth. Data are represented as mean ± SEM. (k) Total number of metastatic nodules at euthanasia (day 18). $^{*}P = 0.05$, $^{**}P = 0.01$, $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple comparisons test (c-d,j and k), or two-tailed unpaired Student's *t*-test (e). In j: $ compares irrelevant antibody vs schedule-2 (days 14 and 18, $P<0.05$); * compares vehicle vs schedule-1 (days 14 and 18, $P<0.01$);
compares irrelevant antibody vs schedule-1 (days 14 and 18, $P<0.001$). Statistical analyses with Šídák multiple comparisons test.

that neutrophils have anti-metastatic property, following previous evidence.[22–25] We validated this paradigm in different tumor models and mouse strains.

Having established an effect of CIN in metastasis, we studied which steps of the metastatic cascade were affected by neutropenia. The lack of neutrophils did not alter the progression of established metastases, thus indicating that cancer patients with overt metastases, and experiencing CIN, are unlikely to present faster progression of secondary tumors.

Data were contrasting on chemotherapy altering CTCs arrest in the lungs in an early stage (24 hours) of metastatic dissemination. As expected, CPA increased this early retention, in agreement with reports that CPA promoted tumor cell arrest in the lung by inducing lung vascular alterations.[13,29] Surprisingly, DXR reduced lung cancer cell arrest compared to control mice or DDP pretreated mice with normal ANCs. Similarly, the antibody-mediated depletion of neutrophils lowered the cancer cell load in the lungs at 24 hours. This validated previous reports of a diminished arrest, in secondary organs, of CTCs in mice lacking neutrophils, which was described as one of the mechanisms of neutrophil-promoted metastasis.[19,21,44] Our data do not exclude that CPA might increase metastasis by favoring the early CTC arrest in the secondary organ but also show that the CPA-related myelosuppression and neutropenia might be another condition to promote metastatic outgrowth.

The observation that prolonged neutropenia promoted a long-term tumor rise in the lungs (6 days after tail vein injection), independently of the neutropenia inducer used, further suggests that a neutropenic condition favors the lung colonization.

Nevertheless, six days after the injection an increase in the tumor load followed by more metastases was observed in mice with CIN and neutrophils depleted. Because of the increased Ki-67 proliferating cancer cells in the lungs, we feel like excluding that CIN acts on the early arrest of CTCs but rather on the interaction with a supportive tumor microenvironment. This is supported by the increased proliferation of cancer cells cultured with neutropenic mouse-derived serum. One can speculate that neutropenia affects the so-called metastatic inefficiency, which is the failure of solitary cells to grow into macroscopic tumors, once extravasated.[3]

We have not dissected the molecular mechanism behind the increased proliferation and further studies are required. Proteomic or RNA sequencing analyses might reveal the different cytokines or differently activated pathways in neutropenic and healthy mouse sera, which might account for the higher proliferation rate.

In mice orthotopically transplanted with Renca-Luc cells, neutropenia delayed the primary tumor growth, while still increasing metastasis. The primary tumor growth delay in the absence of neutrophils indicates that they have pro-tumor activities at the primary site. Neutrophils might induce an immunosuppressive microenvironment that supports tumorigenesis. For example, one report indicated that neutrophils constrain primary tumor formation by inducing the release of interferon-γ by unconventional αβ-T cells.[45] However, pro-tumor neutrophils were also described as promoting angiogenesis, supporting tumor growth.[46] Beyond the role of neutrophils on the primary tumor, here we show that the effect of neutropenia in increasing metastasis was not directly associated with a primary tumor increase. A limitation of this study is the sensitivity of our tumor models to chemotherapy that prevented to study spontaneous metastasis formation under pharmacological treatments. The use of chemotherapy-resistant tumors might offer one way to distinguish the net effect of the CIN on metastasis, avoiding the anti-tumor effect of chemotherapy.

Our study gives translational insights into the context of chemotherapy-induced metastasis. The increase of neutrophils by G-CSF, which boosts their production and release into the circulation,[47] in combination with CPA, prevented the reduction of the ANC and counteracted metastasis. We used G-CSF which is currently offered to patients experiencing chemotherapy-induced neutropenia, instrumental to show that rescuing neutrophils prevents neutropenia-increase metastasis by chemotherapy.

In clinical settings, CIN is associated with drug effectiveness and better overall survival.[36,48] However, the response to debulking cytotoxic therapy might hide an increased risk of metastases. Unfortunately, no clinical data are available to study the correlation between CIN and metastasis. A deep dive into the relationship between chemotherapy-induced neutropenia and the metastasis-free survival is recommended. Our study shows that a side effect of chemotherapy that reduces neutrophils raises to the risk of metastasis. Although the exact mechanism causing this is not understood, we showed that the systemic absence of neutrophils created a profitable metastatic microenvironment for DTC proliferation.

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